

**Interactions between *S. aureus* components and the host immune system**

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###### Title page

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###### Summary

*Staphylococcus aureus* is a significant human pathogen, able to cause a wide range of diseases. Emergence of multi drug-resistant strains puts a significant burden on healthcare providers and highlights a pressing need for alternative control regimes. This project set out to explore the interactions between *S. aureus* and the host immune system and demonstrated a novel role for commensal bacteria and their components in initiation of *S. aureus* infection.

As many staphylococcal infections occur through wounds or with inserted medical devices, there is a likely initial coinfection with commensal bacteria. This hypothesis was tested using animal models of infection. Zebrafish embryos were initially used to inform a murine sepsis model.Firstly, the Gram-positive commensal bacteria *Micrococcus luteus* and *Staphylococcus epidermidis* were found to augment *S. aureus* pathogenesis in all models. This was extended to show that the Gram-negative skin commensal *Roseomonas mucosa* also augmented. Finally, the enteric organism *Escherichia coli* augmented but also benefitted to the detriment of the host. Mechanistic studies showed that peptidoglycan from a range of bacterial species and chemical constituencies augmented. One of the most significant advances of this project was the dramatic reduction in the *S. aureus* infectious dose in the presence of augmenting material, which establishes a more physiologically relevant model of human disease.There are also important wider implications as to how we view and treat infections as all are initiated from a polymicrobial environment.

###### Abbreviations

|  |  |
| --- | --- |
| °C | Degree Celsius |
| × | Times |
| < | Less than |
| ~ | Approximately |
| aa | amino acid |
| Amp | Ampicillin |
| AMPs | Antimicrobial peptides |
| APC | Antigen presenting cell |
| ATP | Adenosine triphosphate |
| BALB/c | Inbred mouse strain |
| BHI | Brain heart infusion |
| C3H/HeN | Inbred mouse strain |
| CA | Community acquired |
| CCL2 | Chemokine (C-C motif) ligand 2 |
| CCL4 | Chemokine (C-C motif) ligand 4 |
| CFU | Colony forming units |
| CXCL1 | Chemokine (C-X-C motif) ligand 1 |
| dH2O | Distilled water |
| DIC | Differential interference contrast |
| DMSO | Dimethyl sulphoxide |
| dpf | days post fertilisation |
| dpi | days post infection |
| EDTA | Ethylenediamine tetra-acetic acid |
| Ery | Erythromycin |
| GFP | Green fluorescent protein |
| GlcNAc | *N*-acetyl glucosamine |
| Gly | Glycine |
| GVBS | Gelatine Veronal buffer saline |
| h | hours |
| HF | Hydrofluoric acid |
| HPE | High performance Elisa buffer |
| hpf | hours post fertilisation |
| hpi | hours post infection |
| HRP | Horse radish peroxide |
| IFN-𝛾 | Interferon gamma |
| IL-12 | Interleukin 12 |
| IL-17A | Interleukin 17A |
| IL-1𝛽 | Interleukin 1 beta |
| IL-6 | Interleukin 6 |
| IL-8 | Interleukin 8 |
| Kan | Kanamycin |
| KC | Kupffer cells |
| L | Litre |
| LB | Luria-Bertani medium |
| LPS | Lipopolysaccharide |
| LTA | Lipoteichoic acid |
| LWT | London Wildtype |
| M | Molar |
| MAC | Membrane attack complex |
| MDP | Muramyl dipeptide |
| mg | Milligrams |
| MIC | Minimum inhibitory concentration |
| min | minutes |
| ml | millilitres |
| mM | millimolar |
| MO | Morpholino oligonucleotide |
| MOI | Multiplicity of infection |
| MQ | Millipore ultra pure water |
| MRSA | Methicillin resistant *Staphylococcus aureus* |
| MSCRAMM | Microbial surface components recognising adhesive matrix molecules |
| MyD88 | Myeloid differentiation primary response gene 88 |
| Neo | Neomycin |
| NF-κB | Nuclear Factor |
| nl | Nanolitre |
| NLR | Nucleotide oligomerisation domain like receptor |
| nM | Nanomolar |
| nm | Nanometre |
| Nod | Nucleotide oligomerisation domain |
| OCT | Optimum cutting temperature compound |
| ODx | Optical density at indicated wavelength x (nm) |
| PAMP | Pathogen-associated molecular pattern |
| PBPs | Penicillin binding proteins |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PGN | Peptidoglycan |
| PGRP | Peptidoglycan recognition protein |
| pPGN | Polymeric peptidoglycan |
| PRR | Pattern recognition receptors |
| PVL | Panton-V alentine leukocidin |
| RBC | Red blood cell |
| RIP2 | Receptor interacting protein 2 |
| RNA | Ribonucleic acid |
| ROS | Reactive oxygen species |
| rpm | Revolutions per min |
| RT | Room temperature |
| SCVs | Small colony variants |
| sPGN | Soluble peptidoglycan |
| *Spp.* | Species |
| t | Time |
| TA | Teichoic acid |
| TAE | Tris-acetate EDTA (buffer) |
| TES | Tris-EDTA NaCl |
| Tet | Tetracycline |
| TLR | Toll like receptor |
| TMB | 3,3',5,5'-tetramethylbenzidine |
| TNFα | Tumour necrosis factor alpha |
| TSS | Toxic shock syndrome |
| TSST-1 | Toxic shock syndrome toxin-1 |
| v/v | Volume for volume |
| Van | Vancomycin |
| VBS | Veronal Buffer Saline |
| VISA | Vancomycin intermediate |
| VRE | Vancomycin resistant enterococci |
| VRSA | Vancomycin resistant Staphylococcus aureus |
| w/v | Weight for volume |
| WT | Wild-type |
| WTA | Wall teichoic acid |
| μg | Microgram |
| μl | Microlitre |
| μm | Micrometre |
| μM | Micromolar |
| Φ | Phage |

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# Chapter 1. Introduction

## *Staphylococcus aureus*

Historically, in 1880 Alexander Ogston isolated a bacterium from pus taken from a surgical wound, noticing a grape-like appearance of the isolated organism, therefore naming them staphylococci (from greek staphyle-cluster and kokkos- berry) (Ogston, 1881). In 1977, this grape-like structure of *Staphylococcus spp.* was explained by phase contrast microscopy: the bacteria divide sequentially in three planes, with cells remaining attached to each other following division (Tzagoloff and Novick, 1977).

In 1884 the German surgeon Anton Rosenbach isolated staphylococcal colonies from the pus of his patients, and further described the bacteria as *Staphylococcus albus,* because of the white colour of the colonies, and *Staphylococcus aureus,* due to the unmistakeable golden-orange colour of the bacteria (Thomer, Schneewind and Missiakas, 2016). Staphyloxanthin was later shown to be responsible for the characteristic golden colour of the colonies (Marshall and Wilmoth, 1981), as well as allowing *S. aureus* to be resistant to oxidative stress (Clauditz *et al.*, 2006).

*S. aureus* is a non-motile and non-spore-forming coagulase-positive bacterium (Galperin, 2013), commonly found tocolonise the human nares and skin of healthy humans (van Belkum *et al.*, 2009a). Alongside humans, many mammals such as rats, rabbits and bovines are naturally colonized by *S. aureus* (van Duijkeren *et al.*, 2008; Williams, Bloebaum and Petti, 2008; Wang *et al.*, 2015)*.*

*S. aureus* is an opportunist pathogen, capable of causing mild and severe infections such as folliculitis and impetigo (superficial skin infections), carbuncles (deep soft tissue infection), and blood infections whereby it can disseminate to all organs (Thomer, Schneewind and Missiakas, 2016).

## Metabolism of *S. aureus*

Humans provide ideal habitats for colonizing bacteria, with each microenvironment, such as the nose, made up of a unique composition of metabolites. *S. aureus* is capable of adjusting its metabolic needs based on availability of nutrients in the environment through a stringent response system (Reiß *et al.*, 2012). Isoleucyl-tRNA synthase plays important roles in the response system and are therefore antibiotic targets. Reiβ *et al.* has shown that addition of mupirocin blocks charging of Isoleucyl-tRNA, which accumulates in the bacterial cell ultimately signalling to turn off the stringent response due to amino acid starvation (Reiß *et al.*, 2012). Krismer *et al.* created a synthetic nasal medium based on metabolites in human nasal secretions, showing that the methionine metabolic pathway is essential for the colonization of the nares (Krismer *et al.*, 2014). Moreover, it has been shown that low levels of amino acids (2.6 mM) and glucose are required for successful colonization of the human nose, while the lungs of cystic fibrosis patients infected with *S. aureus* have a high amino acid concentration (4.3 mM) (Goerke and Wolz, 2010), indicating that metabolite requirements of *S. aureus* change based on the stage of infection. Chaffin and colleagues demonstrated that there is a significant difference in the *S. aureus* transcriptome when grown *in vitro* or introduced into the lungs ofa mouse (Chaffin *et al.*, 2012). In particular, glycolysis was upregulated in the lung infection model, indicating the preference of *S. aureus* to use glucose as a primary carbon source. Overall, the metabolicactivity of *S. aureus* is highly dependent on the environment it is in, and is highly flexible to allow survival in harsh environments such as low-oxygen conditions, due to enabling anaerobic growth dependent on pyruvate(Evans, 1975).

*S. aureus* is extremely efficient in adapting to its conditions, being able to survive even on dry plastic (Coughenour, Stevens and Stetzenbach, 2011). Generally, it is thought that *S. aureus* can tolerate temperatures ranging from 7˚C to 48.5 ˚C, and pH as low as 4.0 and as high as 10.0 (Schmitt, Schuler-Schmid and Schmidt-Lorenz, 1990). However, the optimum growth windows are much narrower, with 30˚C to 37˚C temperature and 6.0-7.0 for pH. Moreover, it has remarkable ability for halotolerance, being able to endure salt solutions of up to 25% w/v (Valero *et al.*, 2009).

## The *S. aureus* bacterial cell wall

*S. aureus,* being a Gram-positive bacterium is surrounded by a thick layer of peptidoglycan, allowing it to withstand internal turgor pressure (Silhavy, Kahne and Walker, 2010). Wall teichoic acids (WTA), composed of anionic polymers span the thickness of the peptidoglycan and are covalently attached to it (Brown, Santa Maria and Walker, 2013). Another abundant cell wall component is lipoteichoic acid (LTA), a polymer with a lipid anchor in the bacterial membrane (Percy and Gründling, 2014), and together with WTA constituting around 60% of dry weight of the Gram-positive cell wall. There are also a multitude of proteins in the cell wall, with their composition constantly changing, dependent on current environmental factors (Pollackt and Neuhaus, 1994).

### *S. aureus* peptidoglycan

Peptidoglycan is a major cell wall component of all Gram-positive bacteria, with the sugar (disaccharide) backbone consisting of alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) residues. However, the peptide stem, attached to every MurNAc varies between bacteria. In the case of *S. aureus,* the peptide stem is composed of L-alanine, D-isoglutamic acid, L-lysine and D-alanine, with a pentaglycine bridge acting as a linking peptide between glycan strands (Vollmer, Blanot and De Pedro, 2008).Of note, many Gram-positive bacteria have a branched peptide stem, whereas *B. subtilis* does not, with its peptide stem closely matching that of *E. coli* (Figure 1.1)(Silhavy, Kahne and Walker, 2010)*.*

Peptidoglycan needs to be constantly degraded and re-built during cell growth and division, and there are currently 4 known classes of hydrolases, based on what bond they cleave. The amine bond between MurNAc and the first stem peptide L-alanine is hydrolyzed by N-acetylmuramyl-L-alanine amidases, essentially separating the conserved sugar backbone from the stem peptides. The glycosidic bonds between MurNac and GlcNAc are broken by N-acetyl-β-D-muramidases (muramidases) and between GlcNAc and MurNac by N-acetyl-β-D-glucosaminidases (glucosaminidases) (Figure 1.1). Amine bonds between amino acids in the stem peptide are cleaved by endopeptidases (Vollmer, Blanot and De Pedro, 2008),for instance lysostaphin that specifically cleaves the glycine-glycine bond in the *S. aureus* cross-linking peptide. However the main autolysin (peptidoglycan hydrolase) in *S. aureus* is Atl, which has both amidase and glucosaminidase activity.This hydrolase is important in cell wall turnover and cell separation after division (Foster, 1995).

|  |
| --- |
|  |
| Figure 1.1 Structure of Gram-positive and Gram negative peptidoglycan  Sites of peptidoglycan digestion dependant on enzyme used (dashed line). Minimal active peptidoglycan structure muramyl dipeptide (MDP) highlighted in the dashed box.  Adapted from (Fournier and Philpott, 2005). |

### Modification of *S. aureus* peptidoglycan

As peptidoglycan is abundant of the cell surface of Gram-positive bacteria, it is a common target for the host immune system. For example, alveolar type II epithelial cells of the lung produce lysozyme to combat *S. aureus* colonization (Nash *et al.*, 2006; Herbert *et al.*, 2007). However other studies have argued that lysozyme kills bacteria via an enzymatic-independent mechanism, as even denatured lysozyme exhibited bactericidal activity (Ibrahim and June, 1996).

Gram-positive organisms, unlike Gram-negative bacteria, do not have an outer membrane to protect them against host enzymes. Therefore *S. aureus* has developed the ability to modify the peptidoglycan layer in such a way, that host lysozyme cannot recognise it. Not only does this prevent the bacterial cell from lysing, but it also limits the amount of bacterial components the host can recognise and in such a way decrease the overall immune response (Davis and Weiser, 2011). *N*-deacetylation of GlcNAc residues on *B. anthracis* has been shown to provide this bacteria with resistance to lysozyme, moreover *N*-acetylating *B. anthracis* caused it to become lysozyme sensitive (Zipperle, Ezzell and Doyle, 1984).

One of the first proteins identified that impacts peptidoglycan modification is *O*-acetyltransferase (OatA), capable of acetylating MurNAc and causing resistance to lysozyme in *S. aureus* (Bera *et al.*, 2006; Sychantha *et al.*, 2017). What is more, the same studies have shown that addition of OatA to lysozyme-sensitive strains of *S. aureus* was enough for the strain to be completely resistant to this peptidoglycan hydrolase. Interestingly, the modification of bacterial peptidoglycan by OatA also results in the bacteria being able to evade killing within the phagolysosome, as well as inhibiting IL-1β production, as for this cytokine to be activated it is essential for peptidoglycan to be digested (Shimada, Park, Andrea J. Wolf, *et al.*, 2010a). Homologues of OatA have been discovered in various Gram-positive bacterial species, such as *S. pneumoniae*, *E. faecalis* and *L. lactis* (Hébert *et al.*, 2007; Veiga *et al.*, 2007)*.*

## *S. aureus* virulence

Bacterial virulence factors are largely defined as microbial genes and their products that are specifically required for infection (Casadevall and Pirofski, 1999).Expression of these factors influence *S. aureus* pathogenicity and can roughly be grouped into three categories. The first group of virulence factors are adhesins, essential for the colonizationof *S. aureus* in the host; second group are mainly exoproteins that cause damage to the host and allow dissemination and proliferation; the final group involves factors responsible for immune evasion.However, many virulence factors can play different roles, depending on the current stage of infection. For instance Protein A (SpA)can upregulate cytokines at sites of infection through binding to TNFR1 and in such as way increase airway epithelial damage during *S. aureus* –induced pneumonia(Gómez *et al.*, 2004). What is more it is also capable of triggering an anti-inflammatory response by activating TNF converting enzyme of the lung epithelial cells, leading to the cleavage of TNFR1 from the cell surface and therefore reducing the amount of TNF signalling and decreasing the overall inflammatory response (Gómez, Seaghdha and Prince, 2007).

A major group of virulence factors of note are MSCRAMMs (Microbial Surface Components Recognising Adhesive Matrix Molecules), which are essential for adhesion of *S. aureus* to host tissues (Foster *et al.*, 2014).ClfA (Clumping Factor A) was one of the first to be identified and since has been shown to bind fibrinogen (Scully *et al.*, 2015), and a pre-clinical murine model has shown a decrease in mouse mortality when treated with antibodies against ClfA.). MSCRAMM proteins are often referred to having a dock, lock and latch mechanism when binding ligands, as docking triggers an altered conformation of the MSCRAMM, ultimately resulting in in a ‘latch’ formation(Foster *et al.*, 2014). However, this mechanism of action is not enough for a protein to be described as an MSCRAMM, as its binding mechanism must also rely on subdomains containing IgG-like folds (Deivanayagam CC *et.al.,* 2002). Other notable members of the *S. aureus* MSCRAMM group are Clumping-factor B (ClfB), thought to be responsible for nasal colonization and adherence (Ganesh VK *et al.,* 2011), Fibronectin-binding proteins A and B (FnBPA,B) that are responsible for tissue invasion and adhesion (Sinha B *et al.,* 2000), as well as Protein A, capable of interfering with opsonisation (Lambris JD *et al.,* 2008). Importantly, it has been shown that attachment of MSCRAMMs and other cell surface proteins to peptidoglycan is a requirement for successful infection of the host by *S. aureus* (Mazmanian *et al.,* 2001). The bond between cell wall proteins and peptidoglycan is facilitated by sortase, an enzyme capable of linking the carboxyl group of threonine to the amino group, typical for peptidoglycan cross-bridges (Mazmanian *et al.,* 2001). Interestingly the *S. aureus* sortase mutant *srtA* is up to 1000 times less effective in forming renal abscesses (Mazmanian *et. al,* 2000), as well as having a notable decrease in the ability to adhere to and invade host tissues (Bolken *et al.,* 2001)

*S. aureus* membrane damaging polypeptides allow for the lysis of host leukocytes and blood cells (Julianelle, 1922), and include haemolysins (α-,β-,γ- toxin), leukocidinsand cytolytic peptides (Vandenesch, Lina and Henry, 2012). While their functions may generally overlap, they are expressed at different stages during infection to target specific cell types. α-haemolysin was one of the first toxins to be described and has been shown to have cell type specificity, as it forms pores in human lymphocytes and monocytes, but not granulocytes, where binding takes place but insertion of the pore-forming proteins into the membrane is perturbed (Valeva *et al.*, 1997). This cell specificity is thought to be governed by ADAM-10, acting as a receptor for α-toxin and the subsequent oligomerization of caveolin-1 to ADAM10 and triggering a conformational change allowing the toxin to enter the host cell and ultimately cause lysis (Wilke and Wardenburg, 2010). Interestingly, studies have shown that α-toxin is important for *S. aureus* infection and bacterial dissemination in healthy individuals, as USA300 *S. aureus* isolates affecting healthy people express the α-toxin in high amounts, while recurrent hospital acquired strains have mutations preventing α-toxin formation (Montgomery, Boyle-Vavra and Daum, 2010; DeLeo *et al.*, 2011).

In contrast to α-toxin, several other *S. aureus* toxins require two different polypeptides to form pores on host cell surfaces and include γ-haemolysin (Cooney *et al.*, 1993), Panton and Valentine (PVL) (Adler *et al.*, 2006), LukED (Reyes-Robles *et al.*, 2013)and LukGH (Janesch *et al.*, 2017). Recently, LukGH has been shown to be present on thebacterial cell surface during late exponential phase of growth, despite not being anchored in the membrane, and is thought to contribute to lysis of cells by *S. aureus* following phagocytosis (Ventura *et al.*, 2010).

*S. aureus* possesses a plethora of strategies to subvert detection and killing by the host immune system,from hampering initial detection to evade rapid clearing, to dampening chemotaxis and disrupting adhesion of neutrophils (De Oliveira, Rosowski and Huttenlocher, 2016). Several virulence factors and strategies have been identified, often expressed at particular stages of infection and targeted to specific immune cells, and are summarised in Table 1.1.

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| **Strategy** | **Virulence factors** | **Source** |
| Prevention of neutrophil migration and prevention of neutrophil activation | CHIPS (Chemotaxis inhibitory protein), FPLR1 (Formylpeptide receptor like-1), staphopain A, Staphylococcal superantigen-like (SSLs), SELX (staphylococcal enterotoxin-like toxin X) | (De Haas *et al.*, 2004), (Prat *et al.*, 2009), (Laarman *et al.*, 2012),(Bestebroer *et al.*, 2010), (Fevre *et al.*, 2014) |
| Evasion of complement- and immunoglobulin -induced phagocytosis | SCIN (Staphylococcal complement inhibitor), Efb (extracellular fibrinogen binding-like), Protein A, Spa (capsule formation) | (Rooijakkers *et al.*, 2006), (Lee *et al.*, 2004),(Forsgren and Sjöquist, 1966),(Nanra *et al.*, 2013) |
| Evasion of intraphagosomal killing | LPG (lysylphosphatidylglycerol synthase), Staphylokinase, superoxide dismutases (Sods) | (Peschel *et al.*, 2001),(Jin *et al.*, 2004), (Papayannopoulos *et al.*, 2010), (Karavolos *et al.*, 2003) |
| Persistence | FnBP(fibronectin binding protein), downregulation of virulence factors, SCV (small colony variant) phenotype | (Tuchscherr *et al.*, 2011), (Von Eiff *et al.*, 2005) |
| Release of bacterial toxins | SEs(staphylococcal enterotoxins), TSST-1 (staphylococcal toxic shock syndrome toxin) , staphylococcal enterotoxin-like toxins (SEIs) | (Silversides, Lappin and Ferguson, 2010) |

Table 1.1 Examples of immune evasion strategies used by *S. aureus*

### Regulation of *S. aureus* virulence factors

As outlined above, *S. aureus* expresses an large array of virulence factors, depending on the stage of infection as well as surrounding immune cells. Generally, there are two stages in *S. aureus* infection : adhesion, during which cell wall virulence factors are upregulated to attach to host tissues, while during the invasion phase secreted factors, such as toxins and hemolysins increase and cell-wall associated ones are downregulated (Dinges, Orwin and Schlievert, 2000; Kong, Vuong and Otto, 2006). The switch between these two cycles depends on the bacteria being able to sense the population density around them and produce autoinducer(AIs) molecules (Rutherford and Bassler, 2012). Accumulation of AIs triggers the quorum-sensing process, allowing a population of *S. aureus* bacteria to produce virulence factors in concert, as well as further increasing the production of AIs in a positive feedback loop (Williams and Cámara, 2009).

In *S. aureus* the QS system is controlled by the *agr* accessory gene regulator (Novick *et al.*, 1995) and involves a peptide signal. In short, the Auto Inducing Peptide (AIP) is sensed by AgrC in the bacterial membrane, causing it to autophosphorylate and subsequentlyactivate cytosolic AgrA by transferring the phosphate group. This in turn causes activation of the *agr* operon RNAII and RNAIII, with RNAIII being essential for the upregulation of α-toxin production and downregulation of cell-surface virulence factors such as protein A (Morfeldt *et al.*, 1995). Moreover, RNAIII represses *rot* (repressor of toxins), a well-known repressor of toxin production by the bacteria (Geisinger *et al.*, 2006).Several studies have demonstrated the necessity of *agr* for successfully infecting a host. For instance, Gong *et al*., has shown that mice deficient in *agr* had a significantly reduced amount of intracranial abscesses during *S. aureus* infection.

Apart from *agr*, 20 core regulators of *S. aureus* virulence have been identified, as well as some of the interactions between them (Figure 1.2). However, not all regulators are expressed, with different *S. aureus* strains carrying combinations of them, as well as sequence variation in the genetic codes, leading to significant alterations between bacterial strains (Priest *et al.*, 2012). However SarA (staphylococcus accessory regulator) family of homologues has been shown to be conserved throughout strains, and such stringent regulation points at its key role in virulence control (Manna, Bayer and Cheung, 1998; Blevins *et al.*, 1999). While SarAhas been shown to increase levels of RNAII and RNAIII therefore influencing *agr* activity, it also acts in an *agr*-independent way through sensing of low O2 or high CO2levels (Chan and Foster, 1998). A change in the bacterial environment results in the upregulation of α- and β-hemolysin production as well as TSST-1 upregulation through association with the *tst* promoter (Andrey *et al.*, 2010).

Several of the virulence regulators are capable of taking on the functions of each other, for instance *saeRS* has been shown to decrease expression of proteases during infection (Mrak *et al.*, 2012) and it has been shown that it is capable of rescuing a *sarA* attenuated mutantin a murine model of biofilm formation (Beenken *et al.*, 2014). However, both Agr and SarA are important for invasive disease, as shown in a mouse model of pneumonia (Heyer *et al.*, 2002), where a knockout of either factor resulted in *S. aureus* being unable to invade the host, demonstrating that time-dependent activation ofvirulence regulators is crucial for a successful colonization of the host.

Virulence factors believed to be essential during pathogenesis have been used as therapeutic targets and include *agr* and *sarA*, as mutants are attenuated (Cheung *et al.*, 1994; Dunman *et al.*, 2001). The small molecule savarin (*S. aureus* virulence inhibitor) successfully inhibits the function of AgrA and therefore downregulates genes often associated with *S. aureus* skin infections such as hemolysins, lipase and protease (Sully *et al.*, 2014). When savarin was co-injected with *agr+**S. aureus* in a skin infection model, bacterial clearance after 24h was significantly higher. Moreover, there were considerably fewer abscesses present on the kidneys of infected animals. Using savarin as an alternative to antibiotics in tissue infection was also proposed, as secondary treatment with savarin did not result in tolerance to this compound.

While SarA is mostly known for its interaction with *agr,* it is also capable of independently causing the upregulation of toxins such as TSST1 and various biofilm associated proteins (Andrey *et al.*, 2010; Arciola *et al.*, 2012). Arya *et al.* have demonstrated that a SarA inhibitor (SarABI) binds with high affinity to bacterial SarA and therefore interferes with transcriptional activation of several virulence factors (Arya *et al.*, 2015).Moreover, mice challenged with a high dose *S. aureus* infection showed no biofilm formation on a graft pre-soaked with SarABI.

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| Figure 1.2 Current view of *S. aureus* virulence regulators and their interactions  Outside of circle: Arrow- upregulated, red T- inhibited, question mark- not known). Shows effect of various virulence factor genes on *S. aureus* adhesiveness (A), toxicity (T), immune evasiveness (E). Absence of a line means interaction as not been investigated.  Adapted from (Priest *et al.*, 2012) |

## Antibiotic resistance

Initially the discovery and application of penicillin for *S. aureus* infections brought down mortality rates from 80% in 1937 to 30% in 1944. However, this number once again rose to around 50% mortality ratein 1954, as *S. aureus* strains developed resistance (Dancer, 2007). As such, evolution of *S. aureus* resistance has been characterised as waves, with an antibiotic-resistant strain emerging after the introduction of a novel antibiotic for clinical use (Figure 1.3).

*S. aureus* became resistant to penicillin due to the secretion of beta- lactamase which can cleave the beta-lactam ring and deactivate the antibiotic (Abraham and Chain, 1940; Chen and Herzberg, 2001). Interestingly, beta-lactamase is not constitutively expressed by the bacteria, but rather is upregulated once *S. aureus* carrying a penicillinase plasmid interacts with a beta-lactamthrough the cell surface receptor BlaR1, causing the downstream dissociation of the DNA repressor BlaI from *blaZ* and therefore the transcription of beta-lactamase (Safo *et al.*, 2005). To combat effects of beta-lactamase, methicillin was synthesized, containing methoxy groups on the phenol ring, resulting in resistance to cleavage by beta-lactams (Stapleton and Taylor, 2002). However shortly after introduction of methicillin for clinical use, methicillin-resistant *S. aureus* (MRSA)was isolated with a novel penicillin binding protein PBP2a encoded by the gene *mecA*, allowing for a resistance to all known beta-lactam class antibiotics. Over time, MRSA not only caused significant problems for hospitals worldwide, but is now also a common cause of community acquired infections (CA-MRSA) (Vandenesch *et al.*, 2003).

Vancomycin is a glycopeptide that can inhibit peptidoglycan synthesis (Watanakunakorn, 1984), by binding to the D-alanine-D-alanine termini present in thepeptidoglycan precursors, perturbing cell wall formation*.* In 1997 an MRSA isolated from a patient was shown to have decreased vancomycin susceptibility, termed VISA (vancomycin intermediate-resistant *S. aureus)*, with a minimum inhibitory concentration to vancomycin around 5 µg/ml (Hiramatsu, 1997).Moreover, VISA was shown to be resistant to treatment with general antibiotics (Liñares, 2001). However vancomycin-resistant *S. aureus* (VRSA) was first isolated in 2002, having obtained a *vanA* (vancomycin resistance) gene from *E. faecalis* (Chang *et al.*, 2003)*.*

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| Figure 1.3 Waves of acquired antibiotic resistance by *S. aureus*   1. Wave of resistance to beta-lactams starting from 1940 and continuing to this day, with the first resistant lineage named phage type 80/81 2. Second wave in response to methicillin- MRSA-I, *S. aureus* acquires Staphylococcal chromosome cassette *mec* I *(*SCCmecI). 3. Increase in virulence of hospital MRSA strains, with MRSA-II acquiring SCC*mec*II and MRSAIII SCC*mec*III. 4. *S. aureus* acquires vancomycin resistance (VISA and VRSA) and MRSA moves into the community (CA-MRSA)   The acquisition of the *mecA* gene by *S. aureus* allowed this organisms to become resistant to β-lactam class of antibiotics. Descendant strains possessed further changes in the cassette, often resulting in increased virulence.  Adapted from (Chambers and Deleo, 2010) |

## Role of *S. aureus* phagocytosis in immune signalling

Phagocytosis is a complex process, involving cell surface receptors sensing an antigen and signalling for the formation of the endocytic vesicle, fusion of the phagosome with the endosome in the phagocyte cytoplasm, causing digestion of intraphagosomal antigens and finally their presentation on the cell surface MHC protein (Doyle *et al.*, 2004).

Sensing invading bacteria or their components through TLRs is essential for cytokine production and initiation of immune cascades, and this alone is not enough to deal with a *S. aureus* infection(Takeuchi, Hoshino and Akira, 2000). Besides recognition, a phagocyte must enable conformational changes within its cytoskeleton to allow engulfment of the organism, therefore upon activation of cell surface receptors, downstream signalling causes an upregulation of actin polymerization, allowing the cell to change shape in order to internalise (Weiss and Schaible, 2015). Once inside the macrophage, the phagosome goes through several steps leading to full maturation, with an important step being the acidification of the phagosome with the help of vATPase protein pump (Weiss and Schaible, 2015). This pump allows for a low pH environment, ideal for the functioning of various hydrolases delivered to the phagosome from the Golgi (Weiss and Schaible, 2015).

Remarkably, studies suggest that phagocytosis of Gram-positive bacteria is essential for the generation of an immune response, while this was not essential in the case of Gram-negatives (Ip *et al.*, 2010). Ip and colleagues have used an *in-vitro* assay with human peritoneal macrophages. They showed that when the ability to internalise bacteria by macrophages was blocked by cytochalasin D, heat killed Gram-negative bacteria did not differ in TNF-α stimulation compared to untreated macrophages, while Gram-positive bacteria failed to stimulate production of TNF-α. These experiments support the notion that stimulation of cell-surface receptors is not sufficient for launching an appropriate immune response against *S. aureus* and other Gram positive organisms*.*

While the TLR family is thought to mostly play a role in the activation of various inflammatory cascades, they have also been shown to play a major role in all stages of phagocytosis.In particular, TLR-2 mediated phagocytoses of *E. coli* and *S. aureus* was shown to increase the amount of bacteria taken up by individual phagocytes, as well as increasing overall the amount of phagocytes taking up *E. coli* (Doyle *et al.*, 2004)*.* This amplification of response was shown to happen through TLR2 mediated Myd88 signalling resulting in the upregulation of scavenger receptors, with IRAK and p38 playing important roles in activation of intracellular pathways.Moreover, prolonged stimulation of TLRs such as TLR 4 and 9 resulted in a significantly higher uptake of *S. pneumoniae* as well as increased rate of intracellular killing compared to unstimulated cells (Ribes *et al.*, 2010). Notably, persistent stimulation of TLR3,4 and 9 results in the upregulation of FCγR responsible for binding antibodies attached to antigens, as well as scavenger receptors (Pincetic *et al.*, 2014).

However, not all TLRs initiate phagocytosis in the same way, with TLR9 thought to elicit the greatest response and TLR3 the weakest (Doyle *et al.*, 2004). What is more, it isbelieved that immune activation is triggered after the pathogen has been phagocytosed, as TLRs recognise the contents of the phagosome, ratherthan merely due to the process of phagocytosis. TLR2 is capable of signalling differently in response to Gram-positive and Gram-negative bacteria (Underhill *et al.*, 1999).

The inflammasome is an essential cell component that reacts to intracellular infection by pathogens such as *S. aureus* (Craven *et al.*, 2009)*.* Inflammasomes are formed in response to NOD – like receptor (NLR) signalling, with each response slightly different dependent on the pathogen encountered. *S. aureus* is thought to be sensed by NLP3 and this causes the formation of a multi-protein unit, composed of NLP3, adaptor apoptosis-associated speck-like protein containing a CARD (ASC), and caspase (Latz, 2010). The assembled and activated inflammasome can be potentially dangerous to the host cell, as it is able to trigger not only cell death, but also excessive release of pro-inflammatory cytokines, as seen in some autoimmune disorders where the inflammasome is persistently active (Agostini *et al.*, 2004).

It is thought that two signals are required for the activation of NLP3 inflammasome. Firstly, TLR 2 or 4 sense a microbial component and signal through Myd88 to initiate the transcription of pro-IL-1β (McGilligan *et al.*, 2013). A second signal, possibly ROS activation, causes the activation of the inflammasome and activates caspase-1. Finally, pro-IL-1β is cleaved by caspase-1 to produce the mature and active IL-1β (McGilligan *et al.*, 2013).

Both *S. aureus* and its cell wall peptidoglycan have been shown to trigger inflammasome activation and production of IL-1β (Wang, Liu and Dziarski, 2000; Mariathasan *et al.*, 2006). To determine whether *S. aureus* peptidoglycan needs to be polymeric, Shimada and colleagues used mouse bone-derived macrophages (MDBMs) and incubated them with polymeric peptidoglycan, digested peptidoglycan and MDP. Result showed that only polymeric peptidoglycan was capable of IL-1β production by macrophages, and suggests phagocytosis of peptidoglycan is essential for inflammasome activation. It is thought that once peptidoglycan is digested within the phagosome this is seen as the second inflammasome activation signal. This was shown to be true, as *S. aureus* which areresistant to lysozyme degradation failed to stimulate IL-1β production, and therefore highlighted that *S. aureus* is capable of peptidoglycan modification to avoid host immune responses.

## Immune protection against *S. aureus* infection

### Innate immune system

The innate immune system is composed of anatomical barriers, chemicals and cells that protect the host from pathogenic bacteria and other toxins that could impact normal functioning.

The first lines of defence against any type of infection are the intact skin and mucosal barriers of the body, including the gastrointestinal and respiratory tracts. Epithelial cells form tight junctions (TJs), that not only act as a barrier to environmental impacts such as bacteria (Anderson and Van Itallie, 2009), but are also capable of dynamically adjusting to cater for physiological needs such as the transport of ions as well as translocation of dendritic cells (Kubo *et al.*, 2009). The mucous layers of both the respiratory and gastrointestinal tracts contain antimicrobial peptides (AMP’s)such as alpha- defencins, lysozyme (Dupont *et al.*, 2015) and cathelicidins (Agerberth *et al.*, 1999). For instance, cathelicidin hCAP-18 is released by epithelial cells in response to inflammation, and leads to the generation of LL-37 peptide, known for its anti- staphylococcal properties (Sørensen *et al.*, 1997).

Due to the controlled chemical composition of the host skin, only a certain diversity of commensal bacteria is able to successfully colonise it without causing inflammation (Lai *et al.*, 2009). This becomes even more important in the occurrence of skin injury, where an upregulated inflammatory response to skin damage could be damaging to overall tissue repair.

However, *S. aureus* has developed an array of strategies to bypass all the initial innate immune defences. For instance, it employs adhesion tactics, such as interactions between staphylococcal wall teichoic acids (WTAs) and SREC-I, a F-scavenger receptor normally found on nasal epithelial cells (Baur *et al.*, 2014). Adhesion allows for the initial step of bacterial colonization and may be critical in the initiation of skin infections, and therefore *S. aureus* has been shown to possess several different mechanisms for adhesion. Another example is *S. aureus* Clumping Factor B (ClfB) attaching to loricin, a known protein of the squamous epithelial cells, present in the nares of humans and mice (Mulcahy *et al.*, 2012). In this study *lor-/-* mice were significantly less susceptible to colonisation by *clfB+* *S. aureus,* as well as *clfB-* *S. aureus* being less effective in colonising the nares of wild type mice.It was therefore suggested that ClfB can be important in adherence of *S. aureus* to skin corneocytes, as atopic dermatitis (AD) patients have an increased incidence of skin colonisation by *S. aureus* (Fleury *et al.*, 2017)*.* Corneocytes of patients suffering from AD have an altered morphology due to skewedlevels of filagrin, leading to the formation of a cornified structures on the cell (Riethmuller *et al.*, 2015), resulting in *clfB+*bacteria attaching to these structures with higher affinity than to healthy corneocytes. Other ligands that are known to bind and promote *S. aureus* adhesion are known and summarised in Figure 1.4.

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| Figure 1.4 *S. aureus* adhesion strategies  **(A)** Clumping factor B (ClfB) and Iron-regulated surface determinant A (IsdA) bind to loricin, involucrin and cytokeratin 10, while wall teichoic acids (WTAs) bind to epithelial receptor SREC-I, on squamous epithelial cells. **(B)** Fibronectin on epithelial cells provides a binding site for fibronectin binding proteins A and B (FnBPs) and triggers activation of α5β1 integrin, ultimately allowing tissue invasion. Phagocytosis of *S.aureus* is thought to be triggered by ClfA interaction with cell-surface annexin A.  Adapted from (Pietrocola *et al.*, 2017) |

#### Complement system

In the initial stages of bacterial colonisation and adhesion, the immune complement system is one of the early obstacles in the way of *S. aureus.* It is composed of plasma proteins, mostly synthesized by hepatocytes in the liver (Alper *et al.*, 1969), that activate each other in an enzymatic cascade. This cascade could be activated by three distinct pathways: classical (CP), lectin (LP) and alternative pathways (AP), with the end goal being opsonisation of antigens, providing chemotactic signals for immune cells and lysis of unwanted cells through formation of the membrane attack complex (MAC) (Lubbers *et al.*, 2017). Each arm of the complement system recognises different cues of bacterial presence, such as opsonised bacteria trigger activation of the CP, mannosepresent in bacterial cell wall trigger the LP and the AP is triggered by presence of LPS or through auto-generation of C3b, called tickover (Thurman and Holers, 2006). A detailed schematic representation could be seen in Figure 1.5, and it is important to note that despite different initial mechanisms of pathway activation, they all converge on the formation of C3 convertase, that is then cleaved into C3a that acts as a chemoattractantand C3b acts as an opsonin. Once C3b amounts reach a certain threshold, they are able to bind to the C3 convertase and this initiates the formation of C5 convertase, that cleaves C5 and C5a, producing C5b and thereby recruiting C6, C7, C8 and 16 molecules of C9 to create the MAC (Lubbers *et al.*, 2017). Importantly, *S. aureus* is capable of activating all three arms of the complement system (Verbrugh *et al.*, 1982).

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| Figure 1.5 Production of complement components by cells of the immune system  The proteins and enzymes involved in classical, lectin and alternative pathways. FB- factor B; FP -factor P; FD-factor D; FH-factor H; C1INH - C1 inhibitor; FIfactor I -(FI), C4BP 5 C4b-binding protein; CR- complement receptor. Inhibitors of the complement pathways are marked in red boxes. (Lubbers *et al.*, 2017) |

##### Interaction between different arms of the complement system and *S. aureus*

Overall studies have shown that mice lacking the ability to activate all three complement pathways are unable toclear bacterial infection and this results in 100% mortality (Celik *et al.*, 2001). Further research has dissected each individual pathway and determined their role in bacterial clearance.

The classical pathway is activated once C1q binds to ligands such as immunoglobulin M (IgM) and petraxins, already bound to *S. aureus* (Beurskens, van Schaarenburg and Trouw, 2015)*.* Attachment to the Fc region of the antibody causes C1q recognition subcomponent to activate the serine proteases C1r and C1s. While it has been thought that the activation of both CP and AP is essential for successful bacterial clearance, studies have shown that CP -/- mice are completely overwhelmed by sepsis at 6 h post infection (hpi), whereas AP knockout mice (fD-/-) successfully manage bacterial numbers at the same time point (Dahlke *et al.*, 2011). What is more, while 24 hpi fD-/- mice did not manage to clear bacterial numbers as efficiently as WT mice, they were not overwhelmed by the infection.Therefore it is of no surprise that *S. aureus* has several strategies to evade immune activation via the classical pathway. For example, the VraXprotein is secreted by *S. aureus* and is capable of binding to C1q, therefore preventing the activation of C1r and C1s (Yan *et al.*, 2017). The lectin pathway is reliant on the classical pathway in that both need to be active for formation of C4bC2a, a C3 convertase. It has been shown that *S. aureus* produces the Eap proteincapable of binding C4b, efficiently inhibiting binding of C2 and in that wayinhibiting both the CP and LP arms of the complement system (Woehl *et al.*, 2014).

*S. aureus* is also capable of inactivating the alternative pathway through staphylococcal complement inhibitors SCIN-A and SCIN-B (Summers *et al.*, 2015). These proteins interfere with C3bBb formation by binding to C3b.

While some staphylococcal proteins target specific arms of the complement system, the majority of anti-complement factors target the central components C3 and C5, thereby suppressing opsonisation, amplification and chemokine signalling. For instance, staphylococcal superantigen – like protein 7 (SSL7) binds C5 (Langley *et al.*, 2005). *S. aureus* chemotaxis inhibiting protein (CHIPS) is capable of binding to C5aR on the surface of neutrophils, successfully blocking chemotaxis (De Haas *et al.*, 2004). Finally, Sbi (*S. aureus* binder of immunoglobulin)protein interacts with the C3 component C3dg, thereby preventing C3b-opsonised *S. aureus* attaching to CR1 on macrophages and red blood cells, effectively blocking not only phagocytosis but also transport of the opsonized bacteria to liver Kupffer cells (Burman *et al.*, 2008). The same study has shown that Sbi causes consumption of complement, as it forms covalent attachments with activated C3b, without the downstream complement activation.

#### Binding of pathogens to host immune cell receptors.

Bacteria and their components are recognised by the host immune system through common pathogen-recognition molecular patterns (PAMPs) that bind to pattern recognition receptors (PRRs), causing pro-inflammatory signalling or phagocytosis (Mogensen, 2009). Several cells of the innate immune system are capable of phagocytosis, such as organ-specific macrophages, neutrophils, monocytes, natural killer T-cells and dendritic cells. These cells usually mediate the immediate and non-specific response to bacteria (Figure 1.6). Once the bacteria are inside the phagocyte,one of the outcomesis digestionwithin the phagolysosome and antigen components are presented to cells of the adaptive immune system (Gregersen and Behrens, 2006).

Toll-like receptor is one of the most studied PPRs and was initially discovered in *Drosophila melanogaster* and shown to be essential in antifungal immune response (Lemaitre *et al.*, 1996). TLRs are transmembrane domains,with an extracellular ligand binding and a cytoplasmic domain, that activates intracellular signalling cascades (O’Neill and Bowie, 2007). There are 10 distinct TLRs in mammals (Figure 1.7), each capable of recognising specific molecules but eventually leading to recruitment of immune cells as well as upregulation of pro-inflammatory cytokines. Not all TLRs can be found in the same region of the cell, with TLR 1,2,4,5,6, 10 mostly expressed on the cell surface, and TLR- 3,7,8,9 mainly seen on endosomes and lysosomes. This allows for cell surface TLRs to recognise bacterial surface components, while intracellular TLRs recognise specific nucleic acids (Iwasaki and Medzhitov, 2004). An important function of all TLRs is that they are capable of recognising multiple ligands through dimerization with other TLRs. For instance, bacterial lipoproteins aresensed by TLR2, and initiates formation of dimers with either TLR1 or TLR6 (Jin *et al.*, 2007; Kang *et al.*, 2009), resulting in the proximity of TIR domains and commencement of downstream signalling. TLRs are also capable of binding to other molecules to facilitate immune reactions. For example bacterial liposaccharide is recognised by myeloid differentiation factor 2 (MD2) that is non-covalently attached to TLR4 (Lu, Yeh and Ohashi, 2008). This causes the dimerization of the MD2/TLR4 complex and downstream signalling.

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| Figure 1.6 Interaction between the innate and adaptive immune systems.  Various interactions between the innate and adaptive arms of the immune system.  PRR- pattern recognition receptors; TLR- toll like receptor; complement proteins (C1q);mannose-binding protein (MBP); c-reactive protein (CRP), T-cell receptors (TCRs);tumour necrosis factor (TNF); interleukin-1 (IL-1); antigen presenting cell (APC); B-cell receptor (BCR).  Adapted from (Gregersen and Behrens, 2006) |

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| Figure 1.7 Known mammalian TLRs and their signalling pathways  A detailed diagram of all currently known interactions between TLRs and their signalling pathways. Toll like receptor (TLR); Toll-IL-1-resistance domains (TIR); myeloid differentiation primary response protein 88 (Myd88); Myd88-adapter-like protein (MAL); TIR domain-containing adaptor protein inducing IFNβ (TRIF); TRIF-related adaptor molecule (TRAM); IL-1R associated kinase (IRAK); TNF receptor associated factors (TRAFs); mitogen activated protein kinase (MAPK); interferon regulated factors (IRFs);cyclic AMP responsive element binding protein (CREB).  Adapted from (O’Neill, Golenbock and Bowie, 2013) |

### Recognition of peptidoglycan by the host immune system

Bacterial components including peptidoglycan can be sensed by pattern recognition receptors (PRRs). The two major classes of PRRs described in the literature for sensing *S. aureus* are TLRs and Nod-like rectors (NLRs). TLR2 has been shown to recognise Gram-positive bacterial components such as lipoproteins and lipoteichoic acids (Underhill *et al.*, 1999; Trianiafilou *et al.*, 2004) and TLR2-/- mice are much more susceptible to *S. aureus* infection compared to wild-type mice (Takeuchi, Hoshino and Akira, 2000). Interestingly, it has long been thought that peptidoglycan is the ligand for TLR2 (Dziarski and Gupta, 2005), however It was later proven that commercially available peptidoglycan is contaminated by lipoproteins and lipoteichoic acids and it is indeed lipoproteins that signal in a TLR2/1 dependant manner (Travassos *et al.*, 2004a; Trianiafilou *et al.*, 2004).

Nod1 and Nod 2 are currently thought to be the main intracellular receptors shown to recognise and bind to peptidoglycan, and though they are structurally similar, they recognise different types of peptidoglycan.Nod1 is known to be present in all cell types and is responsible for sensing the m-DAP portion of Gram- negative peptidoglycan (Figure 4.3)(Inohara *et al.*, 1999; Chamaillard *et al.*, 2003)*.* Nod2 has been shown to be present *in vivo* in phagocytes such as monocytes, macrophages and neutrophils and some studies have also shown it to be expressed *in-vitro* in epithelial cells stimulated by LPS (Gutierrez *et al.*, 2002). Nod2 is capable of sensing intracellular Gram-positive and Gram- peptidoglycan, with MDP(Figure 1.1) being the smallest peptidoglycan fragment that could be sensed(Girardin *et al.*, 2003). The end result of Nod-activation is production of pro-inflammatory cytokines through upregulation of transcription by NF-κB, as well as activation of the ERK and JNK pathways. For instance Nod1 has been shown to cause production of IL-8 chemokine by signalling through the JNK pathway (Ryu *et al.*, 2017), while Nod2 can signal through the ERK pathway (Kobayashi *et al.*, 2005).

MDP occurs naturally due to the remodelling of bacterial cell wall during cell division and growth (Girardin *et al.*, 2003), as well as through proteolysis of polymeric peptidoglycan inside phagocytic cells. It is unknown how MDP is taken up into cells, as it is believed to be too small for phagocytosis, and pinocytotic uptake has been suggested as an alternative(Rejman *et al.*, 2004). Another mechanism of MDP uptake has been proposed by Vavricka *et al.,* via the transporter hPepT1 in the colon (Vavricka *et al.*, 2004)*.* However it is important to note studies showing uptake of MDP by various phagocytic cells often first prime them with LPS, resulting in a reaction profile that may not necessarily occur under physiological conditions (Sugiyama *et al.*, 1996; Monodane, Kawabata and Takada, 1997a; Yang, Tamai, *et al.*, 2001). Therefore the most widely accepted version of the route of MDP into a phagocytic cell is through the uptake of a bacterium by such cells and subsequent digestion of the polymeric peptidoglycan by various host hydrolases, resulting in the formation of MDP (Iyer and Coggeshall, 2011a).

Moreover, it is generally thought that during *S. aureus* infection there is an interplay between NOD and TLR systems.While polymeric peptidoglycan is not regarded to be sensed by TLR2 (Travassos *et al.*, 2004a), studies have shown that in mouse keratinocytes, polymeric peptidoglycan co-localizes with both Nod2 and TLR2, and that knock-out of either of these receptors causes a 50% drop in peptidoglycan-induced cytokine production, suggesting that Nod2 and TLR2 act synergistically and amplify the immune response (Müller-Anstett *et al.*, 2010).

### Other components of the Gram-positive cell wall recognised by the host immune system

Lipoteichoic acid (LTA) is abundant in the Gram-positive bacterial cell wall, but is anchored in the membrane via glycolipid anchors, rather than being attached to peptidoglycan. LTA has been implicated in the severity of septic shock in mice (Kengatharan *et al.*, 1998; Ginsburg, 2002), however to date, the main hypothesis lies with LTA not being immunostimulatory and that previous work used LTA samples contaminated by lipoproteins (Hashimoto, Tawaratsumida, Kariya, Kiyohara, Suda, Krikae, Kirikae and Götz, 2006).

Lipoproteins of the *S. aureus* cell wall attach to the outer leaflet of the bacterial membrane and span the peptidoglycan layer (Mazmanian, Ton-That and Schneewind, 2001). They have also been shown to play an essential role in generating an immune response in the host, as *S. aureus Δlgt* mutants, lacking mature lipoproteins, are unable of immune stimulation in the same extent as wild type strains (Stoll *et al.*, 2005b; Schmaler *et al.*, 2009).

Several mentioned studies have highlighted that peptidoglycan and LTA may be contaminated with lipoproteins, despite the vigorous process of peptidoglycan purification. Therefore currently the only way to isolate *S. aureus* bacterial cell wall components that would not be contaminated by lipoproteins is through the use of a *Δlgt S. aureus* mutant (Shahmirzadi, Nguyen and Götz, 2016). Diacyl-glycerol transferase (Lgt) is essential for the final step of synthesizing pre-lipoprotein into mature lipoprotein (Sankaran and Wu, 1994). Several studies have shown that often immune responses thought to have been in response to peptidoglycan stimulation were actually due to lipoproteins. For example peritoneal murine macrophages failed to produce cytokines in response to stimulation by peptidoglycan purified from an *Δlgt* mutant, emphasizing that it is most likely lipoproteins signalling through TLR’s (Travassos *et al.*, 2004b). Further supporting the notion that lipoproteins are the main immunostimulatory component in contaminated peptidoglycan preparations, is that peptidoglycan isolated from wild-type *S. aureus* caused upregulation of NF-κB levels and therefore an increased cytokine response, whereas lipoprotein-free *S. aureus* peptidoglycan did not (Volz *et al.*, 2010).Interestingly, it has also been shown that LTA isolated from an *Δlgt S. aureus* mutant was 100 times less potent in TLR2 activation than the wild-type*.* Of note, while this study suggests that lipoproteins and not LTA are the prime stimulant of TLR2, the group did not manage to isolate completely inactive LTA, suggesting that either LTA is a weak agonist of this receptor or perhaps there are other contaminants in the preparation that are capable of activating TLR2 (Hashimoto, Tawaratsumida, Kariya, Kiyohara, Suda, Krikae, Kirikae and Gotz, 2006).

Finally, the majority of studies point to the necessity of using cell wall materials derived from bacteria carrying the *Δlgt* mutation, to avoid speculation on whether the observed effect is simply due to lipoprotein contamination.

#### Cytokine response

One of the downstream responses to bacterial sensing by TLRs is the production ofpro-inflammatory cytokines. Cytokines are mediators of pathogenesis and trigger an array of host defence mechanisms. For example TNF-alpha and IL-1β have been shown to cause vasodilation, thereby allowing for leukocytes and macrophages to travel to the site of infection (Johns and Webb, 1998). Moreover, other pro-inflammatory cytokines such as IL-1a, IL-1b and IL-6 are capable of triggering the upregulation of adhesion receptors on the cell surface of leukocytes such as P- and E- selectins and intracellular adhesion molecule 1 (I-CAM1), thereby promoting neutrophil rolling and adhesion and allowing the cells to move out of the circulation and to the sites of damage (Figure 1.8) (Ley *et al.*, 2007). Moreover, the production of pro-inflammatorycytokines results in the secretion of several types of chemokines such as CXC-chemokine ligand 1, 2,5 and 8, primarily for the attraction of neutrophils (Kim and Luster, 2015).

The bactericidal activity of neutrophils is heavily dependent on production of superoxide and reactive oxygen species (Figure 1.8), in a process termed oxidative burst, thought to be closely linked to phagocytosis, causing the assembly of NADPH oxidase multi-subunits on phagosomes (Clark *et al.*, 1990; Quinn, Ammons and DeLeo, 2006). The assembled NADPH oxydase transfers electrons from the NADPH in the neutrophil cytosol to intraphagosomal O2 thereby creating superoxide (O2-) (Chen and Junger, 2012). Superoxide leads to the formation of reactive oxygen species (ROS) such as hydrogen peroxide and hypochlorous acid. Together with ROS, several cytotoxic molecules such as azurohilic and specific granules that aid bacterial killing (Falloon, 1986). In particular, azurophilic granules contain myeloperoxidase (MPO), several proteinases such as cathepsin G and lysozyme (Segal, 2005).

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| Figure 1.8 Neutrophil migration to the site of infection and intracellular killing.  Strategy of neutrophil rolling, adherence and migration to the site of infection, as well as mechanisms for intracellular killing of bacteria.  FcR - Fc receptor, binds Fc region of antibodies; CR- complement receptor; ROS- reactive oxygen species; MPO- myeloperoxidase.  (Rigby and DeLeo, 2012) |

Several *S. aureus* cell wall components and secreted proteins are capable of triggering a cytokine response. LTA has been shown to cause secretion of cell-associated interleukin 8 (IL-8), a known leukocyte chemotactic factor, from human peripheral blood monocytes (PBMs) (Standiford *et al.*, 1994). What is more, *S. aureus* is capable of inducing cytokine productions from various different cells, such as IL-8 from epithelial cells, IL-8 and IL6 from endothelial cells, IL6, IL8, IL-1β and TNF-alpha from monocytes, all in the absence of serum (Soell *et al.*, 1995). This suggests a direct interaction between *S. aureus* and receptors on the cell surface. Finally, C57BL/6J mice missing alphabeta T cells-/-, injected with *S. aureus* at a surgical wound site, had significantly less neutrophil recruitment to the affected area, as well as much lower CXC levels compared to WT mice (McLoughlin *et al.*, 2006). Together, this data points to the possible role of T-cells in the production of neutrophil chemokines in response to *S. aureus* infection.

### *S. aureus* and the adaptive immune system

The adaptive immune system is activated once cells of the innate immune system, usually dendritic cells, present antigens to naïve lymphocytes in the lymph nodes (Figure 1.6). This event triggers the production of antigen-specific antibodies by B- and T-cells, that are not only capable of increasing the action of innate immune system cells, but also ‘remember’ the antigen for a quicker response in the case of a repeated infection (Karauzum and Datta, 2016). It is thought that B-cells are responsible for antibody –mediated adaptive immune response, while T – cells deal with cell-mediated responses (Girardi, 2007)

Activated B cells produce immunoglobulins that are known to have 2 major functions: they either disable the action of the antigen by binding to it, or flag up antigens through opsonisation, making the antigen more visible to macrophages. In this way, both the adaptive and innate immune system collaborate for the clearance of infection (Murphy *et al.*, 2012). Interestingly, patients lacking an appropriate B-cell response due to suffering from X-linked agammaglobulinaemia have not been reported to be more susceptible to *S. aureus* infections (Bruton, 1952). Moreover, mouse experiments where animals lacked B cells did not result in an increased mortality rate in response to *S. aureus* infection, even when B cells were re-activated during infection (Gjertsson *et al.*, 2000; Schmaler *et al.*, 2011).

T-cells are produced in the thymus and have specific T-cell receptors for binding to antigens. There are several different subsets of T-cell, with CD4+ T-cells being one of the most abundant. The further specification of these cells depends on the amount of a specific cytokine when the TCR of the T-cells is activated by an antigen. For instance, TH1 are generated through STAT4 signalling in the presence of IL-12, and this differentiation allows the TH1 cell to produce the cytokine interferon-gamma (IFN- γ) (O’Shea and Paul, 2010). There are conflicting studies in regards to the effects of TH1 cell on *S. aureus* pathogenesis. Some studies claim that in transgenic mice expressing the human lactoferrin protein (involved in T-cell polarization) and suffering from *S. aureus* induced septic arthritis, had an increased survival rate compared to WT mice due to an increase in TH1 amounts (Guillén *et al.*, 2002). Moreover, in a *S. aureus* surgicalwound model of infection, TH1 cells increased the amount of neutrophils at the site of infection and this subsequently resulted in increased tissue bacterial burden, most likely due to the ability of *S. aureus* to infiltrate host neutrophils (McLoughlin *et al.*, 2008). TH2 cells are activated under the influence of IL-4 and mostly produce transcription factor GATA-3, essential in the clearance of extracellular parasite infections (Allen and Sutherland, 2014). TH2 cells have also been shown to produce IL-10 and IL-4 and increase of these cytokines in patients with atopic dermatitis (AD) has been linked to an increased susceptibility to *S. aureus* skin colonization (Bieber, 2010).Upon the decrease of an active immune response following elimination of infection, T-cells undergo apoptosis (Lohman and Welsh, 1998).

Despite the wealth of information on the interactions between the adaptive immune system and *S. aureus,* it is still unknown why recurrent infections are common, despite interaction of this bacteria with B- and T-cells (Montgomery *et al.*, 2014). It is thought that because *S. aureus* is part of the microflora of many people, both bacteria and human immune system have co-evolved into a general tolerance (van Belkum *et al.*, 2009b), with most people not producing any type of protective antibodies (Kolata *et al.*, 2011). Moreover, as *S. aureus* has an incredibly large capability for genetic variability, resulting in a vast array of antigenic targets, makes it near impossible to find vaccine candidates (Golubchik *et al.*, 2013). Nevertheless, individuals already colonized by *S. aureus* experience more frequent infections, however their severity is decreased in comparison to individuals with first-time infections (Verkaik *et al.*, 2009). The latest vaccine development efforts have shown that a four-antigen vaccine, consisting of capsular polysaccharides 5 and 8 (CP5 and CP8) linked to non-toxic diphtheria toxin, and lipoprotein manganase transporter C and a recombinant clumping factor A (ClfA) (Frenck *et al.*, 2017). This allows the targeting of several *S. aureus* virulence factors commonly found in clinical isolates of this bacteria. Remarkably, opsonophagocytic assays of patients receiving this vaccine saw an 80-fold increase in opsonic antibodies against *S. aureus* CP5 and more than a 20 times increase for *S. aureus* CP8 by day 11, with such a robust response suggesting the use of this vaccine as a prophylactic treatment before any type of surgery. Further clinical studies for this vaccine have been initiated.

Therefore it is evident that despite the innate and adaptive immune systems possessing a vast array of defences against *S. aureus* infection, our understanding of how the bacteria evades immune killing is largely lacking.

## Model organisms for *S. aureus* disease

Ultimately, the perfect model organisms for an infection must mimic as closely as possible the human response to the pathogen. *In-vitro* studies have been historically used to inform further *in-vivo* experiments, usually done in the mouse (Andriole, Nagel and Southwick, 1974; An and Friedman, 1998). However often expression of bacterial factors *in-vitro* did not match up with disease-associated virulence factors *in-vivo.* For instance, the P2 promotor of global regulator SarA was inactive *in-vitro*, but highly expressed in an *in-vivo* model of endocarditis (Cheung, Nast and Bayer, 1998). Moreover, Goerke *et al.* demonstrated that upregulation of *hla* expression was significantly higher in an implanted device model of *S. aureus* infection in the guinea pig (Goerke *et al.*, 2001). However using mammals as a primary model for studying *S. aureus* has many disadvantages, from ethical concerns and difficulty of genetic manipulation to the high costs of maintaining the animals.

Despite our common ancestor with invertebrates being as far as 2000 million years ago (Hedges and Kumar, 2003), humans share a remarkable amount of orthologous genes with flies and worms, often encoding the same conserved pathways of innate immune system responses to pathogens (Tatusov *et al.*, 2003). Moreover, creating genetic knock outs and modifications is quick and cheap in models such as *Drosophila melanogaster.* While invertebrates are useful for the study innate immune system responses, these organisms usually lack the adaptive immune system, with one of the common animals sharing it with humans being the zebrafish (Figure 1.9). Nevertheless, knowledge obtained from using invertebrate models is often used to direct experiments in mammals.

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| Figure 1.9 Immune system evolution  Immune system components for invertebrates (pink), jawless vertebrates (green), jawed vertebrates (blue). 1,2,3 R –genome duplication events.  Adapted from (Flajnik and Kasahara, 2010) |

### Invertebrate models

#### *Caenorhabditis elegans*

*C. elegans* is a nematode that normally resides within soil, where it feeds on bacteria*.* It has been used in a wide variety of biological research including epigenetics(González-Aguilera, Palladino and Askjaer, 2014) and neurobiology (Husson, Gottschalk and Leifer, 2013). This organism has a long list of positive characteristics, such as being transparent and therefore allowing the monitoring of disease progression, ithas a short life cycle,and adults do not reach more than 1.5mm in length, allowing for easy manipulation in the laboratory setting (Schulenburg, Kurz and Ewbank, 2004). What is more, some *C. elegans* are hermaphrodytes, allowing for the generation of isogenic lines (Riddle *et al.*, 1997). Sifri *et al.* was one of the first to establish *S. aureus* pathogenesis in *C. elegans,* demonstrating that most *S. aureus* strains had nematocidal activity (Sifri *et al.*, 2003). Further research showed that the knockout of *esp-2* and *esp-8,* essential in the MAP-kinase pathway, in *C. elegans* made them much more susceptible to *S. aureus* infection, demonstrating that innate immune pathways conserved through different species are essential for defence against *S. aureus* (Sifri *et al.*, 2003)*.* Finally, a completegenome DNA sequence of *C. elegans* is available, allowing for further genetic studies.

#### *Drosophila melanogaster*

*D. melanogaster* has been extensively used in biological research (Stephenson and Metcalfe, 2013). *D. melanogaster* not only offers ease of use due to small size and a quick reproductive cycle, but also due to easy genetic manipulation and the fact that 75% of disease – associated proteins and pathways have an ortholog in humans, such as the TLR cascade (Needham *et al.*, 2004). Experiments with *D. melanogaster* have shown the importance of phagocytosis receptor integrin βv for the clearance of *S. aureus* infection (Shiratsuchi *et al.*, 2012).

While invertebrate models are undoubtedly important for studying aspects of *S. aureus* behaviour in an *in-vivo* setting, more complex organisms with evolved immune systems are essential for understanding pathogenesis.

## Vertebrate models

### Zebrafish

The zebrafish were initially used to study embryogenesis and aspects of developmental biology. However, with the onset of available tools for genetic manipulation, the zebrafish became a model organism for infection studies, especially due to the fact that the innate immune system is the only one present in larval zebrafish, with the adaptive immune system maturing only several weeks later (Lam *et al.*, 2004). Moreover, real-time visualization of pathogenesis has shed light on bacterial functions *in-vivo* such as virulence factor expression in *P. aeruginosa* (Díaz-Pascual *et al.*, 2017)*, S. enterica* and *M. marinum* (Benard *et al.*, 2012)*,* as well *as S. aureus* (Prajsnar, Hamilton, Garcia-Lara, Mcvicker, *et al.*, 2012)*.*

Zebrafish housing requires an intricate system of aquaria and water filtering devices, however breeding is exceptionally easy. Fertilised embryos can be collected the next day and are not only optically transparent but also easily genetically traceable through fluorophore-marked cells (Tobin, May and Wheeler, 2012). For instance a macrophage specific marker mpeg-1 allows for real-time observation of macrophage and neutrophil interactions in a zebrafish wound model (Ellett *et al.*, 2011). Several genetic tools are available for zebrafish manipulation such as reverse genetics using morpholinos, allowing the specific targeting of genes of interest (Stainier *et al.*, 2017). One of the more popular morpholinos used in zebrafish immune research is *pu.1,* when injected into the early stage zebrafish embryo, halts myeloid cell creation by the embryos for 48 h (Klemsz *et al.*, 1990; Bukrinsky *et al.*, 2009). However there has recently been reports of off-target morpholino binding, with studies reporting inconsistent or even contradictory results when using a morpholino as opposed to a genetic knockout embryo (Eve, Place and Smith, 2017).

To date, the CRISPR/cas9 (clustered regularly interspaced short palindromic repeats) system isconsidered the best and most reliable tool for zebrafish genetic manipulation, allowing one to specifically and accurately target sites of interest on the DNA (Li *et al.*, 2016). Both targeted knock-ins and knock-outs are possible with the use of CRISPR, with the main mechanism being two short RNAs, one of which identifies and binds to a selected DNA region of the host, then the other one binds the cleaving protein Cas9 that cleaves the double helix (Hille and Charpentier, 2016). Such a technique has allowed incredible opportunities for creation of not only genetic mutant strains, but also several GFP-tagged strains, previously unattainable (Lackner *et al.*, 2015).

However there are also several limitations to the zebrafish model in infection studies, the most obvious being that zebrafish require a steady temperature of 28oC for development and viability, while many bacteria infect hosts with a much higher core temperature and therefore the expression of virulence factors may be varied. Moreover, even inbred zebrafish have a genetic variability of no less than 7% (Guryev *et al.*, 2006), unlike the inbred mouse models available.

### Zebrafish innate immune system

Zebrafish embryos remarkably already have several immune cell types early in their development. Studies have shown that as early as 24 h post fertilisation (hpf) macrophages are present in the embryo yolk sac and by 25 hpf can be identified in the blood (Herbomel, Thisse and Thisse, 1999). Neutrophils are thought to be present in the bloodstream at 48 hours post infection (hpi) (Willett *et al.*, 1999) and detected by their granulocytes, as these scatter light and can be seen by microscopy (Le Guyader *et al.*, 2008). Moreover, eosinophils (Bertrand *et al.*, 2007) and mast cells (Dobson *et al.*, 2008) have also been shown to be presentin the embryo bloodstream approximately 24 hpf. The adaptive immune system does not mature until around 3 weeks post fertilisation and therefore allows unprecedented opportunities to study the innate immune system. For instance Prajsnar *et al.* had discovered a population bottleneck during infection by *S. aureus,* that is mediated by the host phagocytes (Prajsnar, Hamilton, Garcia-Lara, Mcvicker, *et al.*, 2012).

Interestingly, many zebrafish genes are duplicated and have a certain degree of redundancy. Moreover, products of copies of the same gene may have different roles, such as loss or gain of function (Force *et al.*, 1999). This results in a human gene having several orthologues in the zebrafish. This genomic duplication was an evolutionary step during the late Devonian period about 400 million years ago (Meyer and Schartl, 1999). Importantly, pathways involved in immune system response to pathogens are conserved in both zebrafish and humans. These include the TLR signalling pathway via Myd88 and TRIF (Purcell *et al.*, 2006), as well as NOD orthologues signalling in response to bacterial infection are similar to that observed in mammals and human cell lines (Oehlers *et al.*, 2011).

Despite the similarities between immune signalling between zebrafish and human immune system, often the response seen in humans cannot be re-enacted in zebrafish. The zebrafish CXCL12 homologues are not capable of recognising human leukaemia cells, as they do not interact with human CXCR4, making such disease difficult to study (Rajan *et al.*, 2015). However due to ease of genetic manipulation, Rajan *et al.* created a zebrafish mutant capable of expressing human CXCR4. Therefore, despite current limitations of the zebrafish model, continuous work on perfecting the model could make it ideal for infection studies.

## Mammalian

Mammals arguably have the most similarities to humans in the case of infection and disease progression and most studies of *S. aureus* pathogenesis have been conducted in mice and less frequently rats and rabbits.However due to *S. aureus* alsoaffecting large bovines and ovines,studies using these animals are emerging.

### Murine

Having diverged from rodents around 90 million years ago, humans and mice have around fifteen thousand genes with common functions, with 1716 genes having a definitive role in disease (Church *et al.*, 2009). However, many promising pre-clinical trials conducted on mice have failed to translate into humans (Mak, Evaniew and Ghert, 2014), demonstrating that enough genetic variation has occurred to cause difficulties in comparison of immune responses between mice and humans. One of the main differences in immune response of mice is that their method of immune protection is control, rather than eradication of the invading pathogen as in humans (Schneider and Ayres, 2008). For instance humans rely on TLR signalling during most infections, as this triggers the process of eliminating the pathogen (Akira and Takeda, 2004) (Seok *et al.*, 2013), while mice are largely resistant to TLR stimulation, such as with LPS, compared to humans (Glode, Mergenhagen and Rosenstreich, 1976; da Silva *et al.*, 1993). What is more, there are several discrepancies in the bacterial killing mechanisms between mice and humans, with human myeloperoxidase deficient neutrophils being completely ineffective in killing *C. albicans* (Lehrer and Cline, 1969)*,* while myeloperoxidase deficient mouse neutrophils retained some ability to clear infection, with a 60% survival rate at 25 days post infection (Brennan *et al.*, 2001).

However, despite obvious differences in the immune response to pathogens, many successful models of bacterial pathogenesis have been established in the mouse. Some examples include intravenous administration of *E. faecalis,* allowing the investigation of bacterial dissemination in murine organs(Gentry-Weeks *et al.*, 2003); a subcutaneous model for *S. aureus* skin and soft tissue infections allows one to study the interplay of virulence factors during this type of disease (Malachowa *et al.*, 2013); administration of *S. pneumoniae* intra-nasally allowed to mimic a human pneumonia infection (Seitz *et al.*, 2012); intraperitoneal injection of *Shigella flexneri* allowed for the creation of a robust assay to test possible vaccines against this bacterium (J. Y. Yang *et al.*, 2014).

While *S. aureus* naturally infects mice, the doses required for initiation and progression of pathogenesis are significantly higher than thought involved in human infection (Schulz *et al.*, 2017). What is more, mouse strains vary significantly in their response to *S. aureus* infection, with C57BL/6 being the most resistant and A/J mice completely succumbing to infection 24 h post infection (Stapels *et al.*, 2014). On the other hand, there is a large amount of information available on various inbred mouse strains, allowing their molecular comparison (Blake *et al.*, 2003). Additionally, the recent development of successful humanized mouse strains will allow the more precise study of disease progression of pathogens that do not normally infect mice (Rämer *et al.*, 2011).

### Other rodents

In some cases, the use of animal models as an alternative to mice are required. This may be due to desired structural similarities to a human organ or system, or simply a larger size of the animal allowing easier surgical access. For instance, the nose of cotton rats has been shown to bear striking histological similarities to human nasal tissues, and adhesion strategies used by *S. aureus* are comparable (Burian *et al.*, 2010). Moreover, WTA’s have been identified as the essential adhesion molecule during nasal colonisation (Weidenmaier *et al.*, 2004).The mouse nares microbiome has been shown to significantly differ to that found in humans, suggesting that mechanisms of colonisation and invasion may be affected differently depending on the bacterial environment (Chaves-Moreno *et al.*, 2015).

Studying orthopaedic implant biofilm formation requires a large femur, and therefore Sprague-Dawley rats were chosen over mice (Lei, Gupta and Lee, 2017). Larger animals allow for a bigger implant and therefore a higher quantity of biofilm generated and collected for analysis.

Guinea pigs are less often used as a model for *S. aureus* disease, despite having advantages over the mouse model. In particular, regeneration mechanisms underpinning the burn wound model, where an area of the skin is scalded with a hot metal plate and then infected with a dose of *S. aureus,* have been shown to bear strong similarities to those observed in human infected burn wounds (Herndon, Wilmore and Mason, 1978). Moreover, Guinea pigs have been successfully used in several vaccines studies, such as the BacillusCalmette-Guerin (BCG) vaccine (D.W., D.N. and H, 1970), and this animal model has also been used to develop potential new vaccines for Legionnaires disease (Breiman and Horwitz, 1987) and *Chlamydia* (de Jonge *et al.*, 2011). More wide-spread use of this model is currently hindered by a lack of a fully sequenced genome.

### Rabbits

Rabbits have been shown to be natural hosts of *S. aureus,* often present as a commensal together with rabbit-specific microflora (Devriese *et al.*, 1981). Largely rabbits are used for the study of biofilm formation on the surfaces of implanted medical devices (Carmen *et al.*, 2004). Moreover, rabbits, unlike other animals allowed for the creation of a robust knee implant infection model, closely mimicking the infection development in human hosts (Chu *et al.*, 2016). Finally, due to similarities in immune signalling, rabbits have been used in several *S. aureus* sepsis studies, showing that variation in virulence factor expression, such as cytolysins, between strains has an impact on progression and severity of pathogenesis (Spaulding *et al.*, 2012). However due to the relatively large size of the animal it is difficult to have many experimental groups within the same experiment, compared to using smaller rodents.

### Large mammals

Large mammals are most frequently used as models for osteomyelitis, as the surgical implants used are similar to ones used on human patients, allowing for not only more clinically relevant studies but also enabling different groups to replicate and test the procedures in their own experiments.

Historically *S. aureus* osteomyelitis has been studied in canine models (Fitzgerald Jr., 1983), and was used by other group to analyse progression of infection and bone damage in canines through injection of bacteria into the tibial nutrient artery (Fitzgerald Jr., 1983). As implant medical devices became more accessible, the need to treat formation of *S. aureus* biofilms on these implants became crucial, as up to 10% of patients experience life-threatening infections (Puolakka *et al.*, 2001; McPherson *et al.*, 2002).The use of sheep as a model for human orthopaedic trauma implant has been successful, the size of the animal allowing not only for the use of human-sized implants but also for increased blood sampling throughout the experiment (Stewart *et al.*, 2012). Stewart *et al.* has developed a vancomycin implant coating with this model to aid bone regeneration and increase recovery speed, through prevention of *S. aureus* biofilm formation on the implant. Finally, porcine and goat models have been used to demonstrate contamination risks and infection management of open fractures (Curtis *et al.*, 1995; Gaines *et al.*, 2012). Importantly, mastitis cause by *S. aureus* in dairy cows constitutes 30% of all mastitis cases, causing significant economic losses as there is currently no vaccine (Gröhn *et al.*, 1998). Ovines have also been successfully used as model for the development of various therapies for *S. aureus* mastitis (Gabadage *et al.*, 2017).

Evidently, no one animal model of *S. aureus* disease is sufficient for a comprehensive understanding of pathogenesis and ideally various models should be used to get a deeper understanding of disease, with *in vitro* assays. Understanding the differences, drawbacks and benefits of each model will help design the required experiment. It is also important to note that not all *S. aureus* strains are equally pathogenic across all animal models (Coulter *et al.*, 1998).

## Initiation of infection in humans and animal models

The exact mechanism whereby *S. aureus* initiates infection is yet uncharacterised. The required human inoculum is difficult to establish in clinical practice, as patients often present at hospital with progressive bacteraemia or other established infections. Moreover, the current animal models of *S. aureus* infection, such as zebrafish, rodents, rabbits, canines, pigs and goats, vary greatly in virulence, with different doses of inoculum mediating disease dissemination (García-Lara, Needham and Foster, 2005; Reizner *et al.*, 2014). Anecdotally, it has been suggested that the actual dose necessary for initial human *S. aureus* infection is relatively low*,* given that surgical wounds are unlikely to contain large doses of bacteria (Allen *et al.*, 2014). Furthermore, the amount of *S. aureus* bacteria required to form pus in human subdermal infections has been shown to be in the area of 1-2x106 CFU per 100 µl collected (Elek and Conen, 1957). Conversely, further variables within *S. aureus* pathogenesis, such as microbial exposure, mode of host invasion, and a history of previous colonization by *S. aureus,* impact on the amount of bacteria considered necessary to establish infection (Von Eiff *et al.*, 2001).

Initially, *S. aureus* was considered an extracellular pathogen, predominantly found in localised abscesses, which would destroy the tissue surrounding colonized sites with exotoxins and enzymes (Finlay, 1997). It has since been shown how *S. aureus* infiltrates host cells and launches multiple different survival mechanisms. One important example is its ability to downregulate the expression of secreted virulence factors and slow down growth rate (Proctor *et al.*, 2006; Löffler *et al.*, 2014). Furthermore, recent *in-vitro* studies have highlighted that *S. aureus* can evade killing within macrophages because of reduced phagolysosome acidification, suggesting that *S. aureus* can modulate phagosome maturation by intervening with cathepsin D activation (Jubrail *et al.*, 2016a). Therefore it is of utmost importance to explore the interactions between *S. aureus* and the host immune system as well as study the importance of commensal bacteria and their components in initiation of *S. aureus* infection.

## Aims of this project

The project aims to identify the interaction between *S. aureus* and the host by:

1. Using zebrafish and mouse models to identify the role of commensal bacteria in the initiation of *S. aureus* infection
2. Using human cells, zebrafish and mouse models to determine impact of bacterial cell wall components on *S. aureus* disease progression and the host immune system.

# Chapter 2. Materials and Methods

## Media

All media used was made with distilled water (dH2O) unless otherwise stated, and sterilized by autoclaving at 121°C for 20 min, 15psi.

### Brain Heart Infusion Broth (BHI)

Brain Heart Infusion (Oxoid) 37 g l-1

Together with above ingredients 1.5% (w/v) Oxoid No. 1 agar was added to make BHI agar, a highly nutritious general purpose growth media.

### Luria-Bertani Broth (LB)

Tryptone (Oxoid) 10 g l-1

Yeast extract (Oxoid) 5 g l-1

NaCl  5 g l-1

Together with above ingredients 1.5% (w/v) Oxoid No. 1 agar was added to make LB agar, preferable for *E. coli* growth.

### R2A broth

R2A pre-mix (Oxoid) 4 g l-1

Media used preferably for the growth of *R. mucosa.* 1.5% (w/v) Oxoid No. 1 agar was added to make R2A agar

## Human blood

### Collection

After informed consent, venous blood was taken from healthy volunteers and University Medical Centre Utrecht, in accordance to ethical regulations. Blood was collected into 50 ml standard tubes containing citric acid and used immediately.

## Reagents used in ELISA cytokine analysis

### Blocking buffer

Milk proteins (ELK, Utrecht) 40 mg/ml

ELK was topped off with PBS to desired volume to a final concentration of 4% (w/v).

### Coating Buffer

100mM Carbonate buffer made up to pH 9.6 (from PeliKine Toolset Cat.No.M1980).

### Dilution buffer

High performance ELISA buffer (HPE) (from PeliKine Toolset Cat.No.M1940).

### Standard

Stock standard of appropriate cytokine (from PeliKine Toolset Cat.No.M1980).

### Streptavidin-HRP conjugate

Stock supplied (PeliKine Toolset Cat.No.M2032) diluted 1:5000 for working concentration.

### Substrate solution (TMB) (detection buffer)

MQ 5.4 ml

Na Acetate buffer 0.6 ml

TMB 100 µl

Ureumperoxidase 50 µl

### Stop solution

1M H2SO4

### Veronal (VBS) x5 pH 7.4

NaCl 42.5 g

Diethylbarbital 1.875 ml

Diethylbarbitiuric acid 1.875 ml

### GVBS

VBS x 5 10 ml

10% w/v gelatine 0.5 ml

1M CaCl2 25 µl

1M MgCl2 12.5 µl

## Antibiotics

Antibiotics used in this study can be found in Table 2.1. Stock solutions were prepared by dissolving the appropriate amount in suitable solvent, then filter sterilized through 0.22 µm filters and stored in -20°C. When antibiotics were added to liquid media or molten agar, the temperature was always 55°C or lower.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Antibiotic | Stock (mg ml-1) | Solvent | *E. coli* working concentration (µg ml-1) | *S. aureus* working concentration (µg ml-1) |
| Ampicillin (Amp) | 100 | dH2O | 100 | *n/a* |
| Erythromycin (Ery) | 5 | 100% v/v ethanol | *n/a* | 5 |
| Kanamycin (Kan) | 50 | dH2O | *n/a* | 50 |
| Lincomycin (Lin) | 25 | 50% v/v ethanol | *n/a* | 25 |
| Tetracycline (Tet) | 5 | 100% v/v ethanol | *n/a* | 5 |

Table 2.1 Stock solutions and concentrations for antibiotics

## Bacterial Strains

### *Staphylococcus aureus* strains

All *Staphylococcus aureus (S. aureus)* strains that were used are listed in Table 2.2. All strains were grown from bacterial storage beads (Fisher Scientific) stored at -80°C and cultured in either normal BHI plates or plates made with appropriate antibiotics to retain specific strain resistance markers. Before each animal experiment, a fresh plate was grown, else it was stored wrapped in Parafilm® at 4°C, no more than 2 weeks.

Bacteria are grown aerobically in liquid media at 37°C. An overnight culture was created by inoculating 5ml of BHI broth with a single colony and placing it on a rotary shaker at 250 rpm. Exponential phase growth was achieved by inoculating 50ml BHI broth with 0.5 ml of overnight broth in 250 ml conical flask and incubated for around 2 h on a rotating shaker at 250 rpm, 37°C.

|  |  |  |
| --- | --- | --- |
| **Strain** | **Description** | **Reference** |
| SH1000 | Functional *rsbU*+ derivative of 8325-4 | (Horsburgh *et al.*, 2002) |
| USA300 JE2 | Plasmid cured USA300\_FPR3757, MetR | (Fey *et al.*, 2013) |
| NewHG | Newman, repaired *saeS* | (Mainiero *et al.*, 2010) |
| SJF 4308 | SH1000 carrying pMV158-*mCherry* | This study |
| SJF 4405 | SH1000 carrying pMV158-*gfp* | This study |
| SJF 4591 | SH1000 *lgt* EryR | This study |

Table 2.2 *S. aureus* strains used

EryR – erythromycin resistant, MetR -methicillin resistant

### Gram-positive species

*Micrococcus luteus (M. luteus)* and *Curtobacterium flaccumfaciens (C. flaccumfaciens)* were grown at 30°C, *Staphylococcus epidermidis (S. epidermidis)* was grown at 37°C, all were grown aerobically in BHI broth. Storage conditions same as *S. aureus*.

|  |  |  |
| --- | --- | --- |
| Strain | Description | Source |
| SJF 4393 | *Micrococcus luteus,* RifR | Lab stock |
| SJF 449 | *Curtobacterium flaccumfaciens* |
| SJF 4381 | *Staphylococcus epidermidis* strain 138, RifR |
| SJF 1 | *Bacillus subtilis* 168 |

Table 2.3 Gram-positive species used

## Gram-negative species used

*Escherichia coli (E. coli)* grown at 37°C using LB broth, *Roseomonas mucosa (R. mucosa*) grown at 32°C on R2A broth. Storage conditions same to *S. aureus*

|  |  |  |
| --- | --- | --- |
| **Strain** | **Description** | **Source** |
| *Escherichia coli* SJF 4060 | *E. coli* strain W311 | Lab stock |
| *Roseomonas mucosa*  Atypical Dermititis (AD) | Clinical isolate of *R. mucosa* from patient with atypical dermatitis | Supplied by Ian Myles (National Institute of Health) |
| *Roseomonas mucosa*  Healthy volunteer (HV) | Human isolate of *R. mucosa* from healthy volunteer | Supplied by Ian Myles (National Institute of Health) |

Table 2.4 Gram-negative species used

## Buffers and solutions

All buffers were made with dH2O unless otherwise stated and stored at 4°C when applicable.

### Phosphate buffered saline (PBS)

NaCl 8 g l-1

Na2HPO4 1.4 g l-1

KCl 0.2 g l-1

KH2PO4  0.2 g l-1

### 50 mM Tris-HCl pH 7.5

Tris base 6.05 g l -1

7.5pH reached by addition of HCl before autoclaving.

### Tris/EDTA/NaCl buffer (TES) pH 8.0

Tris base 2.42 g l-1

EDTA 1.86 g l-1

NaCl 5.84 g l-1

8.0pH reached by addition of HCl before autoclaving.

### 0.1 M Sodium phosphate buffer pH 5.5

Na2HPO4 (1.0 M) 93 ml

NaH2PO4 (1.0 M) 7 ml

pH adjusted until reaching 5.5 and volume increased to 1 L dH2O before autoclaving.

## Centrifugation

|  |  |  |
| --- | --- | --- |
| **Make** | **Maximum volume** | **Maximum Speed** |
| Eppendorf microfuge 5415D | 2 ml | 13,200 rpm (10,000 x g) |
| Sigma centrifuge 4K14C | 50 ml | 5,100 rpm (5525 x g) |
| AvantiTM J-251 (Beckman)  JA-25,50 | 6 x 50 ml | 25,000 rpm (75,600 x g) |
| AvantiTM J-26XP (Beckman);  JLA 8.1000 | 6 x 1000 ml | 8,000 rpm (15,950 x g) |

Table 2.5 Centrifuges used in this study

## Growth curves

After growing overnight cultures (Section 2.5), 50 ml BHI was inoculated to an OD600of 0.01. Cultures were grown in triplicate in a Grant OLS 200 water-bath at 250 rpm, 37°C, with serial OD600 measurements and direct cell counts at appropriate times.

## Measurement of bacterial numbers

### Spectrophotometry

A Jenway 6100 spectrophotometer was used to quantify bacterial growth in liquid broth at OD600.

### Direct cell counts

Direct cells counts allow the quantification of viable bacteria (CFU ml -1). This is done by plating out 10 µl in triplicates of serially diluted bacterial suspensions (1:10) in PBS, and incubation at an appropriate temperature overnight or longer. Number of formed colony units (CFU) was counted following incubation.

## Zebrafish techniques

### Zebrafish strains

London Wild Type (LWT) zebrafish were used in all studies.

### Zebrafish husbandry

All adult zebrafish were maintained in a re-circulating closed system of aquarium water at a constant temperature of 28°C. The light/dark cycle lasts for 14/10 h respectively. LWT zebrafish embryos were maintained in E3 medium for duration of the experiment (28.5 °C).

### Zebrafish E3 medium (x10 concentration)

NaCl 50 mM

KCl 1.7 mM

CaCl2 3.3 mM

MgSO4 3.3 mM

E3 stock solution was kept at room temperature. Working solution was made by diluting to x1 concentration with dH2O and adding methylene blue (final concentration 1x10-5 %) to prevent fungal growth (Nüsslein-Volhard and Dahm, 2002). 1x E3 media sterilized as usual(section 2.1).

### Methylcellulose

3.0% (w/v) methylcellulose was dissolved in E3 medium. For complete solubilisation, the solution was stirred, frozen and defrosted in several cycles (Nüsslein-Volhard and Dahm, 2002). Upon completion, the methylcellulose was aliquoted into 20 ml syringes and stored at -20°C. Before use, each syringe was thawed at 28.5°C overnight.

### Zebrafish embryo anaesthesia

Tricaine (MS322, Sigma) was kept as a stock solution (0.4% w/v) at -20°C. Working solution was made up to0.02% (w/v) and kept at 4°C in the dark. For anaesthesia 1.5 ml working solution was added to an E3 petri dish containing zebrafish embryos.

### Microinjection needles

Non-filament glass capillary tubes (World Precision Instruments, WPI) were heated and pulled in an electrode puller to produce hollow fine needles.

### Microinjecion of bacteriainto zebrafish embryos

Bacteria were grown from an overnight culture until reaching exponential phase (Chapter 2.9). Then 40 ml of culture was centrifuged for 10 min at 5100 rpm, 4°C, supernatant discarded and pellet resuspended in 40 ml PBS and centrifuged as before. The washed pellet was then resuspended in PBS to a required concentration. Quantification of bacteria was achieved by 1:4 dilution of the sample in PBS, with 10 µl plated in duplicate on agar plates and incubated for the appropriate time and temperature.

Dechorionation of zebrafish embryos was performed 2-4 h prior to injection. At the beginning of experiment zebrafish embryos were immersed in E3 tricaine working solution before being transferred onto 3% (w/v) methylcellulose for immobilization. 10 µl of bacterial suspension was loaded into microcapillary needles and inserted into a micromanipulator (WPI) attached to a pneumatic micropump (WPI, PV820). Calibration was achieved using a graticule slide to produce 1 nl drops. Immobilized zebrafish embryos were placed under a light microscope (Leica) and injected into the circulation valley.

Following injection, zebrafish embryos were removed from methylcellulose and placed back into petri dishes containing 30 ml fresh E3 in an incubator with a constant temperature of 28.5°C for 1 h, allowing the methylcellulose to dissolve from embryos. At the end of the experiment zebrafish were placed in individual wells of a 96-well plate.

### Microinjection of morpholino oligonucleotides into the zebrafish embryo

#### Morpholinos

Morpholino oligonucleotides are used to inhibit translation of RNA transcripts *in vivo.* PU.1 morpholino was purchased from Gene tools.

|  |  |  |  |
| --- | --- | --- | --- |
| Morpholino target | Sequence 5’-3’ | Quantity | Reference |
| *pu.*1 | GATATACTGATACTCCAT TGGTGGT | 0.5 pmole | (Rhodes *et al.*, 2005) |

Table 2.6 Morpholino used for zebrafish work

#### Injection of morpholinos into zebrafish embryos

1 mM concentration of *pu.*1 morpholino (in dH2O) was injected into yolk sac of 1-4 stage embryos using the same method as above (section 2.11.7)

## Murine sepsis model

In this study female Balb/c or C57BL/6J mice were purchased from Charles River (Margate, UK) or Envigo (Cambridgeshire, UK) at the age of 6-7 weeks and maintained at Biological Services (University of Sheffield) in accordance to Home Office husbandry techniques.

### Injection of material and bacteria into murine model

1 µm unmodified polystyrene beads were obtained from Sigma. Beads were diluted in PBS to necessary concentration from stock (1.8x 1011 beads ml-1) and sonicated to avoid clumping (Soniprep 150, MSE, UK). Bacterial stocks for injection were made by growing bacteria to exponential phase in BHI broth, washing and resuspending to required dose in endotoxin-free PBS with 10% (w/v) bovine serum albumin (BSA) and aliquots stored at -80°C. Prior to injection, aliquots of the bacterial suspension were thawed and appropriate bacterial doses made up. Mice were injected with 100 µl of bacterial suspension into the tail vein. Serial dilutions and plating out of the bacteria suspension allowed for quantification of viable bacterial numbers. In cases where purified peptidoglycan was used, prior to being resuspended in endotoxin-free PBS, peptidoglycan was sonicated for 30 sec with 1 min chilling on ice, three times.

Following injection daily weight checks were performed and recorded for all mice. They were also checked twice daily for well-being and any sign of discomfort noted for the duration of 72-h experiment. Mice were sacrificed in accordance to Schedule 1 via anaesthetic overdose or concussion, followed by cervical dislocation if mouse severity limits were reached or at the end of the experiment. Immediately following Schedule 1, mice were dissected and organs harvested.

Bacterial numbers present in the organs were quantified by serial dilutions of homogenized organs onto agar plates, incubated for an appropriate amount of time and temperature.

### Histological preparations of murine organs

Following Schedule 1, murine organs were placed in embedding cubes filled with 5mm of optimal cutting temperature (OCT) medium, then topped up with OCT medium to cover the organ. Liquid nitrogen was added until the organs were completely frozen and stored at -80°C. Sectioning was performed by Histology Core Facility (University of Sheffield). Organs sections were prepared in 200 µm cuts and stained with hematoxylin and eosin (H&E) stain.

### Murine serum cytokine level determination

At different time points defined by experimental protocol, a small amount of blood was taken from the mouse tail vein by venesection, not exceeding 15% blood volume over 4 weeks. Microcentrifuge tubes containing collected blood were left 2 h, allowing blood samples to clot. Following this, samples were centrifuged for 10 min at 5000 x g, separating clotted blood from serum. Serum was then carefully removed and stored at -20°C until FACS analysis by technical staff at University of Sheffield (cytometric bead assay on FACSArray Bioanalyzer).

## Peptidoglycan purification

### Breakage of cell walls

10 litres of the required bacterial strain was grown to a mid exponential phase in BHI broth and centrifuged at 1100 x g for 10 min at 4°C, with the supernatant discarded and bacterial pellet resuspended in a minimally possible amount of 50mM ice-cold Tris-HCl at pH 7.5. This was then boiled at 100°C for 10 min and the cells broken using the Braun homogenizer (Braun, Germany) by homogenizing in Braun bottles filled with 50 g of sterile glass beads and around 7 ml bacterial resuspension. Each bottle was shaken for 1 minute 6 times with intermittent bursts of liquid CO2. A vacuum sintered glass filter was used to separate beads from broken cells, with the cell debris centrifuged at 18000 rpm for 10 min at 18°C and the supernatant discarded.

### Extraction of cell wall peptidoglycan

Following centrifugation the pellet was resuspended in 30 ml of 50mM Tris-HCl (pH 7.5) with 2% (w/v) SDS and incubated at 50°C for 15 min, centrifuged as above each time re-suspending and washing pellet with water. Pronase (2 mg ml-1) in TES was added to the washed pellet for 1 h at 60°C to remove any covalently attached proteins. Following centrifugation under the same conditions as before, the washed pellet was resuspended in 30 ml of 50mM Tris-HCl (pH 7.5) with 3% (w/v) SDS, 50 mM dithiothreitol (DTT) and 1mM EDTA, boiled for 10 min, centrifuged as before and repeated again. Following the last centrifugation step, the pellet was resuspended and washed five times in dH2O. Hydrofluoric acid (HF) was added to the pellet (10 ml of 48% v/v HF) and incubated at 2°C for 24 h. Following incubation, the suspension was centrifuged for 5 min at 18000 rpm, 18°C and pellet resuspended and washed with alternating50 mM Tris-HCl buffer (pH 7) and ice-cold dH2O until pH reached neutral. Peptidoglycan was resuspended in endotoxin-free PBS, aliquoted into Eppendorf tubes, autoclaved and stored at -20°C.

### Hydrolysis of peptidoglycan

Mutanolysin was made up to a stock concentration of 250 µg ml-1 in 50 mM sodium phosphate buffer (pH 5.5)

### Quantification of peptidoglycan

It is considered that 1x10 7 *S. aureus*  bacterial cells contain 0.1 µg of peptidoglycan (Timmerman *et al.*, 1993).

*S. aureus* was grown to an OD600 of 1 from an overnight culture as described in Chapter 2.9. At reaching the required OD, 1 ml of culture was added to a microcentrifuge tube, harvested at 13,000 rpm for 2 min, 4°C and supernatant discarded. This was repeated until 10 ml of culture were spun down in the same microcentrifuge tube. The pellet was then frozen by immersion of the tub into liquid nitrogen and placed in a freeze-dryer (ScanVac Cool Safe 55-4 Pro 3800) overnight, sublimating any liquid present in the sample. Having weighed the microcentrifuge tube before adding bacterial solution, the tube was re-weighed with the sample inside.As the amount of bacteria in the tube was known to be 2 x 108 CFU ml-1, and it is estimated that peptidoglycan makes up 10% w/v of the cell mass (personal communication, Dr. Stephane Mesnage), it was calculated that 0.026 mg of peptidoglycan per ml of culture was isolated, therefore 26 µg of peptidoglycan was isolated from 2x108 CFU.

## Isolating human polymorphonuclear (PMN) and mononuclear (MNC) cells from whole human blood.

10 ml of Ficoll-paque was layered on top of 12 ml Histopaque in a 50ml Falcon tube. Blood was anti-coagulated after being collected from healthy human volunteers by on-site phlebotomist and gently layered on top of Ficoll-paque, then PBS was added in equal proportion to blood. The tube was then centrifuged 400 x g for 20 min. Following centrifugation the top plasma layer was removed and MNC layer collected with a Pasteur pipette and transferred to a tube containing RPMI 1640 with albumin. The Ficoll faction was then removed to access PMC cells, transferred to a tube containing same RPMI buffer as above. Both MNC and PMC containing tubes were centrifuged for 10 min at 400 x g, 4°C, supernatant removed and cells re-suspended in RPMI buffer as above. Cell counts were performed using Luna-IITM fully automated cell counter (Logos Biosystems). All the above was performed aseptically.

## Stimulation of MNC cells by peptidoglycan

Human MNC cells isolated as per Chapter 2.14 and made up to 3 x 106 cells/ml in RPMI albumin buffer. 100 µl of cells per well were then put into 96 flat-bottom NUNC plates and incubated for 1 h,37°C, 5% v/v CO2. Following incubation cells washed with buffer to remove non-adherent cells and 300 µl of peptidoglycan made up with buffer at 30 µg/ml added to cells and diluted to 0.234 µg/ml. MNC cells with peptidoglycan were left to incubate overnight 37°C, 5% v/v CO2. Following incubation plates were centrifuged for 10 min at 400 x g, supernatant collected and stored at -20°C. Prior to use peptidoglycan samples were sonicated as described in Chapter 2.12.1.

## Determination of IL-8 and TNF-α production by human mononuclear cells in response to peptidoglycan stimulation

This assay was done in accordance to PeliPairTM human cytokine ELISA reagent set. Nunc maxisorp microtiter plates were coated with 50 µl of appropriate coating antibody diluted 1 : 100 in coating buffer (for this buffer and all below see Chapter 2.3), plate covered with plastic sealer (included by Elisa kit provider) and incubated overnight at 2-8°C.Plate was then washed three times with PBS + 0.05 % Tween20 and then blocked with 100 µl blocking buffer (Chapter 2.3.1) per well, covered and incubated 1 h at room temperature (TRM). Washing was repeated as above. Previously stimulated plasma by peptidoglycan (Chapter 2.15) was thawed and diluted in dilution buffer (Chapter 2.3.3), specific to the cytokine standard sample was also diluted in dilution buffer. 50 µl of samples were then transferred into duplicate wells, plate sealed and incubated 1 h at TRM. Biotinylated antibody (included by Elisa kit provider) was diluted 1:100 in dilution buffer and 50 µl transferred to appropriate duplicate wells, plate sealed and incubated 1 h at TRM. The plate was then washed as above and 50 µl of streptavidin-HRP conjugate added to appropriate wells, and the plate sealed and incubated 30 min at TRM. The plate was washed as above. 50 µl of substrate solution (Chapter 2.3.6) transferred to all wells and plate sealed and incubated 30 min at TRM in the dark. The enzymatic reaction was then stopped by 100 µl of stop solution and OD450 measured. HPE dilution buffer (Chapter 2.3.3) was used as negative control and standard dilution buffer (Chapter 2.3.3) as positive control.

## C3b complement activation by MNC cells stimulated with peptidoglycan

This assay was designed in the University Medical Centre (UMC) Utrecht (The Netherlands) and carried out under the supervision of Dr. Kok van Kessel (UMC Utrecht). Plates for this assay were prepared by diluting mannan (from *Sacchsromyces cerevisiae*) (stock concentration 10 mg ml-1) to working concentration 20 µg ml-1 in 0.1M Na-carbonate buffer (pH 9.6) and adding 50 µl to the wells of a Nunc maxisorp microtiter plate and incubation overnight at TRM. Wells were left empty to check for nonspecific complement activation. Following incubation, the plate was washed with washing buffer (PBS + 0.05% Tween-20), and plate was blocked with 1% w/v BSA dissolved in washing buffer, 80 µl per well for 1 h at 37°C. The plate was then washed three times as above and human serum (Chapter 2.15) was diluted to concentrations of 4, 3, 2, 1, 0.8, 0.6, 0.4 and 0% v/v serum in GVBS (Chapter 2.3.9). 50 µl of each dilution was added to Nunc wells and incubated for 1 h at 37°C. The plate was then washed three times as above. Mouse anti C3 antibody (‘WM-1’ DIG, provided by UMC) diluted to 0.1 µg ml-1 in washing buffer with 1% w/v BSA, and 50 µl added to each Nunc plate well, incubated as above. The plate was then washed thrice as above. Anti-DIG-PO (for C3dig detection) was diluted as above, 50 µl added to each well and incubated as above. After washing as above, detection was carried out by addition of 50 µl of fresh substrate solution (Section 2.3.6) and the colour reaction stopped by adding 50µl 1M H2SO4. Samples were measured at OD450.

## Tri-colour flow cytometry assay

Flow cytometry is used for the measurement of a given parameter on a large number of cells, over a relatively short amount of time. The Tri-colour assay allows for three different antibodies with varying fluorescence markers to be present at the same time, allowing to observe 57 different cell-surface receptor dynamics in a single experiment. Fluorescent intensities must be calibrated to avoid over-spilling of readings and ultimately incorrect results.

### List of monoclonal antibodies and their fluorescence channels.

Monoclonal antibodies were kindly provided by UMC (Utrecht, The Netherlands) and this assay was done with the help of Dr. Kok van Kessel (UMC, Utrecht, The Netherlands). Table 2.8 lists all the monoclonal antibodies used and their respective fluorescence channels.

### Activation of cells for Tri-colour assay

Antibody mixes were added to 96 well plate on ice in accordance to Table 2.1 and incubated in 4°C room on shaking platform at 250 rpm. PMC and MNC cells were isolated as described in Section 2.14. MNC and PMN cells were diluted to a concentration of 5 x 106 cells/ml in PBS and combined. A peptidoglycan sample was sonicated as before and added to the tube containing MNC and PMN cells at a concentration of 3 µg/ml and the mixture was incubated 30 min at 37°C, 5% CO2. The mixture was then transferred to the plate containing antibodies and incubated a further 45 min at 4°C, on a shaking platform at 250 rpm. Following incubation, 200 µl/well PBS was added and plate centrifuged for 8 min at 320 x g, 4°C. Supernatant was discarded from the plates and 150 µl of 1% v/v paraformaldehyde added to each well and plate incubated for 30 min at 4°C. Unstimulated cells were used as a control. Data was analysed using FACS canto II (BD Biosciences) and data collected using BD FACSDiva software (BD Biosciences).

### Gating strategy

Neutrophils, lymphocytes and monocytes were gated based on their forward and side scatter (Table 2.7). FlowJo 10.4.1 software was used to gate cell populations in all samples.

|  |
| --- |
|  |
| Table 2.7 Gating strategy for Tri-colour assay  Flow cytometric analysis of human blood collected from healthy volunteers; **SSC**- side scatter; **FSC** – forward scatter. Number beside each gate represents percentage of specific cells out of total number of leukocytes |

### Analysis of results

FlowJo 10.4.1 software was used to calculate mean fluorescence intensity (MFI) for all samples. Due to inaccurate setup of 2 repeats (by UMC technical staff), statistical analysis was not possible and therefore differences between MFI of non-stimulated and stimulated cells were used as a suggestion for further experiments. Example of data acquisition is shown in Figure 2.1.

|  |
| --- |
| CD11b gated on Neutrophils |
| Figure 2.1 Example of MFI applied to Flow cytometry data  Changes in Cd11b expression in human neutrophils stimulated by peptidoglycan from SH1000𝛥*tarO* and SH1000𝛥*tarO* digested by mutanolysin. Unstimulated cells were used as control (buffer). MFI calculated for each sample is shown. Analysis was performed using flow cytometry FlowJo software. |

|  |  |
| --- | --- |
| Table 2.8 Monoclonal antibodies used in Tri-colour assay.  Allophycocyanin (APC) (blue heading) has far-red fluorescence, excitation maximum of 650, emission peak at 660 nm (ThermoFisher, 2013); Fluorescein isothiocyanate (FITC) (green heading) has a broadfluorescence emission range, emission peak at 525 nm, excitation maximum of 490 nm (ThermoFisher, 2018a); R-phycoerythrin (PE) (red heading) has an excitation maximum of 496and emission maximum of 578 (ThermoFisher, 2018b) |  |

## Ethical permissions

Murine work was carried out according to UK law in the Animals (Scientific Procedures) Act 1986, under project licence PPL 40/3699 (*Staphylococcus aureus,* pathogenesis to therapy). An amendment was granted to this licence on 12.02.16, allowing to include other Gram-positive species. Personal licence PIL (SAB/SCT-W13/66) (Categories A, B, C).

Zebrafish embryos used were under 5 days post fertilization and therefore are not protected by the Animals (Scientific Procedures) act 1986 as they are not capable of independent feeding and therefore do not require a personal licence. However, all zebrafish work was carried out under Project Licence PPL 40/3574.

## Data and statistical analysis

The Kaplan- Meier method was used in zebrafish embryo survival experiments, with the log-rank (Mantel Cox) test performed to compare significance between survival curves.

All statistical analysis was performed using GraphPad Prism version 7.0.All zebrafish experiments are representative of no less than 3 repeats, as data cannot be combined due to variation in each group.

Mouse bacterial counts were analysed using Mann-Whitney U test or chi-square test with Yates correction. Non-parametric samples were compared using Kruskal Wallis test applied with Dunns’s multiple comparison test. Species evenness was analysed by the Shannon diversity index.

Chapter 4 – murine work involving peptidoglycan was conducted in collaboration with Dr. Emma Boldock.

# Chapter 3. Live commensal bacteria augment *S. aureus* pathogenesis in zebrafish and murine models of infection.

## Introduction

As humans we heavily rely on the vast array of commensal microorganisms that reside on our skin (microbiome), and those inside of us mainly found in the gastrointestinal tract (microbiota) (Turnbaugh *et al.*, 2007). It is therefore not surprising that a number of human diseases are caused by the dysregulation of the immune response to the microbiome or microbiota. For instance, inflammatory bowel disease (IBD) in humans is thought to be triggered by an altered intestinal microbiome leading to chronic inflammation (Lane, Zisman and Suskind, 2017). Particular interest is also paidto the skin microbiome, as it provides a provides a wide range of niches with diversechemical and physical attributes, allowingfor colonization by a vast array of microorganisms (Grice and Segre, 2011a). For instance, *Propionibacterium acnes* colonizes the sebaceous glands of the skin by making use of the fatty acids present (Marples, Downing and Kligman, 1971). However the skin serves not only as a physical barrier, but also an immunological one, with tolerance mechanisms to commensal bacteria developed, such as desensitization of epithelial cell TLRs to the constant presence of certain bacteria (Fukao and Koyasu, 2003).

Many skin commensal organisms such as *S. epidermidis* provide an additional layer of protection against pathogenic bacteria by producing antimicrobial peptides (Cogen *et al.*, 2010). However, when the skin barrier is breached such as during a wound or surgical intervention, commensal bacteria can invade and on occasion cause severe disease (Frank *et al.*, 2009).

*S. aureus* is an opportunist pathogen (Wertheim *et al.*, 2005), causing various life-threatening diseases. Currently, there is a large range of animal models available.However, do these reflect human infection? This is important when one considers prophylaxis such as vaccine development, where current approaches have failed in phase III clinical trials, despite promising animal data.It must be remembered that within any environment *S. aureus* is likely only a small proportion of the microbiome and thus initial infection will intuitively occur with a mixture of *S. aureus* and other, likely commensal, flora. It was therefore of interest to explore the role of other commensal bacteria in a polymicrobial infection with *S. aureus.*

Initial exciting results suggested commensal Gram-positive organisms are able to augment *S. aureus* pathogenesis in the zebrafish model of infection, and that low dose *S. aureus* together with commensals lead to death. These findings set the scene for further work. To define the possible role of other commensal organisms during *S. aureus* infection, a range of common Gram-positive and Gram- negative bacteria were chosen. *Roseomonas mucosa* is a Gram-negative commensal present on the skin of most individuals (Han *et al.*, 2003; Romano-Bertrand *et al.*, 2016). However, it has recently been found in several cases of catheter infections, as well as reports of *Roseomonas* spp. involved inarthritis and peritonitis (Bibashi *et al.*, 2000; Fanella *et al.*, 2009).

While *R. mucosa* is mostly a human organism and is not normally found in zebrafish, most animals including fish are colonised with *E. coli,* a common human gastrointestinal commensaland archetypical Gram-negative bacterium (Huggins and Rast, 1963; Palmer *et al.*, 2007)*.* Interestingly, some studies have shown that *in-vitro* there is a reduced uptake of *E. coli* by human granulocytes in the presence of *S. aureus,* due to opsonin depletion in the serum, moreover, granulocytes that had already ingested *S. aureus* showed a reduced ability for *E. coli* phagocytosis and intracellular killing (Dijkmans *et al.*, 1985). What is more, the same study showed that *in-vivo* recovered *E. coli* numberswere greater when co-injected with *S. aureus* into the mouse thigh muscle. This is in agreement with other studies claiming that the killing of Gram-negative organisms by immune cells is decreased in the presence of *S. aureus* (Wade, Kasper and Mandell, 1983)*.*

*S. epidermidis,* though closely related to *S. aureus,* is mostly a harmless skin commensal, with some work even suggesting it having pro-biotic properties as it interferes with *S. aureus* colonization on the skin (Duguid *et al.*, 1992). However, there are now numerous studies pointing to the fact that *S. epidermidis* causes a great number of infections, in particular those associated with inserted medical devices (Davies, 1985; Vuong and Otto, 2002). Several studies have looked into the interactions between of *S. aureus* and *S. epidermidis* as they occupy similar biological niches in the host, for example, in the nose *S. epidermidis* produces the *Esp* protease, successfully inhibiting *S. aureus* biofilm formation (Fredheim *et al.*, 2014). However, no work to date has explored co-infection of these bacteria.

*M. luteus* is considered to be a commensal primarily occupying human skin, however It has also been isolated from water and soil (Kocur M, Kloos W E and Schleifer K-H, 1992). There is little evidence to suggest that *M. luteus* can cause infection, however there have been some cases demonstrating that this bacteria may be involved in meningitis (Fosse *et al.*, 1985), peritonitis (Kao, Chiang and Huang, 2014), septic shock (Albertson, Natsios and Gleckman, 1978) and endocarditis (Seifert, Kaltheuner and Perdreau-Remington, 1995).

## Aims

1. To determine whether Gram- negative bacteria are capable of augmenting *S. aureus* pathogenesis in the zebrafish model of infection
2. To determine whether commensal Gram - positive and negative organisms are capable of augmenting *S. aureus* pathogenesis in the murine sepsis model

## Results

### Can Gram-negative organisms augment *S. aureus* infection in the zebrafish embryo model of disease?

Zebrafish have been previously used as a model of intestinal *E. coli* infection (Wiles *et al.*, 2009), as both fish and mammals in this case rely on innate immune mechanisms of defence (Mulvey *et al.*, 2000). *R. mucosa* is currently known to be a human associated commensal and has not yet been tested for pathogenicity in zebrafish embryos.

Therefore the ability of commensal Gram-negative organisms to augment *S. aureus* pathogenesis was tested. In order to choose the lowest dose that does not impact embryo survival, dose-dependent experiments were conducted with *R. mucosa* or *E. coli* alone (Figure 3.1 A, C), and doses of 2500 CFU and 3000 CFU *E. coli* gave less than 20% killing at 96 hpi. *E. coli* was chosen as it is an archetypical Gram-negative organism. Augmentation was tested by injecting a low dose of *S. aureus* (150 CFU) alone or together with either *R. mucosa* 2000CFU(Figure 3.1 B) or *E. coli* W311 3000 CFU(Figure 3.1 D). *Roseomonas mucosa* was chosen as it is a common skin commensal and it was kindly provided by Ian Myles (NIH), and was originally isolated from a patient suffering from atypical dermatitis. In the first experiment, low dose *S. aureus* alone or *R. mucosa* (RMAD)alone caused little embryo death, however when co-injected, caused a significant (p = 0.0158) drop in embryo survival. Injection of either *S. aureus* or *E. coli* alonecaused minimal host death, while co-injection caused significant host mortality (p < 0.0001) and augmentation of pathogenesis.

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| Figure 3.1 *S. aureus* virulence is augmented by Gram- negative commensal flora in the zebrafish embryo infection model  Survival curves of zebrafish embryos (n=30) injected with **(A)** *R. mucosa* (RMAD) (2500, 4000, 6000, CFU); **(B)** *R. mucosa* alone (2000 CFU), *S. aureus* alone (150 CFU), or a combination of *S. aureus* (150 CFU) and *R. mucosa* (2000 CFU) ; **(C)** *E. coli* (EC) (2000, 3000, 5000 CFU); **(D)** *E. coli* (EC) alone (3000 CFU), *S. aureus* alone (150 CFU), or a combination of *S. aureus* (150 CFU) *E. coli* (EC) (3000 CFU) ; SH1000 (1500 CFU) was used as positive control in (A) and (C).  \*\*\*\* p < 0.0001, \*\*\* p < 0.001, \* p = 0.05 ; ns – non significant. |

### Augmentation of *S. aureus* infection in the murine sepsis model with Gram-positive organisms

Initial analysis was to determine appropriate dosing levels for the commensal bacteria. Therefore *S. epidermidis* was injected IV at 1.3x107, 1.9x108 and 4.7x 108 CFU into female BALB/c mice. *S. epidermidis* 138, used in all following experiments is a clinical isolate that had been serially passaged on agar plates with rifampicin, in order to introduce a spontaneous RifR mutation. This was important to differentiate between *S. aureus* (NewHGKanR)and *S. epidermidis* isolated from the same organ in future experiments. Only the highest dose caused weight changes in the injected group, with up to 20% weight loss, while mice receiving lower doses did not lose weight from the beginning of the experiment (Figure 3.2 A). Doses up to 1.9 x108 CFU led to few bacteria recovered (up to 104 CFU) from kidneys and liver. With a dose of 4.7 x 108 CFU there were substantial numbers (up to 108 CFU) recovered from kidneys and liver (Figure 3.2 B, C). It was therefore concluded from this pilot study that 1x108 CFU *S. epidermidis* is the optimal dose for further augmentation experiments.

As there was no previous data on the effect of co-injection of *S. epidermidis* and *S. aureus* live bacterial species into mice, a pilot experiment was conducted, with 5 female Balb/c mice as control. *S. epidermidis* RifR was injected alone (1x108 CFU), *S. aureus* NewHG KanR (1x106 CFU) alone or together with low dose *S. aureus* NewHG KanR (1x106 CFU) (Figure 3.2 B). The co-injected group lost the most weight (p=0.0238) compared to either *S. epidermidis* or *S. aureus* alone, however recovered kidney bacterial numbers showed few *S. aureus* or *S. epidermidis* and this data was non-significant between the groups. Interestingly, recovered *S. aureus* bacterial numbers from the livers of the mixed group showed a significant increase compared to *S. aureus* injectedalone (p = 0.0079) (Figure 3.2 F, black circles). Moreover, there was no significant difference in the amount of *S. epidermidis* recovered from either mixed or single *S. epidermidis* group (Figure 3.2 F, red circles), showing no alteration in clearance of this organism. This suggests that *S. epidermidis,* unlike *S. aureus,* does not benefit from the presence of another bacterial species in this model of infection. Next, the same experiment was repeated but this time using 10 mice per group (Figure 3.3) and similarly there was significant weight loss in the mixed dose group (Figure 3.3 A), recovered *S. aureus* was significantly higher in the mixed dose group compared to *S. aureus* alone, and *S. epidermidis* did not seem to benefit from co-infection (Figure 3.3 B). Moreover, bacterial numbers in kidneys were low for all groups (Figure 3.3 C).

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| Figure 3.2 *S. epidermidis* dose titration and initial co-injection with *S. aureus*  Low dose *S. aureus* alone (1.4 x 106CFU), *S. epidermidis* alone (1.3 x107, 1.9 x108, 4.7 x108 CFU)or a combination of *S. aureus* (1.4 x 106CFU) and *S. epidermidis* (1 x108 CFU) was injected IV. Weight loss **(A, D)** and CFU in kidneys **(B, E)** and liver **(C, F)** were recorded; n=5, \*\* p <0.01, \* p = 0.05 ; ns – non significant. Red circle – *S. epidermidis*; black circle – *S. aureus*. |
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| Figure 3.3 *S. epidermidis* can augment *S. aureus* infection in the murine sepsis model  Low dose *S. aureus* alone (1 x 106CFU), *S. epidermidis* alone (1 x108 CFU)or a combination of *S. aureus* and *S. epidermidis* was injected IV (n=10). Weight loss **(A)** and CFU in kidneys **(B)** and liver **(C)** were recorded; n=10, \*\* p <0.01, \* p = 0.05 ; ns – non significant. Red circle – *S. epidermidis*; black circle – *S. aureus*. |

*M. luteus* was used as an alternative Gram-positive augmenting organism. Therefore as a pilot study, *M. luteus* doses of 3.8x107, 1.9x108 or 2.9x108 were injected into Balb/c mice with 5 animals per group, in order to determine an optimal dose for further augmentation experiments (Figure 3.4 A). None of the doses administered caused any change in animal weights or health and at the end of the experiment all *M. luteus* bacteria were cleared from livers and kidneys of injected mice. It was therefore decided to use *M. luteus* 2x108 CFU as the optimal for augmentation experiments.

To check for augmentation, a pilot experiment with 5 Balb/c mice per group, where *M. luteus* was injected alone (2x108 CFU), low dose *S. aureus* (2x106 CFU) alone or co-injection of both. Mice in the mixed group lost significantly more weight than those in the control *S. aureus* alone group (p= 0.023) (Figure 3.4 B). What is more, *S. aureus* bacterial numbersrecovered from the livers of the mixed inoculum group were significantly higher than the control (p = 0.024), with all *M. luteus* bacteria cleared at the end of the experiment (Figure 3.4 D).There was no significant difference between control and mixed groups seen in *S. aureus* bacterial kidney numbers, with *M. luteus* completely cleared from this organ (Figure 3.4 C). This experiment was repeated with 10 Balb/c mice for *S. aureus* alone (1x106 CFU) or *S. aureus* (1x106 CFU)mixed with *M. luteus* (1x107 CFU) per group, and 20 Balb/c mice for *S. aureus* (1x106 CFU)mixed with *M. luteus* (2x108 CFU)(Figure 3.4 E, F, G). This experiment showed significant decrease in weights when *S. aureus* was co-injected with 2 x 108 CFU *M. luteus*, but not when co-injected with the lower dose of 1x107 CFU (Figure 3.4 E). However, the lower *M. luteus* dose when co-injected with *S. aureus* augmented in the liver, with a significantly higher bacterial load than in the liver of *S. aureus* control mice (Figure 3.4 G).

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| Figure 3.4 *M. luteus* augments *S. aureus* pathogenesis in the murine sepsis model  Balb/c mice (n=5) were injected into the tail vein with: *M. luteus* alone (3.8x107, 2.8x108,1.9x108 or 2.9x108 CFU), *S. aureus* alone (2x106 CFU), or a combination of *S. aureus* (2x106 CFU) and *M. luteus* (2x108 CFU). Weight loss **(A, B,)** as well as kidney **(C)** and liver **(D)** CFUs were recorded; Balb/c mice (n=10) and Balb/c mice (n=20) were injected into the tail vein with *S. aureus* alone (1x106 CFU), or a combination of *S. aureus* (1x106 CFU) and *M. luteus* (2x108, 1x107 CFU). Weight loss **(E)** as well as kidney **(F)** and liver **(G)** CFUs were recorded; \*\*\*\* p < 0.0001, \*\*\* p < 0.001, \* p = 0.05, ns – non significant. |

Having established that commensalbacteria such as *M. luteus* are capable of augmenting *S. aureus* infection, it was of interest to see If the dose of either *S. aureus* or *M. luteus* could be lowered to equate the numbers found on normal human skin. Grice *et al.* (2011) reported commensal density of around 1x107 CFU cm-2 on the skin surface of healthy volunteers(Grice and Segre, 2011b) and therefore *S. aureus* was injected at various doses (1x106, 2.1x105, 2x104, 8.9x102 CFU) alone or mixed with 1x108 CFU *M. luteus* (Figure 3.5A, B, C). Remarkably, a 1000- fold difference in injected bacterial numbers between *S. aureus* (2.1x105) CFU and *M. luteus* (1x108 CFU)led to augmentation of infection in murine livers (Figure 3.5 C). The experiment was repeated with a dose titration of *M. luteus* (1x107, 1x106, 1x105 CFU) together with low dose *S. aureus* (1x106 CFU) (Figure 3.5 D, E, F), showing that an equal quantity of *M. luteus* (1x106 CFU) are capable of augmenting *S. aureus* (1x106 CFU) pathogenesis in murine livers(Figure 3.5 F), as there was significant difference between bacteria isolated from livers in *S. aureus* alone group (1x106 CFU) and a mix of *S. aureus* (1x106 CFU) and *M. luteus* (1x106 CFU).

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| Figure 3.5 Dose titration of *S. aureus* and *M. luteus* infectious dose  Balb/c mice (n=10) injected with: *S. aureus* alone (9.3x104, 1.7x103, 1x102, CFU) or a combination of *S. aureus* (2.1x105, 2x104, 8.9x102 CFU) and *M. luteus* (1x108 CFU). Weight loss **(A)** as well as CFU from kidneys **(B)** and liver **(C)** was recorded.  ; *S. aureus* alone (1x106 CFU) or a combination of ; *S. aureus* (1x106 CFU) and *M. luteus* (1x107, 1x106, 1x105 CFU). Weight loss **(D)** as well as CFU from kidneys **(E)** and liver **(F)** was recorded. \*\* p < 0.01, \* p < 0.05, ns- not significant. |

### Augmentation of *S. aureus* infection in the murine sepsis model with Gram-negative organisms

As both *E. coli* and *R. mucosa* are able to augment *S. aureus* pathogenesis in the zebrafish model, they were then examined in a model of mouse sepsis. A pilot experiment was conducted where female Balb/c mice were injected with different doses of *E. coli* (2x107, 1.8x106, 2.3x105, 1.8x104, 2.3x103 CFU), with 3 mice per group (Figure 3.6 A). Only the highest dose resulted in up to 10% weight loss, with all other groups remaining at approximately their starting weight. All animals were apparently healthy and did not show signs of illness. Therefore, a dose of 1x106 CFU *E. coli* was chosen for further augmentation experiments.

To check for augmentation, 10 female Balb/c mice per group were injected with 1.2x106 CFU *E. coli* alone or in combination with low dose *S. aureus* (5x106). Low dose *S. aureus* was used as control. Results show that mixed group lost more weight compared to the *S. aureus* alone control (p < 0.0001) ( B). Moreover, both *S. aureus* and *E. coli* recovered from the livers of the mixed groups were significantly higher compared to *S. aureus* or *E. coli* alone controls (p = 0.0003 and p < 0.0001) ( D). This suggests a previously undescribed effect of co-injection where both organisms benefit during infection.

Similarly, an *R. mucosa* dosing experiment was conducted, resulting in complete clearance of *R. mucosa* from liver and kidneys of the mice when injected at doses up to 2x108 CFU, as well as no change in weight loss (data not shown). Therefore, mice were injected with *R. mucosa* alone (2x108 CFU), low dose *S. aureus* alone or a combination of low dose *S. aureus* (1x106 CFU) and *R. mucosa* (2x108 CFU). *S. aureus* low dose alone was used as control (). While there was no significant difference in the weights ( E), there was a higher amount of *S. aureus* isolated from the livers of the mixed group compared to control (p = 0.0397) ( F). *R. mucosa* was completely cleared from the organs examined (results not shown).

Both *E. coli* and *R. mucosa* are able to augment *S. aureus* infection in the murine sepsis model (). Interestingly, *R. mucosa* is completely cleared, whereas *E. coli* benefits from the interaction.

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| **% of initial weight** |
| Figure 3.6 Augmentation of *S. aureus* pathogenesis by Gram-negative commensals  Balb/c mice (n=3) injected with **(A)** *E.coli* (EC) alone (2x107,1.8x106, 2.3x105, 1.8x104, 2.3x103 CFU); Balb/c mice (n=10) injected with **(B, C, D)** *E.coli* (EC) alone (5x106 CFU), *S. aureus* NEWHGkanR alone (1.2x106 CFU), or a combination of: *S. aureus* NEWHGkanR (1.2x106 CFU) and *E.coli* (5x106 CFU); Balb/c mice (n=10) injected with **(E, F)** *R. mucosa* (RMHM) alone (2x108CFU), *S. aureus* NEWHGkanR alone (1x106 CFU), or a combination of: *S. aureus* NEWHGkanR (2x106 CFU) and *R. mucosa* (RMHM) (2x108CFU).  \*\*\*\* p < 0.0001, \*\*\* p < 0.001, \*\* p <0.01, \* p = 0.05 ; ns – non significant. |

## Discussion

It has widely been thought that *S. aureus* overwhelms the host through a plethora of virulence factors such as toxins (Otto, 2014), leading to bacterial propagation and eventual death of the host.What is more, animal models of infection have required a large initial inoculum to mimic *S. aureus* sepsis, though the reason for this has remained undetermined. Recently research into this area has gained some insight, with studies showing that out of all the bacterial inoculum injected into a fish embryo, only select few bacteria manage to escape killing by host immune mechanisms and go on to establish abscesses to ultimately cause host mortality (Prajsnar, Hamilton, Garcia-Lara, McVicker, *et al.*, 2012; McVicker, Tomasz K. Prajsnar, *et al.*, 2014). This phenomenon also happens in the mammalian sepsis model in terms of clonality abilities of the bacteria (McVicker, Tomasz K. Prajsnar, *et al.*, 2014). Such findings brought into question the role that the rest of the bacterial inoculum plays during infection and whether it simply acts as a distraction or ‘fog –screen’ to the immune system. This may be in order to allow a few single bacteria to remain viable and found future bacterial populations within the host.

Previous findings in our laboratory by Dr. Emma Boldock have shown the ability of Gram-positive bacteria, but not beads, to cause augmentation of *S. aureus* infection in the zebrafish. My experiments have demonstrated the same can be achieved with Gram-negative organisms such as *R. mucosa* and *E. coli.* Similarly, in the murine model of infection several Gram-positive and Gram-negative organisms managed to augment *S. aureus* infection. Such results may start to reveal the potential mechanism of infection initiation, as it has been suggested that most *S. aureus* infections are opportunistic, and require the entry of the bacteria through a wound or together with a surgical appliance such as an intravascular device (Naber, 2009). It is evident that due to the presence of a rich microbiome, it is highly unlikely for the wound to contain pure *S. aureus* alone(Grice and Segre, 2011a)*.* What is more, it is possible that the other bacteria present at the site of infection do not contribute to disease severity, but rather are used by *S. aureus* to propagate infection. Moreover, while the typical dose for sepsis in a similar murine model is 1x107 CFU *S. aureus,* the co-injection model has been able to bring down the infectious dose of *S. aureus* almost 1000 fold, due to co-administration ofcommensal organism *M. luteus,* suggesting that we are closer to a more physiological model of *S. aureus* disease.

Surprisingly, there are few studies looking to establish the mechanics of a polymicrobial infection. However, some have looked into cases were colonization by commensal bacteria, such as *S. epidermidis,* is capable of providing immunity against *C. albicans* infection through eitherCD-8+ T cellactivation or IL-17A upregulation, as when either was depletedthe protective effect was completely abolished (Naber, 2009). What is more, *Ruminococcus obeum,* a common intestinal commensal, when introduced into gnotobiotic mice, provided protection against *Vibrio cholerae* (Hsiao *et al.*, 2014)*.* This may suggest that the timing of bacterial administration during polymicrobial infection is crucial. This opens up the complexity of how our immune system interacts with pathogens and commensal organisms.

The difference between commensal and pathogenic bacteria is often hard to describe. *S. aureus* is thought to colonise the nose, however when given the opportunity will trigger an invasive infection. Similarly, both *M. luteus* and *S. epidermidis* have been thought to be harmless skin commensals until they were also shown to cause opportunistic infections in the host (Wakabayashi *et al.*, 1991; Yang, Sugawara, *et al.*, 2001; Coates, Moran and Horsburgh, 2014).

# Chapter 4. Effect of cell wall material on host responses and *S. aureus* infection

## Introduction

Previous work in our laboratory supports the hypothesis that *S. aureus* infiltrates cells to evade the host immune system, but in such a way as to disseminate effectively throughout different tissues. For instance, the zebrafish embryo model revealed complete phagocytosis of *S. aureus* occurs immediately following injection of bacterial inoculum into the fish circulation valley.While complete phagocytosis of extracellular bacteria occurred, some *S. aureus* survived, proliferated and formed a lesion large enough to eventually kill embryos (Prajsnar *et al.*, 2008). Subsequently, it was shown that the source of this overwhelming infection in the fish was *S. aureus* that managed to withstand intraphagosomal killing (Prajsnar, Hamilton, Garcia-Lara, McVicker, *et al.*, 2012).

It is known that *S. aureus* is often isolated in conjunction with other pathogens in unwell patients. For instance, while commensal *S. pneumoniae* carriage negatively impacts the amount of commensal *S. aureus* isolated in nasal swabs of healthy subjects, this correlation is lost in sick patients, particularlythose with immunodeficiency disorders (Regev-Yochay *et al.*, 2004; McNally *et al.*, 2006; Quintero *et al.*, 2011). Similarly, the presence of both *S. aureus* and *P. aeruginosa* in cystic fibrosis patients is often associated with a more severe inflammatory response (Wolter *et al.*, 2013; Wong, Ranganathan and Hart, 2013). *S. aureus* and *P. aeruginosa* display synergistic interaction in an *in-vitro* wound infection model, enhancing each other’s antibiotic tolerance when co-cultured *in-vitro* (DeLeon *et al.*, 2014).

Overall, *S. aureus* behaves differently in polymicrobial infection than during infection alone, by establishing cooperative interactions with other bacteria. This pairing hypothesis has been demonstrated with *C. albicans* (El-Azizi, Starks and Khardori, 2004; Harriott and Noverr, 2009)*, E. faecalis* (Flannagan and Clewell, 2002; Ray *et al.*, 2003)and *H. influenzae* (Margolis, Yates and Levin, 2010)*.* Conversely, *S. aureus* still entertains interactions of a more competitive nature with a number of other microorganisms, but this often results in small colony variants, with increased antibiotic resistance and virulence (Margolis, Yates and Levin, 2010).

While polymicrobial infections are quite common, it is often found that commensal bacterial colonization precedes infection. For example, a skin wound contaminated with *S. aureus* is likely to test positive for skin commensal organisms such as *S. epidermidis.* Such skin and soft tissue commensals, are known to inhibit *S. aureus* colonization. For instance, *S. epidermidis* autoinducing peptide has been shown to inhibit the production of *S. aureus α* - andβ- toxins through inactivation of *agr-1Sa and agr-3Sa* (Dufour *et al.*, 2002). Notwithstanding this benefit, commensal *S. epidermidis, M. luteus* and *Corynebacterium* *spp*. have also been linked to invasive bacteraemias (von Eiff *et al.*, 1996; Cheung and Otto, 2010; Ghide *et al.*, 2010; Miltiadous and Elisaf, 2011).

It is important to understand the difference between *S. aureus* bacteraemia, sepsis and septic shock. While there are no definitions set in stone, in 2016 the understanding behind these terms have been revised and recommendations on their use put into place (Singer *et al.*, 2016). As such, bacteraemia is referred to the presence of bacteria in the bloodstream without a threat to the host, and is usually cleared by the immune system. However, bacteraemia could progress to septicaemia and sepsis, where bacterial load in the bloodstream becomes uncontrollable for the immune system leading to a dysregulated immune response, cytokine storm and organ dysfunction. While sepsis is deemed life-threatening, if left untreated patients go on to develop septic shock, characterised by severe circulatory disruptions such as microvascular oedema, leading to organ failure and death (Pravda, 2014).

Mouse models of *S. aureus* sepsis are quite common and animals receiving 1x107-1x108 CFU *S. aureus* Newman results in 100% mortality 48 h after injection into the tail vein, as well as histopathological lesions on recovered organs (Cheng *et al.*, 2010). What is more, the first signs of sepsis can be seen a mere 3-4 h following infection, with animals displaying signs of disease such as isolation, hunched back and ruffled fur. Interestingly, a recent study has been able to demonstrate the hallmarks of sepsis and septic shock in the zebrafish through an immersion model of LPS infection (Philip and Wang, 2017). Philip *et al.* had shown that 3dpf zebrafish embryos receiving 200 µg/ml LPS had 100% mortality rate within the first 8 h following infection, while embryos receiving 100 µg/ml LPS succumbed to sepsis-like symptoms, including vasculature leakage due to dysregulated junction proteins, upregulated cytokine response and an increase in ROS production.

Recent work carried out within the Florey Institute, University of Sheffield (Boldock, 2016) has shown that *S. aureus* infection could be augmented by bacterial cell wall material, in the zebrafish embryo model, without causing immediate septic shock.

This observation was therefore further tested to establish whether inflammation can be augmented using bacterial components in the zebrafish embryo and whether this material is capable of augmenting *S. aureus* ina murine model of infection.

## Aims

1. To understand the effect of cell wall material on the zebrafish host.
2. To understand the role of cell wall augmenting material in the murine host and its combined role with the pathogen.

## Results

### Effect of bacterial cell-wall material on the zebrafish host

#### Co-injection of peptidoglycan and lipoteichoic acid

Synergistic interactions between PGN and other components of the bacterial cell wall have been demonstrated in rats, where they have shown to cause septic shock and multiple organ failure, i.e. LTA (lipoteichoic acid) from *S. aureus* was capable of synergising with *B. subtilis* PGN (De Kimpe *et al.*, 1995; Kengatharan, De Kimpe and Thiemermann, 1996; Kengatharan *et al.*, 1998; Morath *et al.*, 2002). This indicates that conserved motifs of PGN, such as the glycan backbone can further stimulate the immune system in addition to the response caused by LTA alone. It was therefore possible that a synergistic effect between *S. aureus* PGN and *S. aureus* LTA could be seen.

In order to explore this further, 30 zebrafish embryos were injected with varying doses of LTA to check for mortality (Figure 4.1 A). With all the different amounts of LTA alone tested, none showed any impact on host mortality. It was therefore tested whether the combination of PGN and LTA would synergise to cause host death ( B). To date, peptidoglycan had not been injected into the zebrafish and therefore the attempt was to inject an amount of peptidoglycan equivalent to bacterial CFU. Previous findings by Timmerman *et al.* have shown that 1x107 *S. aureus* bacteria is roughly equivalent to 0.1 µg of peptidoglycan (Timmerman *et al.*, 1993). Keeping with these findings and the notion that peptidoglycan constitutes approximately 10% of the bacterial cell wall (personal communication, Dr. Stephane Mesnage), *S. aureus* grown from an overnight culture to 2x108 CFU/ml yielded 26 µg of peptidoglycan. Therefore 1500 CFU of *S. aureus* could be roughly attributed to 0.2 ng of peptidoglycan. However, due to the rough approximation, 2.5 ng of peptidoglycan was used in all zebrafish experiments unless otherwise stated. With PGN tested with varying concentrations of LTA, none showed an increase in host mortality. *S. aureus* SH1000 injection was used as a positive control in both experiments.

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| LTA (2.5 ng)  LTA (5 ng)  LTA (15 ng)  SH1000 (1654 CFU)  PGN + LPS (5 ng)  PGN + LPS (2 ng)  PGN + LPS (0.5 ng)  PGN (2.5 ng)  PGN + LTA (2.5 ng)  PGN + LTA (5 ng)  PGN + LTA (15 ng)  SH1000 (1482 CFU)  LPS (0.5 ng)  LPS (2 ng)  LPS (5 ng)  SH1000 (1482 CFU)  SH1000 (1300 CFU)  HK SH1000(3000 CFU)  HK SH1000(5000 CFU)  HK SH1000(6000 CFU)  SH1000 (1278 CFU)  HK SH1000(7000 CFU)  HK SH1000(9000 CFU)  SH1000 (1379 CFU)  SH1000 (150 CFU)  SH1000 + HK SH1000 (7000 CFU)  SH1000 + HK SH1000 (3000 CFU)  SH1000 + HK SH1000 (1500 CFU)    Figure 4.1 Effect of bacterial components on zebrafish survival |
| Survival curves of zebrafish embryos (n=30) injected with: **(A)**LTA (2.5, 5, 15 ng); **(B)** PGN or PGN + LTA(PGN: 2.5 ng, LTA: 2.5, 5, 15 ng); **(C)** LPS (0.5, 2, 5 ng); **(D)** PGN + LPS (PGN: 2.5 ng, LPS: 0.5, 2, 5 ng); **(E,F)** Heat-killed *S. aureus* SH1000 (3000, 5000, 6000, 7000, 9000 CFU); **(G)** *S. aureus* SH1000 low dose (150 CFU) alone or together with heat-killed (HK) *S. aureus* SH1000 (1500, 3000, 7000 CFU).  In all experiments *S. aureus* SH1000 (1500 CFU) was used as positive control for fish mortality.  \*\*\*\* p < 0.0001, \*\*\* p < 0.001, \* p = 0.05, ns – non significant |

#### Combination of peptidoglycan and lipopolysaccharide

A number of bacterial components are known to increase the host inflammatory reaction to bacterial infection, such as LPS (lipopolysaccharide)(Ferrante *et al.*, 1984).What is more, LPS has been repeatedly shown to cause upregulated inflammation through synergistic action with MDP, a structural subunit of PGN (Takada and Galanos, 1987; Wolfert *et al.*, 2002). It was therefore investigated whether the same synergy would be observed in the zebrafish model.

To test this, 30 zebrafishembryos were injected with varying doses of LPS to check for mortality. Previous studies have demonstrated that LPSinjection in the form of pure material or heat-killed Gram-negative bacteria is only lethal if delivered into the yolk sack of the zebrafish embryo (Clatworthy *et al.*, 2009; L. L. Yang *et al.*, 2014). Here, LPS was injected at 0.5, 2, 5 ng into the circulation valley of the zebrafish. LPS alone ( C) or co-injection with PGN ( D) did not cause any significant impact on host mortality.

#### Combination of *S. aureus* and heat killed *S. aureus*

Heat-killed (HK) Gram positive bacteria have been shown to induce immune system activation, such as complement activation, TNF- alpha upregulation and tissue damage in rabbits(Wakabayashi *et al.*, 1991). Furthermore, this response was comparable to that of *E. coli* injection in the same animal model. It was therefore investigated whether heat killed *S. aureus* would kill in the zebrafish model alone or with low dose of live *S. aureus.*

To test this, 30 zebrafishembryos were injected with heat killed SH1000 at 3000, 5000, 6000,7000, 9000 CFU ( E, F), with minimal host death. It was not possible to go above 9000 CFU as this blocked the needle and creating a needle with a larger diameter damaged the embryos, causing them to bleed out following injection (data not shown). After establishing that the zebrafish embryos could tolerate large HK bacterial CFU injections, they were co-injected with a low dose (150 CFU) of *S. aureus* SH1000 (live bacteria) and 7000, 3000, 1500 CFU of HK bacteria. In these experiments the highest CFU was 7000 as combined with live bacteria, a higher CFU blocked the needle. Following 96 h after injection, there was no significant difference between co-injected fish and those only with low dose *S. aureus* (Figure 4.1 G).

### Effect of phagocyte depletion on the response of zebrafish embryos to cell wall components

Temporary genetic knock-down methods have been developed for zebrafish embryos. For example, morpholinos disable initiation of translationand therefore act through a RNAse-H- independent mechanism resulting in accurate and predictable *in-vivo* targeting with few side effects (Summerton, 1999). To determine the role of phagocytes in the response to bacterial cell wall components, the PU.1 morpholino was used to temporarilydeplete embryos of myeloid stem cell progenitors (Klemsz *et al.*, 1990). As a result of PU.1 injection at the 1-4 cell stage of development, neutrophils can be recovered only after 36 h and macrophages after 48h post fertilisation. Previous work has shown that PU.1 injected embryos have approximately a 90% mortality rate within 26 h (Prajsnar, Hamilton, Garcia-Lara, McVicker, *et al.*, 2012) after 1500 CFU *S. aureus* injection.

#### Combination of lipoteichoic acid and *S. aureus*

LTA (lipoteichoic acid) has been identified in playing a role in initiating ROS (reactive oxygen species) release in macrophages and neutrophils once bound to the CD14 receptor on these cells (Triantafilou, Triantafilou and Dedrick, 2001). What is more, LTA is also known for binding to metHband stimulationof ROS production by metHb-POX, potentially harmful not only to bacteria but also to host blood cells (Lee *et al.*, 2015). It was therefore investigated whether LTA could synergise with *S. aureus* PGN in the absence of macrophages and neutrophils, to affect host mortality.

First, LTA was administered to PU.1 pre-injected embryos at 5 and 15 ng to check for host lethality. SH1000 (1500 CFU) was used as a positive control and etox-PBS was used as a negative control in all experiments. On its own LTA is not capable of causing host lethality in PU.1 embryos (Figure 4.2 A). It was then further tested if a combination of 2.5 ng *S. aureus* PGN and 5 or 15 ng LTA would synergize in the PU.1- treated zebrafish embryos. However, no significant difference between injection of bacterial components and the PBS control group (Figure 4.2 B).

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| LTA (5 ng)  PBS (endotoxin-free)  LTA (15 ng)  SH1000 (1567 CFU)  PBS (endotoxin-free)  PGN + LTA (15 ng)  PGN + LTA (5 ng)  SH1000 (1400 CFU)  PBS (endotoxin-free)  LPS (2 ng)  LPS (5 ng)  SH1000 (1488 CFU)  PGN + LPS (15 ng)  PGN + LPS (5 ng)  PBS (endotoxin-free)  SH1000 (1552 CFU)  HK SH1000 (5000 CFU)  HK SH1000 (7000 CFU)  PBS (endotoxin-free)  SH1000 (912 CFU)  PGN + HK SH1000 (5000 CFU)  PGN + HK SH1000 (7000 CFU)  PBS (endotoxin-free)  SH1000 (1165 CFU)  Figure 4.2 Bacterial components alone or in combination with PGN do not impact zebrafish embryo survival when myeloid cell progenitors are depleted (PU.1 morpholino injection) |
| Survival curves of PU.1- treated zebrafish embryos (n=30) injected with: **(A)**LTA (5 and 15 ng); **(B)** PGN + LTA(PGN: 2.5 ng, LTA: 5 and 15 ng); **(C)** LPS (2 and 5 ng); **(D)** PGN + LPS (PGN: 2.5 ng, LPS: 2 and 5 ng); **(E)** Heat-killed *S. aureus* SH1000 (5000 and 7000 CFU); **(F)** PGN +HK *S. aureus* SH1000 (5000 and 7000 CFU).  In all experiments *S. aureus* SH1000was used as positive control and endotoxin-free PBS as negative control. \*\*\*\* p < 0.0001, \*\*\* p < 0.001, \* p = 0.05, ns – non significant |

#### Combination of lipopolysaccharide and *S. aureus* peptidoglycan

Recent finding have suggested that zebrafish possess homologues of mammalian TLR-4, zTLR4 and hTLR4, responsible for LPS signalling (Meijer *et al.*, 2004). It was therefore tested whether the absence of myeloid progenitor cells would impact host survival, when challenged with LPS or combination of LPS and PGN.

To investigate this, 5 and 15 ng of LPS was injected into PU.1 pre-treated zebrafish embryos. SH1000 (1500 CFU) was used as a positive control and endotoxin- free PBS as a negative control. In both experiments, there was no significant difference in host mortality of LPS treated embryos compared to etox-PBS treated group (Figure 4.2 C). What is more, co- injection of 5 or 15 ng LPS together with 2.5 ng of PGN also showed no significant difference to PBS-only treated group (Figure 4.2 D).

#### Combination of heat-killed bacteria and *S. aureus* peptidoglycan

Activators of the immune system such as LTA and LPS did not have an effect on zebrafish embryo host mortality and therefore it was tested if any components of the bacterial cell wall are capable of augmenting within the embryo in the absence of myeloid cell progenitors. To investigate this, SH1000 was heat-killed by incubation for 30 min at 70°C. Injection of 5000 and 7000 heat-killed CFU into the circulation valley of PU.1 pre-treated zebrafish did not impact host mortality and there was no statistical difference between groups injected with heat-killed bacteria compared to etox- PBS (Figure 4.2 E). Similarly, co- injection of heat-killed bacteria up to 7000 CFU and PGN had no impact on host survivability.

To test the role of different peptidoglycan structures in the phagocyte depleted zebrafish embryo, PGN from *C. flaccumfaciens, M. luteus* and *B. subtilis* (Figure 4.3)was tested in wild-type and PU.1 treated embryos as described above.

Peptidoglycan from *C*. flaccumfaciens, *M. luteus* or *B. subtilis* had no impact on zebrafish embryo health, in the presence (Figure 4.4 A) or absence (Figure 4.4B) of macrophages and neutrophils.

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| Figure 4.3 Various structures of peptidoglycan from Gram-positive bacteria.  Glycan backbone and peptide chain from **(A)** *M. luteus,* **(B)** *S. epidermidis,* **(C)** *S. aureus,* **(D)** *C. flaccumfaciens,* **(E)** *B. subtilis* |

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| *C. flaccumfaciens* (2.5 ng)  *B. subtilis* (2.5 ng)  *M. luteus* (2.5 ng)  SH1000 (1534 CFU)  *C. flaccumfaciens* (2.5 ng)  *B. subtilis* (2.5 ng)  *M. luteus* (2.5 ng)  SH1000 (1468 CFU)  Figure 4.4 Peptidoglycan from other bacterial species does not impact zebrafish embryo health  Survival curves of wild type zebrafish embryos (n=30) wild-type **(A)** or PU.1 treated **(B)** injected with PGN from: *C. flaccumfaciens* (2.5 ng), *B. subtilis* (2.5 ng), *M. luteus* (2.5 ng)  SH1000 was used as a positive control. \*\*\*\* p < 0.0001. |
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#### Role of *S. aureus* lipoproteins in host-pathogen interaction

Lipoproteins are known to be immunostimulatory and have beenimplicated in NF-kB activation in zebrafish embryos(Norgard *et al.*, 1996; Rawadi *et al.*, 1999). It was therefore of interest whether an *S. aureus* mutant devoid of a diacylglyceryl transferase (*lgt)* would be of any significant difference in pathogenicity compared to wild type *S. aureus,* capable of producing mature lipoproteins (Stoll *et al.*, 2005b). *S. aureus* SH1000*∆lgt* mutant was injected into WT (Figure 4.5 A) and PU.1 pre-treated (Figure 4.5 B) embryos. SH1000*∆lgt* washeat-killed by incubation at 70°C for 30 mins. There was no difference in lethality for zebrafish between SH1000 and SH1000*∆lgt* strains, irrespective of presence or absence of myeloid progenitor cells.

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| SH1000𝛥*lgt* (1318 CFU)  HK SH1000𝛥*lgt* (1670 CFU)  SH1000(1696 CFU)  HK SH1000𝛥*lgt* (1670 CFU)  PBS (endotoxin-free)  SH1000𝛥*lgt* (1687 CFU)  SH1000(1450 CFU)    Figure 4.5 There is no difference in host lethality of zebrafish embryos injected with *S. aureus* or *S. aureus* lacking lipoproteins.  Survival curves of zebrafish embryos (n=30) wild-type **(A)** or PU.1 treated **(B)** injected with: SH1000*∆lgt* (A: 1318 CFU, B: 1687 CFU)*,* HK SH1000*∆lgt* (1670 CFU).  SH1000 was used as a positive control. \*\* p < 0.01, \*\*\* p < 0.001, ns- not significant. |
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### Investigation of the role of peptidoglycan in the murine systemic model of infection

In order to test the role of peptidoglycan alone and with other bacteria or cell wall components, it was essential to first conduct dosing experiments to determine its effect on murine host health. It has been well documented that LPS is a major driver of Gram-negative sepsis (Morrison and Jacobs, 1976; Kohn and Kung, 1995). Similarly, peptidoglycan has been demonstrated as being the major player in inducing Gram-positive sepsis and organ failure ina rat model of infection (Kengatharan *et al.*, 1998). Moreover, it has been shown that LTA and PGN from *S. aureus* synergise to increase TNF-α and INF-γ expression and therefore cause septic shock in rats (De Kimpe *et al.*, 1995). Additionally, cell wall preparations of several bacteria have been shown to cause a significant inflammatory response in murine models of infection (Tuomanen *et al.*, 1985; Carlsen *et al.*, 1992). In particular, cell wall preparations from *M. luteus* caused rapid death in mice primed with MDP (Monodane, Kawabata and Takada, 1997b). In contrast, a previous study demonstrated that a PGN injection up to 4mg per mouse caused no serious impact on murine health (Šverko *et al.*, 1994).

#### The effect of peptidoglycan from *S. aureus,* *B. subtilis, M. luteus, C. flaccumfaciens, B. cereus* and *S. epidermidis*

Following the doses administered to mice and rats in previous studies, a range of PGN from 200 µg to 1mg was administered to 6-7 week old female BALB/c mice. Weight loss was judged as a marker of health. Interestingly, *S. aureus* PGN was more potent than *M. luteus* PGN, as after administration of 1mg into mice, they had to be culled 24 h into the experiment (Figure 4.6 A). All other PGN, including that isolated from *B. subtilis, C. flaccumfaciens, S. epidermidis, B. cereus* and *M. luteus,* resulted in stable mouse health throughout the 72 h experiment. It is of utmost importance that administered peptidoglycan cause minimal weight loss in the animal model when looking for *S. aureus* increased pathogenesis by peptidoglycan in order to avoid additive effects. Therefore 200µg of *S. aureus* and 500µg of *M. luteus* PGN was chosen for further experiments.

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| Figure 4.6 Murine weight loss as an indication of health state in response to different doses and types of peptidoglycan.  Recorded weight loss in mice (n=5) injected with **(A)** A range (200 µg-1mg) of PGN from *M. luteus* or *S. aureus ;* **(B)** either 200 µg *S. epidermidis* PGN, or 500 µg PGN from different bacterial species (*S. epidermidis, B. subtilis, C. flaccumfaciens, M. luteus, B. cereus).* |

#### Role of wall teichoic acids

Wall teichoic acids are one of the most abundant polymers linked to the PGN of Gram-positive bacteria (Neuhaus and Baddiley, 2003), and have been shown to be important in recognition by the host, as mannose-binding lectins and Immunoglobulin favour binding toWTA’s, triggering complement activation (Park, Lee and Choi, 2014). Therefore, it was important to determine the role of WTAs. To test this, PGN was made as standard, however the 48% v/v HF treatment step was omitted (unstripped). 2 groups of BALB/c mice were injected with standard (HF-stripped) or unstripped *S. aureus* PGN (Figure 4.7). Results demonstrate that there is no statistical difference in mouse health based on weight between the experimental groups.

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| Figure 4.7 Effect of removal of wall teichoic acids from the peptidoglycan preparation on murine weight loss.  Recorded weight lossin mice (n=5) of mice injected with 500 µg of PGN treated with HF (HF-stripped) or HF step omitted (unstripped). |
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### Investigating the effect of peptidoglycan administration on *S. aureus* pathogenesis in the murine sepsis model

Mouse models have historically been used to study *S. aureus* pathogenesis (Kim, Missiakas and Schneewind, 2014), as 80% of mouse genes have been shown to have anorthologue in the human genome (Chinwalla *et al.*, 2002). Literature describes several successful murine models of *S. aureus* infection, such as skin lesions and abscesses (Kennedy *et al.*, 2010), bacteraemia (Cheng *et al.*, 2009), sepsis (Stearns-Kurosawa *et al.*, 2011) and arthritis (Bremell *et al.*, 1991). Moreover, previous work in our lab has used data obtained from the zebrafish to inform further mouse experiments (McVicker, Tomasz K Prajsnar, *et al.*, 2014). However, mouse models of infection require considerably higher bacterial inoculum than fish, with doses ranging from 1x107to 1x108.

In all the following work, the limit of bacterial detection in the kidneys was 20 CFU and 30CFU for livers. Statistical significance was established using non-parametric tests, with p values outlined for each individual case.

The NewHG KanR *S. aureus* strain was used in all experiments, unless otherwise stated, as this has been previously tested in a mouse model of infection (McVicker, Tomasz K Prajsnar, *et al.*, 2014). It is a derivative of Newman (Lorenz and Duthie, 1952), with *saeS* repaired, meaning that unlike Newman, NewHG does not express abnormally high levels of toxins (Mainiero *et al.*, 2010), and is comparable to other *S. aureus* strains. Moreover, the used NewHG carries a kanamycin resistance cassette (KanR), allowing the ability to enumerate it compared to other bacteria isolated from murine organs (McVicker, Tomasz K. Prajsnar, *et al.*, 2014).

Previous work from our group by Dr. Emma Boldock has shown that some peptidoglycans were capable of augmenting *S. aureus* pathogenesisin a zebrafish model of infection (Boldock, 2016). It was therefore tested if the same can be replicated in the murine model of infection. It had been established during pilot experiments that a high dose of *S. aureus* (1x107 CFU) caused significant health disturbances in the mouse, while a low dose (1x106 CFU) was almost cleared by the host at the end of 72h. Therefore, all augmentation experiments use *S. aureus* SH1000 at 1x106 (low dose), unless otherwise stated. In most experiments 6-7 week old female BALB/c mice were injected with 100 µl of inoculum into the tail vein. Bacteria and peptidoglycan were resuspended in endotoxin-free PBS, with peptidoglycan being sonicated alone prior to administration or mixing with bacteria. Mice were weighed daily and assessed for health at least twice each day throughout the 72-h experiment. Following dissection, relevant organs were harvested and bacterial numbers established. Murine work involving peptidoglycan conducted in collaboration with Dr. Emma Boldock (University of Sheffield).

#### Combination of *S. aureus* bacteria and *S. aureus* peptidoglycan

First, 200 µg of peptidoglycan isolated from *S. aureus* SH1000 was injected alone or in combination with *S. aureus* NewHGKanR (1.5x106 CFU). Low dose *S. aureus* alone (1.5x106 CFU) was used as control, with weight loss and recovered CFU’s from kidneys and livers recorded (Figure 4.8 A, C, E). Mice injected with the combined inoculum lost significantly (p < 0.0001) more weight than with bacteria or PGN alone, with two mice in the mixed group had to be culled 10 h before the end of the experiment (blue colour) due to reaching severity limits. Peptidoglycan or *S. aureus* alone did not cause any weight loss.

Significantly higher numbers of bacteria were recovered from the kidneys of mixed group mice (p=0.017), suggesting that PGN caused increased pathophysiological changes in this organ.

The most surprising result can be seen in the livers, where exceptionally high numbers of bacteria were recovered, up to 109 CFU, 1000 times higher than the original inoculum. This was also significantly different (p<0.0001) to bacteria recovered from the *S. aureus* only group, with most hosts having cleared the infection at 72h (Figure 4.8).

PGN isolated without 48% v/v HF- treatment, and therefore retaining its WTA acids, when co-injected with *S. aureus* still caused significant augmentation in murine kidneys (p = 0.03) and livers (p = 0004) (Figure 4.8 D,F), but not weights (Figure 4.8 B), when compared to *S. aureus* alone. It is likely that WTA’s, normally anchored to peptidoglycan via phosphodiester bonds, do not play a significant role in augmentation of *S. aureus* pathogenicity by peptidoglycan.

Therefore, the hypothesis that augmentation of *S. aureus* pathogenesis is possible by peptidoglycan was tested.

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| Figure 4.8 Effect of *S. aureus* peptidoglycan on *S. aureus* pathogenesis in the murine sepsis model  Recorded weight loss in mice (n=10) receiving: **(A)** 200 µg *S. aureus* insoluble peptidoglycan (pPGN) alone or together with low dose *S. aureus* (1.5x106 CFU), or low dose alone (1.5x106 CFU); **(B)** Low dose bacteria (6.5x105 CFU) alone, low dose bacteria (5.5x106 CFU) plus 200µg *S. aureus* insoluble peptidoglycan (pPGN), where **PGN was not treated with 48%** **v/v HF.** Kidney **(C,D)** and liver **(E,F)** CFU were recorded. Blue shapes- mice culled at 62h post infection due to reaching severity limits.  \*\*\*\* p < 0.0001, \*\*\* p < 0.001, \* p = 0.05, ns – non significant | |

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#### Lipoproteins are not responsible for *S. aureus* augmentation with PGN

Lipoproteins have been shown to be highly immunostimulatory in the mouse infection model by binding to TLR2and causing immune activation(Oliveira-Nascimento, Massari and Wetzler, 2012). To investigate whether lipoproteins play a role in pathogenicity, a mutant missing the lipoprotein diacylglyceryl transferase (*lgt)* was used*.* This mutant is unable to complete the first step in lipoprotein biosynthesis, due to lacking the transferase responsible for it. This results in the release of unmodified prelipoproteins and the *lgt* mutant triggering considerably less proinflammatory cytokines and chemokines (Stoll *et al.*, 2005a) *in vitro*. However, in more physiological conditions, such as whole blood preparations, the *lgt* mutant was comparable to wild-type *S. aureus* strains in cytokine induction. This suggests that there is a much more complex interaction of factors necessary for eliciting an immune response than simply lipoproteins. Therefore, to exclude the possibility that lipoproteins are responsible for the apparent augmentation between *S. aureus* and cell wall peptidoglycan, *S. aureus* SH1000*∆lgt* peptidoglycan was used (Figure 4.9). SH1000*∆lgt* peptidoglycan and SH1000 peptidoglycan similarly augmented *S. aureus* pathogenicitywith significant weight loss (Figure 4.9 A) (p =0.02 and p<0.0001), significantly high CFU numbers in livers (Figure 4.9 B) (p = 0.01,p = 0.0004) and in kidneys (Figure 4.9 C) (p = 0.036). Compared to *S. aureus* polymeric peptidoglycan*,* digestion of the glycan backbone of peptidoglycan by mutanolysin completely abolished the augmentation effect, suggesting that an intact sugar backbone is essential.

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| Figure 4.9 Lipoproteins are not responsible for *S. aureus* augmentation with PGN  Recorded weight loss in Balb/c mice (n=10) receiving: 200 μg peptidoglycan from *S. aureus,* *S. aureus Δlgt*, soluble *S. aureus Δlgt* as well as *S. aureus* (1x106 CFU) alone or peptidoglycan in combination with *S. aureus* (1x106 CFU). Weight loss **(A)**, as well as CFU from kidneys **(B)** and liver **(C)** were recorded. \*\*\*\* p < 0.0001, \*\*\* p < 0.001, \* p < 0.05, ns – non significant. |

#### Combination of *S. aureus* and *B. subtilis* peptidoglycan

Peptidoglycan from *B. subtilis* (BS PGN)differs from peptidoglycans in many other Gram-positive bacterial species in that it bears similarities to peptidoglycan found in Gram-negative bacteria, due to containing mesodiaminopimelic acid (m-DAP) at position 3 (Schleifer and Kandler, 1972) (Figure 4.3).

Therefore, to test whether peptidoglycan from *B. subtilis* is capable of augmenting *S. aureus* infection despite a different side chain structure, *S. aureus* (7.9 x105 CFU) was injected alone or co-injected with 500 µg *B. subtilis* PGN. Combination of *B. subtilis* peptidoglycan with *S. aureus* caused a significant difference in weight loss between the groups (p<0.0001) (Figure 4.10 A) and a high amount of bacteria were isolated from mixed group livers (p<0.0001) and kidneys (p=0.0003)(Figure 4.10 B, C). One mouse in the mixed group reached severity limits at 44 hpi and had to be culled.

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| Figure 4.10 Effect of *B. subtilis* peptidoglycan on *S. aureus* pathogenesis  Low dose (7.9x105 CFU) bacteria alone or low dose (1x106 CFU) bacteria plus 500 μg *B. subtilis* peptidoglycan were injected intravenously (n=10). Weight loss **(A)** and CFU in liver **(B)** and kidneys **(C)** were recorded. Red shapes indicates a mouse culled at 44 hpi due to reaching severity limits. \*\*\*\* p < 0.0001, \*\*\* p < 0.001. |

#### Combination of *S. aureus* and *C. flaccumfaciens* peptidoglycan

In contrast to other organisms from which PGN has been extracted, *C. flaccumfaciens* is a typical plant pathogen(Zaumeyer and Rex Thomas, 1957), however recently it has been shown to be isolated fromsome patient samples and involved in septic arthritis (Francis *et al.*, 2011). Its PGN structure is different to that of other Gram- positives in that L-glycine replaces L-alanine as the first amino acid in the peptide side chain (Schleifer and Kandler, 1972) (Figure 4.3). Therefore it was tested whether *C. flaccumfaciens* peptidoglycan (CF PGN) is capable of augmenting *S. aureus* pathogenesis.

To test this, groups of 10 mice each were injected with either low dose *S. aureus* (6.5x105 CFU) alone or co-injected with 500 µg of *C. flaccumfaciens* PGN. Surprisingly, the weights showed no significant difference (Figure 4.11 A) between the groups, while there was significant bacterial CFU recovered from both livers and kidneys (Figure 4.11 B, C) in mice receiving the mixed inoculum. This discrepancy between weights and recovered bacteria had not been shown in any other augmentation experiments.

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| Figure 4.11 Effect of *C. flaccumfaciens* peptidoglycan on *S. aureus* pathogenesis  Low dose (6.5x105 CFU) *S. aureus* alone or low dose (5.6x105 CFU) *S. aureus* plus 500 μg *C. flaccumfaciens* insoluble peptidoglycan were injected intravenously (n=10). Weight loss **(A)** and CFU in liver **(B)** and kidneys **(C)** were recorded. \*\* p < 0.01, \* p < 0.05, ns - non significant. |

#### Combination of *S. aureus* and *S. epidermidis peptidoglycan*.

*S. epidermidis* is a common human commensal and is highly abundant on the skin and important for the maintenance of local homeostasis (Grice and Segre, 2011a). However, clinical observations have suggested that some strains of *S. epidermidis* are capable of invasive disease (Schulin and Voss, 2001). *S. epidermidis* peptidoglycan (SE PGN) is structurally similar to *S. aureus* PGN, and therefore 200µg of *S. epidermidis* PGN was injected alone, or co-injected with 1.2x106 CFU *S. aureus* (Figure 4.12)*. S. aureus* low dose alone was used as control. As with previous experiments, there was a significant difference in weight loss between the groups (p=0.001) (Figure 4.12 A) and the number of bacteria recovered from livers (p<0.0001) and kidneys (Figure 4.12 B, C).

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| Figure 4.12 Co-injection of *S. aureus* and *S. epidermidis* peptidoglycan.  Low dose (1.1x106 CFU) bacteria alone, low dose (1.2x106 CFU) bacteria plus 200 μg *S. epidermidis* peptidoglycan or 200 μg *S. epidermidis* peptidoglycan alone were injected intravenously (n=10). Weight loss **(A)** and CFU in kidneys **(B)** and liver **(C)** were recorded \*\*\* p < 0.001, \*\*\*\* p < 0.0001. |

#### Combination of *S. aureus* and microscopic beads

Having observed that peptidoglycan from different bacterial species, as long as it is polymeric, causes augmentation of *S. aureus* in the mouse sepsis model, it was paramount to determine whether particulate matter would be able to cause the same effect. Zebrafish imaging has shown that 1µm latex polystyrene beads are phagocytosed in a similar way to *S. aureus* (Boldock, 2016). Therefore, mice were injected with 1x108 latex beads alone or together with low dose *S. aureus* (8.5x105 CFU). *S. aureus* alone (7.9x105 CFU) was used as control (Figure 4.13). There was no significant difference between the groups and that co-injection of bacteria with beads does not cause augmentation of infection. However, it is of note that in this experiment there were uncommonly high bacterial numbers isolated from the kidneys of *S. aureus* alone group.

Therefore, the mere presence of phagocytosed particles is not enough to cause augmentation of *S. aureus* disease. This suggests that in these experiments, the phagocytes are not simply overloaded with phagocytosed material and unable to successfully kill bacteria, but rather that there is a more intricate cellular pathway involved.

#### Titration of *S. aureus* infectious dose in the presence of *M. luteus* peptidoglycan

It had been demonstrated in previous experiments that *M. luteus* peptidoglycan causes augmentation of disease when co-injected with 1x106 CFU *S. aureus* NewHG. It was therefore of interest to determine the lowest dose of bacteria at which augmentation could still be seen.

Firstly, *S. aureus* NewHG doses between 1x106 and 1x104 were tested alone or together with 500µg *M. luteus* PGN(Figure 4.14 A). Despite there not being significant difference in weights and kidney CFU numbers in the lowest dose, a significant number of bacteria were isolated from livers of mice receiving a dose of peptidoglycan and 1x104 *S. aureus* NewHG bacteria (p<0.01). As expected, the decrease of bacterial dose correlated with the decrease in bacteria within organs. Decreasing the bacterial dose further down to 1x102 CFU (Figure 4.14 B) showed no significant difference between groups, however it is of note that groups receiving a co-injection of bacteria and peptidoglycan had a few mice with a bacterial load of up to 1x106, whereas there was complete clearance of bacteria in *S. aureus* alone groups. Further, it was investigated whether the dose of *M. luteus* peptidoglycan capable of augmenting *S. aureus* could be lowered. Co-injecting 250 µg of peptidoglycan with low dose bacteria (Figure 4.15) resulted in significant recovery of bacteria from the livers (p=0.016), and while there was no statistical difference between the groups co-injected with 50µg PGN, there is a trend of higher bacterial numbers isolated from the livers than low dose bacteria alone.

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| Figure 4.13 Co-injection of latex beads does not augment *S. aureus* pathogenesis*.*  Low dose (7.9x105 CFU) bacteria, low dose (8.9x105 CFU) bacteria plus 1x108 1 μm polystyrene latex beads or 1x108 latex beads alone were injected intravenously (n=10). Weight loss **(A)** as well as kidneys **(B)** and liver **(C)** CFU were recorded. \* p < 0.05, ns - non significant.   |  |  | | --- | --- | |  |  | | Figure 4.14 Determination of lowest possible dose of *S. aureus* pathogenesis for augmentation with *M. luteus* peptidoglycan  Mice (n=5) injected with a range of *S. aureus* doses: **(B)** (1x102, 7x102, 5x103 CFU), **(A)** (1x104, 1.2x105,1.2x106 CFU) alone or in combination with *M. luteus* peptidoglycan (500 μg). With weights as well as liver and kidney CFU recorded. \* p <0.05; \*\* p < 0.01.   |  | | --- | | A  C  B | | Figure 4.15 Titration of *M. luteus* pPGNfor augmentation of *S. aureus* pathogenesis.  Low dose *S. aureus* (1x106 CFU), with or without *M. luteus* PGN at either 500 µg, 250 µg or 50 µg was injected intravenously in the murine sepsis model (n=5). Weight loss **(A)** and CFU in kidneys **(B)** and liver **(C)** were recorded.\* p < 0.05, \*\* p < 0.01. | | | |

#### Effect of peptidoglycan structure on augmentation of *S. aureus* pathogenesis

Polymeric peptidoglycan structure has been shown to be important for triggering immune events both *in vitro* and *in vivo.* PGN from *B. subtilis* and *C. flaccumfaciens,* not known to cause disease in humans, managed to induce a cytokine response, but only when the glycan backbone was polymeric, both in whole human blood and in a rat model, with this effect completely abolished once the peptidoglycan was digested with mutanolysin (Myhre *et al.*, 2004). Moreover, peptidoglycan from *S. epidermidis* has been shown to cause cytokine release in a mouse endothelial cell line (Mattsson *et al.*, 1993; Robertson *et al.*, 2010).

The requirement of the polymeric nature of peptidoglycan was questioned and polymeric peptidoglycan was digested with the hydrolytic enzyme mutanolysin. Such cleavage occurs after each MurNAc residue thus creating soluble peptidoglycan.Low dose of *S. aureus* (1.3x 106 CFU) was injected alone or together with 500µg of soluble or polymeric *M. luteus* peptidoglycan (Figure 4.16). Results clearly demonstrate that when the PGN backbone is cleaved, pathogenesis is abolished. There was no significant difference in the bacteria isolated from livers (Figure 4.16 B) or kidneys (Figure 4.16 C) of mice injected with *S. aureus* alone or in combination with PGN. Moreover, there was a significant difference between the weights of the two groups (p = 0.0288) with the group receiving *S. aureus* with sPGN losing less weight.

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| Figure 4.16 Soluble peptidoglycan does not augment *S. aureus* pathogenesis  Low *S. aureus* (1.2x106 CFU) was injected alone or combination with 500 µg polymeric *M. luteus* PGN (pPGN) or 500 µg of peptidoglycan digested with mutanolysin (sPGN). 500 µg pPGN or sPGN was injected alone as control.Weight loss **(A)**; CFU in livers **(B)** and kidneys **(C)** was recorded. \*\*\*\* p < 0.0001, \*\*\* p < 0.001, \* p < 0.05. |

### Is augmentation of *S. aureus* pathogenesis by peptidoglycanbacterial strain specific?

In all previous murine experiments, NewHG was used as the infecting bacterial strain. Therefore in order to determine whether the augmentation effect is strain specific, it was tested with a clinical strain background USA300, JE2. Pilot dosing data on this strain was already collected (Dr. Pollitt, University of Sheffield), and JE2 5x105CFU was used, with 5 mice per group (Figure 4.17 A,C,E).

*M. luteus* peptidoglycan was used to augment *S. aureus* infection. Groups of 10 mice were injected with either 500µg *M. luteus* PGN alone or co-injected with 4x105 CFU JE2. Interestingly, while there was significant difference between the weights (Figure 4.17 A), bacterial liver numbers recovered were not significantly different between the groups (Figure 4.17 C). Moreover, a surprisingly high amount of bacteria were recovered from the kidneys of the co-injection group, being significantly higher than the bacteria alone (Figure 4.17 B).

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| Figure 4.17 *S. aureus* strain specificity during augmentation  Mice injected with *S. aureus* JE2 (5x105 CFU) alone or together with *M. luteus* pPGN(500 µg), with weight loss **(A)**, as well as kidney **(B)** and liver **(C)** CFU recorded.  \*\*\* p < 0.001, ns - non significant. NB: One mouse reached severity limits at 48 hpi (red circle). |

### Is augmentation of *S. aureus* pathogenesis by peptidoglycan mouse strain specific?

Despite being used extensively for the study of infectious diseases, mice are not natural hosts for human *S. aureus* (Holtfreter *et al.*, 2013)*.* However,researchers argue that it is indeed lack of epidemiological studies leading to this conclusion, and several groups have reported colonisation of mice by *S. aureus* even in specific pathogen free conditions (SPC) (Mrochen *et al.*, 2017; Schulz *et al.*, 2017). What is more, different mouse strains show varying susceptibility and immune response to the same pathogen (Nippe *et al.*, 2011). It was therefore important to test whether the augmentation effect could be seen in a different mouse strain under the same experimental conditions.

Previous research done has shown that in a tail-vein injection model of 4x107 CFU *S. aureus,* C57BL/6 mice were the most resistant to infection with only 10% death at the end of 14 days, whereas strains such as A/J, DBA/2 and Balb/c had a 100% death rate at 1,3 and 7 days respectively. Other strains, including C3H, C57BL/6and CBA had medium mortality rates, with 50-60% survival rate at the end of 14 days (von Köckritz-Blickwede *et al.*, 2008).

C57BL/6 mouse strain was therefore chosen to test the augmentation effect as it has proven to be the most resilient within this model of infection. Firstly, a pilot experiment with 5 mice per group was performed to determine the optimal dose for injection. NewHG was injected at doses of 1x107 and 4.5x107 CFU, as it had been suggested to be similarly virulent to SH1000.Mice receiving 4.5x107 CFU NewHG required culling due to reaching severity limits (red circles, Figure 4.18 A, B). Mice infected with 1x107 CFUlooked healthy throughout the experiment despite losing up to 25% their initial weight (Figure 4.18 A). Both groups had high amounts of bacteria isolated from kidneys and livers (Figure 4.18 B).

The pilot experiment therefore failed to provide us with necessary data, i.e. dose of infection that does not affect the health of injected animals. This was an unanticipated outcome, as literature suggests that C57BL/6 mice are more resilient to *S. aureus* infection than other models. Therefore, while there is no data in the literature suggesting NewHG to be more effective in mouse infection that SH1000 it is a possibility. What is more, strains of mice purchased from different suppliers are bound to have variations. Therefore, it was decided that a dose of 4x106 CFU should be tested.

Similarly to *S. aureus* doses, *M. luteus* (500µg, 1 mg, 2mg)and *S. aureus* (500µg, 1 mg)PGN was also titrated to test for host susceptibility (Figure 4.18 A) with 500 µg of PGN causing minimal changes to mouse weights.

Taking into account the pilot data, C57BL/6 (10 mice per group) were injected with 1mg *M. luteus* PGN alone or in combination with 1.3x106 CFU *S. aureus* NewHG. 3.6x106 CFU NewHG alone was used as control (Figure 4.18 C, D, E). Mice in the co-injected group showed a higher and faster decline in weights and was culled at 44hpi due to reaching severity limits (Figure 4.18 C,D,E). The *S. aureus* alone group was culled at 67 hpi. Bacterial load in the livers of both groups was quite high, ranging from 107-108 CFU and no statistical difference was seen between the groups. Surprisingly, there was a difference in the bacterial numbers isolated from kidneys, with significantly higher numbers (p=0.033) present in the co-injection group (Figure 4.18 D).

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| Figure 4.18 Augmentation of *S. aureus* pathogenesis by *M. luteus* pPGN with C57BL/6 mice  C57BL/6 mice (n=4-5) injected with *S. aureus* NewHG alone (4x106,1x107, 4.5x107 CFU), *M. luteus* PGN alone(500 µg, 1mg, 2 mg), or a combination of *S. aureus* NewHG (4x106 CFU) together with *M. luteus* pPGN (1mg). Weight loss **(A, C)**, as well as kidney **(B, D)**and liver **(B, E)** CFU recorded. Red circles – mice culled at 60 hpi, green circles – 44 hpi, due to reaching severity limits. |

### Histopathological analysis of murine livers

It was observed that co-injection of low dose *S. aureus* bacteria and peptidoglycan results in a specific liver presentation. Livers appeared to be ‘peppered’ with multiple small abscesses compared to livers harvested from animals injected with low dose bacteria alone (Figure 4.19 A, B).

To further study this, microscopic histopathological analysis was performed on livers harvested from experimental groups injected with a low dose *S. aureus* (9.9x105 CFU) or co-injected together with peptidoglycan (500µg *M. luteus* PGN +8.5x105 CFU NewHG) or peptidoglycan alone. Each organ collected was cryogenically frozen, cut into 200µm slices and stained with hematoxylin and eosin (H&E).

Liver tissue from peptidoglycan alone group was histologically normal and did not present with any pathological changes (Figure 4.19 A, C, D). Mixed group mice presented with multiple abscesses in the liver, including some lysed PMN with *S. aureus* leaking out of the cells (Figure 4.19 E, F, arrow 3), as well as an increased density of PMNs (Figure 4.19 F, arrow 4). In the case of high dose bacteria alone (data not shown) at 9.8x106 CFU, kidneys rarely had a large abscess and almost never any in the livers.

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| B  A |
| C (x10)  D (x100)  E (x10)  F (x100)  2  100µm  100µm  10µm  10µm  1 |
| **Figure 4.19 Effect of augmentation of *S. aureus* pathogenesis by *M. luteus* pPGN on liver morphology**  Macroscopic images of a liver harvested from a mouse injected with **(A)** 500µg *M. luteus* PGN **(B)** low dose *S. aureus* (9.6x105 CFU) and 500 µg *M. luteus* (2 abscesses are visible); H&E stained livers from a mouse injected with 500 µg *M. luteus* PGN **(C, D)** or co-injected with *M. luteus* PGN (500 µg) and 9.6x105 CFU *S. aureus* **(E,F), (E)** shows an abscess (central dark region), **(F)** shows escaped extracellular bacteria. Arrows highlight **(1)** liver hepatocytes, **(2)** liver abscess.  **N.B:** C, E imaged at x10 magnification, region of interest (white box) magnified in D,F and imaged at x100 magnification. |

### Population dynamics of mixed inoculum

Previously we have examined *S. aureus* in both the zebrafish and mouse models of infection (Prajsnar, Hamilton, Garcia-Lara, McVicker, *et al.*, 2012; McVicker, Tomasz K Prajsnar, *et al.*, 2014). This has established that in both animal models there is an immunological bottleneck such that very few (or even single) organisms found the lesion or abscess that are characteristic of *S. aureus* disease. The bottleneck is associated with host phagocytes and results in clonal expansion of the population. Augmentation of *S. aureus* disease with peptidoglycan results in increased bacterial numbers and so it was tested how the addition of peptidoglycan to *S. aureus* inoculum alters population dynamics and clonal expansion.

In the murine sepsis model subjects were injected with a *S. aureus* inoculum made up of a 1:1:1 ratio of three antibiotic resistance marked strains (McVicker, Tomasz K Prajsnar, *et al.*, 2014). Animals were injected with low dose *S. aureus* NewHG (1:1:1) alone or in combination with 500 µg *M. luteus* PGN. At experimental timepoints of 30 min and 10, 40, 70 h post infection 8 animals from each group were culled, and the liver, kidneys and spleen harvested. In agreement with findings by McVicker et al. (2014), almost immediately after injection most of the bacterial numbers were present in the livers, with the addition of peptidoglycan not altering this tropism. Using weight as one of the assessments for murine health, it was shown that at 40 and 70 hpi animals with the mixed inoculum lost significantly more weight compared to low dose *S. aureus* alone (Figure 4.20 A). CFU’s extracted from collected livers and spleens showed that already at 10hpi there was significantly more bacteria recovered from the mixed group of bacteria and peptidoglycan, with significance increasing over time post infection (Figure 4.20 B,C,D). Of note, in the mixed group two animals at 52hpi (red circle) and one animal at 69hpi (green circle) had to be culled due to reaching severity limits.

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| Figure 4.20 Effect of augmentation with peptidoglycan on bacterial population dynamics and organ distribution during *S. aureus* pathogenesis.  8 Mice (Balb/c) injected IV into tail vein with: Low dose *S. aureus* (1 x 106 CFU) alone or co-injected with 500 μg *M. luteus* peptidoglycan. Recorded weight loss at 30 mins and 10, 40, 70 h post infection **(A),** as well as CFU from kidneys **(B)**, livers **(C)** and spleen **(D)**. \*\*\* p < 0.001, \*\* p < 0.01, \* p < 0.05, ns - not significant. Green circle – mouse culled at 69 hpi, red circle- mouse culled at 52 hpi, due to reaching severity limits. |

As the bacterial inoculum consisted of three marked *S. aureus* strains resistant to Erythromycin (red), Tetracycline (blue) or Kanamycin (green), strain variance during infection was observed (Figure 4.21). If the total bacteria recovered from an organ was low (lower than 2000 CFU for kidneys and 3000 CFU for livers), it was removed from the assay and marked as <LoD (less than limit of detection). Data is displayed as pie charts, with each colour representative of the proportion of each strain in the left kidney (L), right kidney (R), liver (V) or spleen (S). The number in the middle of each pie chart is the log­­­10(CFU) for each mouse (Figure 4.21).

At 30 minutes post infection, the bacterial dose in the livers of all mice closely represents that of the original inoculum- with all strains in equal proportion to each other. By 10 hours post infection in livers total bacterial numbers in both mixed and *S. aureus* alone groups could be seen decreasing, with no evidence of strain predominance at this time point. At 40 hours mice receiving low dose bacteria alone have cleared most of the infection, with 72 hours even less CFU. Those organs that are colonized show a clear dominance of a single strain in the recovered organs and is representative of data observed previously (McVicker, Tomasz K. Prajsnar, *et al.*, 2014). The dynamics of infection are quite different in the mixed group, with clonal expansion of particular strains already observable at 40hpi in the kidneys and to a lesser extent in the spleen, this being more pronounced at 72 hpi. The liver also shows signs of strain predominance by 40 hpi.

To analyse whether there is significant clonality in the liver of the mixed inoculum animals, species evenness was calculated (Lozupone and Knight, 2008). Species evenness is based on the assumption that each population of bacteria injected has an equal chance of becoming dominant, and therefore quantifies how equal the whole bacterial population is. At the beginning of the experiment, when there is an equal amount of CFU’s in all strains in the liver, species evenness is set as 1 (even). If a species becomes dominant over all others, this value is then 0 (not even). Therefore with the progression of the experiment, species evenness values can be calculated and a linear regression can be plotted (Figure 4.22 A). Calculating the R2 value to be 44% (p<0.0001) shows that there is a 44% chance for the strains to be even, therefore clonality is highly likely. This assay requires for CFU’s to be recovered from organs at all points, and due to kidneys in both groups and most organs in the low dose group alone being below limit of detection, analysis was not possible.

Therefore based on clonal expansion in the kidneys and liver of the mixed inoculum group, as well as liver species evenness results (Figure 4.22) it is possible to conclude that clonal expansion occurs when *S. aureus* infection is augmented in the presence of *M. luteus* peptidoglycan. This likely happens through an immunological bottleneck.

Previous work in our lab conducted by Dr E. Pollitt looked at bacterial clonality during a high *S. aureus* dose (1.8x107 CFU). Therefore, clonality of the mixed inoculum group was compared to clonality of high dose *S. aureus* alone. Similarly to the previous findings, livers were harvested from both groups of animals at 72 hpi, analysed for species evenness and compared (Figure 4.22 B). Results demonstrate a similar pattern of clonality in both groups, suggesting that a similar immune bottleneck might occur in both cases.

Clonality in livers in the mixed inoculum group was less than that observed in kidneys and thus it was hypothesized that perhaps individual abscesses in the livers are clonal and would contain bacteria from only one strain. In order to test this, low dose *S. aureus* consisting of a 1:1:1 ratio antibiotic resistant strains, was co-injected with 500 µg *M. luteus* PGN*.* At 72 hours post infection livers were harvested and individual abscesses dissected out. These were separately homogenized and plated out on selective antibiotic media plates (Figure 4.22 C). Results confirm that each individual abscess is indeed clonal, suggesting that it has been founded by a single bacterium.

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| 1  6  5  4  3  2  8  7  LK  RK  Liv  S  0.5 hpi  Low  Mix  LK  RK  Liv  S | LK  RK  Liv  S  LK  RK  Liv  S  1  6  5  4  3  2  8  7  10 hpi  Mix  Low |
|  |  |
| LK  RK  Liv  S  LK  RK  Liv  S  1  6  5  4  3  2  8  7  Low  Mix  40 hpi | 1  6  5  4  3  2  8  7  72 hpi  LK  RK  Liv  S  LK  RK  Liv  S  Low  Mix |

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| **Figure 4.21** *S. aureus* population dynamics in mice injected with bacteria alone or with *M. luteus* peptidoglycan.  8 mice(Balb/c) per group injected with low dose *S. aureus* 1x106 CFU NewHG(L) alone or co-injected with 500 µg *M. luteus* peptidoglycan (M). At 0.6,10,40 or 72 hpi, 8 animals per group were taken for clonality analysis. CFU in left kidney (LK), right kidney (RK), liver (Liv) and spleen (S) were analysed for each mouse and pie charts for each organ created. Each chart shows proportion of EryR (red), KanR (green), TetR (blue) strains in each organ, with total log10(CFU) within the piechart. In the case when bacteria recovered from the organs were below limit of detection (2000 for kidneys and 3000 for livers), clonality was not measured and marked by <LoD. Mice removed from the experiment due to reaching severity limits were marked as “ - ”. |

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| **A** | **B** |
| **C** | |
| **Figure 4.22** Strain population dynamics during *S. aureus* infection in the presence of *M. luteus* peptidoglycan.  **(A)**Development of clonality over time during infection. Bacterial species evenness in livers was calculated for mice receiving co-injection of low dose S. aureus (1x106 CFU) and 500 µg M. luteus peptidoglycan, with each blue circle representing evenness value for an individual liver; **(B)** Comparison of species evenness between groups of mice co-injected with low dose *S. aureus* and 500 µg *M. luteus* peptidoglycan or high dose *S. aureus* alone; **(C)** clonality of individual abscesses in mice co-injected with low dose *S. aureus* (1x106 CFU) 1:1:1 and 500 µg *M. luteus* peptidoglycan. | |

### Effect of augmentation on cytokine production during *S. aureus* infection in the murine sepsis model.

A significant role in immune regulation is played by cytokines. They are mostly produced by macrophages in response tostimuli, such as bacteria (Duque and Descoteaux, 2014). Initially cytokines have been shown to be upregulated *in vitro* in response to *S. aureus* cell wall components, such as peptidoglycan and lipoteichoic acid (Bhakdi *et al.*, 1991; See and Chow, 1992; Heumann *et al.*, 1994), as well as suggesting that this contributes to the chronic nature of staphylococcal infections (Lee *et al.*, 2006).Several groups have gone further to show that uncontrolled production of cytokines, as often seen during *S. aureus* sepsis causes multiple organ failure (Schulte, Bernhagen and Bucala, 2013). Therefore, several groups have attempted to regulate the host cytokine response during sepsis. For example, Micheliolide (MCL) decreased the production of cytokines such as IL-6 and TNF- alpha in both PGN- and LPS- evoked sepsis response of human monocytes (Qin *et al.*, 2016). Moreover, *in vivo* experiments on C57BL/6 J mice showed that protection against *S. aureus* MRSAlethal dose (1x108 CFU/ mouse) can be achieved by co-injecting bacteria with MCL (Jiang *et al.*, 2017). Mice receiving a co-injection had a 70% survival rate by 72hpi, compared to 0% when injected with bacteria alone, as well as significantly lower cytokine expression in the blood. However despite promising results in animal models, anti-cytokine therapies in human patients suffering from bacterial sepsis has been largely unsuccessful (Abraham *et al.*, 1995; Dinarello, 2001).

Therefore, it was hypothesized that when *S. aureus* is co -injected with peptidoglycan, this may perturb control of cytokine production by the host and subsequently lead to more bacteria escaping killing. Murine cytokines were measured 3 days prior to infection in order to determine base cytokine levels in mouse serum. This was then repeated 6 and 48 h post injection. For accurate comparison, 4 groups of mice (10 per group) were used, with animals injected with low dose *S. aureus* (1.7x106 CFU), high dose *S. aureus* (1.8x107 CFU), 500 µg *M. luteus p*eptidoglycan alone or co-injection of peptidoglycan with low dose *S. aureus* (1.6x106 CFU). At each experimental timepoint, venesection of the tail vein was performed and blood collected. Serum from the blood was stored at -20°C until cytokine analysis at the University of Sheffield Core Flow Cytometry Unit.

In accordance to previous literature, both pro- and anti-inflammatory cytokines and chemokines were measured, such as IFN- gamma, IL-1 beta, IL-6, IL-10,IL-12, CXCL1, IL17-A, NF, CCL2 and CCL4. Moreover, results were in line with previous findings that high dose *S. aureus* caused un upregulation of IL-6 and CXCL1 levels (Figure 4.21 B, F)at 6hpi (van den Berg *et al.*, 2013). It was of significance that peptidoglycan alone did not cause any difference to cytokine levels in the serum, with the levels staying similar to baseline throughout the experiment (Figure 4.21 A-F). Most cytokine levels remained unchanged between groups receiving *S. aureus* alone or co –injected with peptidoglycan. However interestingly, at 6hpi levels of pro-inflammatory cytokines IL-17 and TNF were significantly lower in the serum of animals receiving a mixed inoculum, compared to bacteria alone (Figure 4.21 G, H). Furthermore, results show that the mixed group had significantly higher amounts of chemokines CCL2 and CCL4 (Figure 4.21 I, J).

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| Figure 4.21 Cytokine induction during *S. aureus* pathogenesis and the effect of augmentation.  Balb/c mice (n=10) were injected with high dose *S. aureus* (1.8x107 CFU), low dose *S. aureus* (1.7x106 CFU),500 µg *M. luteus* peptidoglycanalone or peptidoglycan with low dose *S. aureus* (1.6x107 CFU). 3 days prior to injection, 6 and 48 h post injection, blood was taken from mouse tail vein and cytokines measured **(A-J) :** IFN-gamma, IL-b, IL-6, IL-10, IL-12, CXCL1, IL-17A, TNF- alpha, CCL2, CCL4. \*\*\*\* p < 0.0001, \*\*\* p < 0.001, \*\* p <0.01, \* p = 0.05 ; ns – non significant. |

## Discussion

### Bacterial cell wall components alone are not enough to cause death in the zebrafish model

Work done by a colleague (Boldock, 2016) has shown that in the zebrafish model of infection, *S. aureus* PGN is capable of augmenting infection in the presence of live bacteria. Therefore, it was of interest to see whether a combination of bacterial cell wall components are capable of causing shock in the zebrafish model of infection.

Combinations of LTA and LPS did not cause shock in the zebrafish embryo model even when co-injected with heat-killed *S. aureus.* While zebrafish embryos have a number of advantages to study *S. aureus* pathogenesis, there are several drawbacks. For example, while several LPS receptors have been discovered for the zebrafish and are known to be expressed early in development (Meijer *et al.*, 2004), they lack CD14, shown to be crucial in the mammalian LPS response (Iliev *et al.*, 2005). A similar situation might occur with other cell wall bacterial components such as LTA, however to date there is no literature describing detailed zebrafish response to Gram-positive cell-wall bacterial components.

### Peptidoglycans from different bacterial species can enhance pathogenesis of *S. aureus* in mice

Overall it was shown that commensal Gram-positive and Gram-negative bacteria are capable of assisting the initiation of *S. aureus* infection (Chapter 3). In this case, the commensal bacteria are not able to cause disease in the host, but rather augment *S. aureus* infection when co-injected. It was therefore explored what molecular moieties may be involved. Interestingly, the cell wall component peptidoglycan, irrespective of peptide side chain polymers, was able to augment *S. aureus* pathogenesis, but not when the conserved glycan backbone was lysed, neither when latex beads were co-injected with *S. aureus.* This hints at a wide spread phenomenon of infection initiation, as *S. aureus* augmentation of pathogenesis by commensal bacteria or peptidoglycan was not mouse strain specific or bacterial strain specific, but rather reliant on the presence of an intact peptidoglycan backbone. Importantly, it was also shown that lipoproteins were not involved in the observed augmentation effect, as they are known to be potent immunostimulants (Schmaler *et al.*, 2009).

Having shown that *S. aureus* peptidoglycanis capable of augmenting *S. aureus* infection, it was of interest to see whether various different peptidoglycan structures would allow for augmentation. For this reason, *C. flaccumfaciens, B. subtilis, M. luteus, B. cereus* and *S. epidermidis* were chosen due to the differences in their PGN structure (Figure 4.3). This would allow to demonstrate the importance of particular peptidoglycan features to be determined in the augmentation process. Perhaps, *S. aureus* is capable of utilising the by-products of commensal bacteria in order to enhance its own pathogenesis, in particular via the conserved glycan backbone of peptidoglycan.

Interestingly, while peptidoglycan from all tested bacterial species was able to augment pathogenesis in the murine model, the level of this effect was different. *S. epidermidis,* having the closest structure to *S. aureus* peptidoglycan was the most potent in augmenting pathogenesis. Most likely this is due to the fact that during infection *S. aureus* has been shown to release peptidoglycan into the intracellular space (Doyle, Chaloupka and Vinter, 1988; Mayer, 2012; Becker *et al.*, 2014), perhaps self- augmenting, and it is unsurprising that a peptidoglycan close in structure to the native one would be as effective.

An unexpected effect was seen when co-injecting *S. aureus* and *B. subtilis* pPGN, where mice in this group seemed to have ‘fallen asleep’. This could be linked to previously described somnogenic effects of MDP, where mice injected intravenously with muramyl dipeptide showed induced sleep behaviours, similar to the ones observed in current experiments (Krueger, Pappenheimer and Karnovsky, 1982; Krueger *et al.*, 1984).This did not occur when mice were injected with *B. subtilis* peptidoglycan alone.

However, it was noted that when co-injecting *S. aureus* with PGN from other bacterial species apart from *B. subtilis*, no sleeping effect was seen.This might hint that the presence of m-DAP in peptidoglycan is important for somnogenic properties. It would therefore be of interest if PGN isolated from Gram-negative bacteria would still show this effect.

### *S. aureus* infection is not augmented by lipoproteins, wall teichoic acids or particulate material *per se*

It was important to find out whether particulate matter, biological or inert was capable of augmenting *S. aureus* pathogenesis in the mouse model of infection. What is more, the action of phagocytosis by immune cells triggers several intracellular and extracellular immune cascades essential for overall response to an invading pathogen (Shimada, Park, Andrea J Wolf, *et al.*, 2010; van Kessel, Bestebroer and van Strijp, 2014).

Several bacterial cell wall components have been shown to activate the immune system. For instance, bacterial WTAs are recognised by human anti-WTA IgG, in turn triggering the activation of the classical complement pathway (Kurokawa, Takahashi and Luel Lee, 2016). Moreover, WTA has been shown to activate CD4+ T cells via MHC-ll mechanism, promoting abscess formation (Weidenmaier, McLoughlin and Lee, 2010). Similarly, lipoproteins are thought to be involved in triggering inflammation through activation of cell-surface TLR-2 receptors (Stoll *et al.*, 2005b) and shown to be behind wrongly assigned receptor activation by peptidoglycan (Travassos *et al.*, 2004b).Therefore, the fact that the SH1000*∆tarO* mutant nor SH100*∆lgt* did not abolish augmentation is an important step towards understanding the molecular mechanism behind it.

A number of non-bacterial particles have been shown to be pro-inflammatory, such as silica crystals and aluminium salts, capable of triggering NALP3 inflammasome activation through the production of PGE2and IL-1β (Kuroda *et al.*, 2011). Asbestos has also been shown to trigger activation through the NALP3 inflammasome (Dostert *et al.*, 2008). The use of inert latex beads demonstrates that it is not simply the presence of particulate matter that that overwhelms the ability of phagocytes to deal with *S. aureus* infection that causes augmentation.

### The output of augmentation is a dramatic increase in bacteria within murine livers.

Microscopic images of livers harvested from mice receiving a mixed inoculum of bacteria and peptidoglycan suggest that co-injection somehow affects how the host immune system deals with infection. In the presence of PGN, *S. aureus* creates a focus of infection, surrounded by a dense infiltrate of PMNs. Previous research (Cheng *et al.*, 2009) has shown that high *S. aureus* dose of 1x107 CFU Newman causes few abscesses in murine kidneys,shown a dense number of polymorphonuclear leukocytes, eventually surrounded by a pseudocapsule. At the centre of this capsule escaped staphylococcal communities have been observed.

Thus, peptidoglycan somehow alters the tissue tropism of the mouse model leading to liver abscesses.

### The presence of peptidoglycan also allows the infectious dose of *S. aureus* in the sepsis model to be lowered significantly

Bacterial doses as low as 5000 CFU *S. aureus* and 500 µg *M. luteus* peptidoglycan leads to augmentation of *S. aureus* pathogenesis. This is an astonishingly low amount, as other mouse models of *S. aureus* infection required doses of 106- 108 CFU to establish an IV bacterial infection in the mouse (Kokai-Kun, Chanturiya and Mond, 2007; McAdow, Kim, Andrea C DeDent, *et al.*, 2011; Horst *et al.*, 2012; Jenkins *et al.*, 2015). It is evident that in the presence of bacterial cell wall components, such as polymeric PGN, it is possible to considerably lower the amount of *S. aureus* required for an overwhelming infection. This has serious implications for our understanding of how disease is initiated, as it seems that small doses of *S. aureus* bacteriaare capable of utilising the cell wall products of other commensal bacteria to facilitate infection. Moreover, co-injection of bacteria and peptidoglycan may present a more clinically relevant model of *S. aureus* infection. In order to rule out strain specificity effects it was important to use an alternative bacterial and mouse strain to observe augmentation.

Augmentation in the C57BL/6 mouse model resulted in a different tissue tropism than seen in the Balb/c infection model. In particular, there was an increased bacterial load in the kidneys compared to livers, as seen with Balb/c mice.

### Murine cytokine response

Understanding cytokine signalling changes during *S. aureus* co-infection with peptidoglycan may point us to a possible mechanism of augmentation. Of the cytokines chosen, it was expected that IL-6, an early cytokine seen during infection (Ahmed *et al.*, 2011), as well as CXCL1, a known early chemoattractant for neutrophils (Olaru and Jensen, 2010) would be upregulated in the first few h following infection even in the mixed inoculum group, suggesting that the natural infection progression has not been impaired. What is more, knowing that peptidoglycan alone does not cause any difference in the baseline cytokine response supports the notion that co-injection with *S. aureus* does not simply overload host defence mechanisms. Downregulation of TNF-alpha in the co-injected group is peculiar as TNF-alpha is known to be essential for the clearance of *S. aureus* in murine models of infection, as *tnf -/-* mice subvert overwhelming bacteraemia(Nakane *et al.*, 1995). However, conflicting studies have shown that *S. aureus* protein A induces the shedding of a TNF-alpha precursor TNFR1, undermining the host immune response (Giai *et al.*, 2013). Therefore my data suggests that the presence of peptidoglycan together with *S. aureus,* influences the downregulation of a potent pro-inflammatory cytokine. Another downregulated cytokine was IL-17A, know to be important in local defences against *S. aureus* rather than systemic control of infection (Henningsson *et al.*, 2010). This study shows that in IL-17A KOmice mortality was not increased during *S. aureus* infection, however the mice developed more severe arthritic bone changes. Such data suggests that IL-17A is essential for micro – controlling local bacterial clearance, and it is therefore of interest that the levels of this cytokine are decreased when *S. aureus* bacteria are co-injected with peptidoglycan, compared to *S. aureus* alone.

The significant upregulation of chemoattractant cytokines after mixed inoculation administration was surprising, as both CCL2 and CCL4 are known to be essential in clearing *S. aureus* infections(Tekstra *et al.*, 1999; Esen *et al.*, 2004). CCl2 (also known as monocyte chemotactic protein 1, MCP1) is also produced by osteoblasts during cases of osteomyelitis in murine models of infection, and it is thought that this upregulation may actually increase inflammatory damage to the bone (Marriott *et al.*, 2005). However, while upregulation of both CCL2 and CCL4 is substantial in response to mixed *S. aureus* and peptidoglycan inoculum, it is much higher in the high dose *S. aureus* group alone.

It is therefore of interest as to why CCL2 and CCL4 in particular would be upregulated during the first 6 h of infection. Bacteria are phagocytosed, and this is generally supported by the fact that there are few detectable bacteria in the bloodstream following infection (Tsao, Hsu and Yin, 2006; Duan *et al.*, 2016). The clonal expansion theory suggests that the immune bottleneck occurs inside the phagocyte. What is more, bacteria that manage to survive and grow within a macrophage eventually lyse the host cell (Jubrail *et al.*, 2016b). Such cycling between phagocyte hosts would perhaps need upregulation of immune cell recruitment as it would be beneficial for the survival and dissemination of the bacteria. This theory is further supported by findings that CCL2 overexpressing mice were much more susceptible to infections with *M. tuberculosis*, with the author suggesting that increased immune cell infiltration contributed to disease dissemination (Hasan *et al.*, 2009)*.*

Interestingly, there have been reports of the direct effect of peptidoglycan on CCL2 production. For example, mice challenged intranasally with peptidoglycan from *S. pneumoniae,* digested with lysozyme, triggering CCL2 production in a Nod2 dependant manner(Davis, Nakamura and Weiser, 2011).Therefore, it is important to keep in mind that cytokine upregulation seen in the mixed *S. aureus* and peptidoglycan group may be due to Nod2 activation.

However, my experiments have shown that augmentation seems to be taking place predominantly in the murine livers, and therefore ideally measuring local organ changes of cytokine levels would be essential. For example, within the resident macrophages of the liver there are certain cytokines, such as TNF-alpha, that are already transcribed at a basal level and are ready for release in the case of inflammation triggering (Collart, Baeuerle and Vassalli, 1990). However other important pro-inflammatory cytokines such as IL-6cannot be pre-transcribed as constant activation of this signalling pathway may lead to the development of liver tumours (Schmidt-Arras and Rose-John, 2016). Nevertheless, in the case of infection, IL-6 is rapidly produced by the cell. Therefore, it is quite possible that measuring the overall systemic cytokine levels in the serum results in missing potential subtle changes in cytokine signalling at the individual organ level.

### Clonality

Measurement of bacterial clonality allows insight into progression of infection as well as its dynamics. Experiments suggest that up to 10 h post infection there is unobservable clonal growth occurring, as CFU’s recovered from organs of mice receiving either a low dose alone or together with peptidoglycan are the same. This may also point to the control of bacterial numbers by the host immune system. However, in a mixed inoculum bacterial numbers do not decrease, suggesting that there is an interference with bacterial killing via host defence mechanisms, with the rapid increase in bacterial numbers post the 10hpi time indicating a rapid onset of augmentation. These escaped bacteria then go on to found abscesses in the liver, with many more seen in livers harvested from mice receiving a mix of *S. aureus* and peptidoglycan, suggesting that bacteria manage to evade host control in a more efficient manner.

# CHAPTER 5. Effect of peptidoglycan on human phagocyte response.

## Introduction

While the mouse model has been successfully used for studying the innate immune system response to several pathogens (Gentry-Weeks *et al.*, 2003; Cooper, 2014; J. Y. Yang *et al.*, 2014), important cellular pathways between humans and mice significantly differ (Zschaler, Schlorke and Arnhold, 2014). For instance, signalling through TLR3 receptors in mice involves upregulation of NFkB and MAPK activation, as well as secretion of TNF-α and IL-6, while human primary cells have no equivalent response (Lundberg *et al.*, 2007). Having identified a number of cytokines dysregulated during augmentation of *S. aureus* disease in the mouse model, it was investigated whether human phagocytes respond to peptidoglycan from a range of bacterial species.

### Role of cytokines and complement in inflammation and disease

Cytokines are small secreted proteins, that can have an autocrine effect (on the cells that secrete them), paracrine effect (on nearby cells) and endocrine effect (on cells further away from secreting cell). Cytokines can be both pro- and anti- inflammatory, with pro-inflammatory cytokines known to be produced mostly by macrophages, as an up-regulation response to infection (Dinarello, 2000) as well as anti-inflammatory cytokines that control the effect of pro-inflammatory cytokines (Opal and DePalo, 2000).

While cytokines are essential for regulating inflammatory-immune response and tissue damage during infection, a consequence of overactive cytokines is sepsis, where the burden of infection caused by bacteria or viruses leads to an uncontrolled cytokine storm. This causes multi-organ dysfunction, septic shock and often death (Studnek *et al.*, 2012). TNF-α was initially chosen as it is considered to be one of the central regulators of the innate immune response to infection (Maini *et al.*, 1995)

Gram positive organisms have been found to cause septic shock, and PGN has been shown to be implicated in contributing to the development and progression of sepsis (Spika *et al.*, 1982; De Kimpe *et al.*, 1995; Wang *et al.*, 2004). What is more, it has been hypothesized that only specific parts of the peptidoglycan molecule are responsible for the pro-inflammatory properties, however there have been varying reports on the structural requirements necessary for triggering inflammation. It has been shown that PGN synergises with LTA in order to amplify the immune response caused by LTA (Kengatharan *et al.*, 1998); that stem peptides are responsible for inflammatory upregulation; and that the integrity of the glycan chain is essential for cytokine stimulation (Myhre *et al.*, 2004).

The complement system is another integral part of innate immunity. It is made up of more than 30 types of protein, commonly found in the plasma and on cell surfaces (WALPORT, 2001). These proteins allow for the identification of pathogenic and ‘non-self’ molecules, triggering downstream cascades resulting in generation of C3b opsonins that lead to formation of MAC (membrane attack complex), as well as production of anaphylotoxins. What is more, opsonisation of antigens leads to their phagocytosis via C3b and C3bi receptors on phagocytes (Tohyama and Yamamura, 2006).

Several studies have linked Gram-positive peptidoglycan to the complement cascade activation through various pathways, and conflicting studies reported on the ability of solubilized PGN to activate complement (Greenblatt, Boackle and Schwab, 1978; Janusz, Eisenberg and Schwab, 1987; Kawasaki *et al.*, 1987). However, there is now considerable controversy as to the role of peptidoglycan due to the presence of potentially contaminating molecules (Travassos *et al.*, 2004a). This could bring into question previous work and so it is important to verify the role of peptidoglycan in immune system activation.

### Cells of the immune system

All the cells present in human blood are derived from a pluripotent hematopoietic cell (HCs) in the bone marrow (Kondo, 2010). These cells are responsible for upholdingadequate levels of circulating blood cells, asthese have a relatively short lifespan ranging from h to days. (Kotzin *et al.*, 1950; Fulcher and Basten, 1997). Therefore HCs either stay in G0of the cell cycle or differentiate into myeloid progenitor (MP) and lymphoid progenitor (LP) cells (Figure 5.1) (Uchida *et al.*, 1997). Once committed to a LP cell fate, B cells and T cells differentiate in the bone, however T cells migrate to the thymus to mature, and B cells finish maturation within the bone (Charles A Janeway *et al.*, 2001). MP cells differentiate into neutrophils, basophils, eosinophils and monocytes, all similar in their ability to take up bacteria but some are responsible for their killing (neutrophils) while others for secreting bactericidal factors as well as histamine and heparin (eosinophils and basophils) (Charles A Janeway *et al.*, 2001). Monocytes, upon activation, migrate out of the blood flow and into organs to become macrophages and dendritic cells. A further classification of leukocytes stems from the nature of their nucleus, with lymphocytes and monocytes being mononuclear cells, while neutrophils, basophils and eosinophils are polymorphonuclear cells, therefore allowing to split these cells due to their densities (Figure 5.2). As neutrophils, monocytesand lymphocytes are one of the first responders to a bacterial infection (Aderem, 2003; Wagner *et al.*, 2008; Witter, Okunnu and Berg, 2016), these cells were tested for their cell-surface behaviours in response to peptidoglycan.

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| Dendritic cell  Macrophage  Monocyte  Basophil  Eosinophil  Neutrophil  Platelets  RBCs  MPC  LPC  NKC  HPC  T-cells  B-cells |
| Figure 5.1 Simplified diagram of cells of the adaptive and innate immune system  HP- hematopoietic progenitor cell; MP- myeloid progenitor cell; LP-lymphoid progenitor cell; NK- natural killer cells; RBC- red blood cells. Green- cells of the innate immune system, Red- cells of the adaptive immune system. |

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| Plasma layer  Lymphocytes  Monocytes  Polymorphonuclear  cells  Erythrocytes  Mononuclear cells  Eosinophils  Neutrophils |
| Figure 5.2 Fragmentation of human cells from blood  A diagram of how human blood fractionates after centrifugation. |

## Aims

1. To investigate the effects of PGN on cytokine responses in human phagocytes
2. To determine which phagocyte extracellular receptors are affected by presence of PGN

## Results

### TNF-α production

While TNF-α is secreted by many cells in the human body, it is mainly thought to be produced by cells of the monocytic lineagesuch as various tissue specific macrophages (Pfeffer *et al.*, 1993; Flynn *et al.*, 1995)

Previous work on how *S. aureus* evades the immune systemhas shown that the bacterium persists within host cells, including human macrophages, leukocytes and neutrophils (Rogers and Tompset, 1952; Voyich *et al.*, 2005; Kubica *et al.*, 2008; Koziel *et al.*, 2009). In turn, this allows for the dissemination from the site of infection to other parts of the body.

Moreover, it has been demonstrated that not only is *S. aureus* capable of surviving insidemonocyte-derived macrophages for several days without detection (Kubica *et al.*, 2008), but it purposefully blocks staurosporine-induced apoptosis once inside host cells.

Therefore, the hypothesis was, that if during initiation of infection, the aim of *S. aureus* could be to invade host phagocytes to influence cytokine production by host cells.

To test this, peptidoglycan from different strains of *S. aureus, B. subtilis* and *M. luteus* was prepared in different ways: peptidoglycan was either treated with HF to strip WTA’s, not stripped with HF (US) or digested with Mutanolysin (M).Different preparations were then incubated with human mononuclear cellsat concentrations up to 30 µg/ml. After stimulation, the cells were recovered by centrifugation and supernatant removed. An ELISA assay was used to test TNF-⍺ levels. All PGN preparations stimulated TNF-α production except for preparations treated with Mutanolysin (Figure 5.3 A-E). Data suggest that an intact backbone was essential for TNF-α production by human monocytes, as Mutanolysin treatment abolished the response. What is more, in all examples there is a characteristic bell-curve of TNF-⍺ response, suggesting that human monocytes react to PGN when it is at concentrations approximately between 1≤PGN≤10 µg/ml, while concentrations of ≈30 µg/ml were perhaps not in the physiological range. Moreover, in all the PGN samples, there is no difference in response to HF and US PGN, suggesting that WTA’s are not involved in the TNF-⍺ response, confirming previous work (Majcherczyk *et al.*, 2003). This is further supported by the fact that SH1000*∆tarO* (lacking WTA’s) stimulated TNF-⍺ production in a similar way to all other samples (Figure 5.3 C). Furthermore, the absence of lipoproteins in the SH1000*∆lgt* mutant PGN did not significantly impact TNF-⍺ production by human monocytes (Figure 5.3 B), suggesting lipoproteins are not essential for TNF-⍺ production, in contrast to findings by other groups (Stoll *et al.*, 2005a).



Figure 5.3 TNF- α production by human mononuclear cells is stimulated by peptidoglycan

Production of TNF-α by human monocyte cells in response to stimulation by peptidoglycan preparations from **(A)** SH1000, **(B)** SH1000Δ*tarO,* **(C)**SH1000Δ*lgt,* **(D)***M. luteus,* **(E)** *B. subtilis.* HF- peptidoglycan treated with HF; US- unstripped peptidoglycan; M- peptidoglycan digested with Mutanolysin.

### IL8 production

Once resident cells, such as epithelial cells sense PAMPs, they immediately start secreting chemokines to attract immune cells to the site of infection. IL-8 is a major chemokine, known to be strongly chemotactic for neutrophils (Miller *et al.*, 1992; Hammond *et al.*, 1995). Moreover, bacterial components such as PGN and LTA evoke a strong pro-inflammatory response in the host, resulting in an overabundance of IL-8 (Wang *et al.*, 2000; Kumar, Zhang and Yu, 2004). It was therefore hypothesised that IL-8 may be involved in the augmentation effect previously seen, and therefore PGN digested with Mutanolysin might evoke a smaller IL-8 response in human monocytes.

To test this, preparations of peptidoglycan from different strains of *S. aureus, B. subtilis* and *M. luteus* was incubated with human monocyte cells at concentrations up to 30 µg/ml and supernatant tested for IL-8 levels by ELISA as before (Chapter 5.3.1).

Interestingly, all PGN preparations including Mutanolysin digested material evoked an IL-8 response in human monocytes, suggesting that integrity of the glycan backbone is not essential for monocytes to recognise and influence chemokine production in response to PGN stimulation (Figure 5.4 A-E). What is more, unlike TNF-α, there was no decrease in the amount of IL-8 at higher peptidoglycan concentrations.

Results inFigure 5.4 A-C suggest that it is indeed *S. aureus* PGN causing IL-8 production, rather than lipoproteins or WTA, as PGN from SH1000 (Figure 5.4 A), SH1000*∆tagO* (Figure 5.4B) and SH1000*∆lgt* (Figure 5.4C)evoked similar IL-8 responses. Moreover, IL-8 response to PGN from other bacterial species (Figure 5.4 D,E) was similar to that of *S. aureus,* suggesting that it is the conserved glycan backbone that may be recognised by monocytes, as all the PGNs have different side chains (Figure 4.3)



Figure 5.4 IL-8 production by human mononuclear cells in response to PGN stimulation

Production of IL-8 by human monocyte cells in response to stimulation by peptidoglycan preparations from **(A)** SH1000, **(B)** SH1000Δ*tarO,* **(C)**SH1000Δ*lgt,* **(D)***M. luteus,* **(E)** *B. subtilis.* HF- peptidoglycan treated with HF; US- unstripped peptidoglycan; M- peptidoglycan digested with Mutanolysin. \*\* p < 0.01, ns- not significant.

### Lectin pathway is activated by PGN

*S. aureus* has been shown to activate complement pathways (Wilkinson *et al.*, 1978; Verbrugh *et al.*, 1979; Kawasaki *et al.*, 1987; M Bredius *et al.*, 1992; Neth *et al.*, 2002). However, it also has the ability to interfere with complement activation, in particular through C3b pathways. For example, the Efb protein has been shown to bind to C3 and block further opsonisation, causing a diminished complement effect and facilitating *S. aureus* persistence(Lee *et al.*, 2004). It was therefore hypothesized that the augmentation effect may be linked to complement activation, and therefore digestion of PGN by Mutanolysin would abolish this effect. C3 was chosen for experiments as it is where all 3 arms of the complement system converge (Fritzinger *et al.*, 2008).

To test this, peptidoglycan preparations from different strains of *S. aureus, B. subtilis* and *M. luteus* were incubated for 1h in dilutions of human serum. After stimulation, the serum was transferred into ELISA plates containing anti C3 antibody.

Interestingly, all SH1000 strains(Figure 5.5 A-C) as well as PGN from *M. luteus* (Figure 5.5 E)and *B. subtilis* (Figure 5.5 D) evoked similar complement responses, suggesting that peptidoglycan, rather than WTA or lipoproteins plays an active role in complement C3b activation. Moreover, structural integrity of the glycan chain of peptidoglycan does not seem to be essential for complement activation.

This suggests that complement activation by PGN may not be involved in the augmentation effect was seen in previous experiments.



Figure 5.5 Activation of C3b (lectin pathway) complement in human mononuclear cells by PGN

Activation of lectin arm of complement pathway in human mononuclear cells in response to stimulation by peptidoglycan preparations from **(A)** SH1000, **(B)** SH1000Δ*tarO,* **(C)**SH1000Δ*lgt,* **(D)***M. luteus,* **(E)** *B. subtilis.* HF- peptidoglycan treated with HF; US- unstripped peptidoglycan; M- peptidoglycan digested with Mutanolysin. \*\* p < 0.01, ns- not significant.

### PGN causes downregulation of several cell surface receptors on lymphocytes, monocytes and neutrophils

It has been previously suggested that Gram-positive sepsis often involves the mass-production of pro-inflammatory cytokines (Verhoef and Mattsson, 1995; Wang *et al.*, 2000), however the cell-surface receptors involved in initiating cytokine responses has remained largely unknown. It was therefore hypothesized that the upregulation or downregulation may give us a hint in relation to what cellular signalling pathway may be involved in the augmentation effect we see in the animal model.

To check if PGN upregulates or downregulates any particular cell surface receptors, PGN preparations from *S. aureus* (SH1000*,* SH1000*∆tarO*)*, B. subtilis* and *M. luteus* was screened against 57 different receptors on human lymphocytes, monocytes and neutrophils. Each PGN was prepared in 3 distinct ways: HF- stripped (lacking WTA), unstripped (WTA present) and digested with Mutanolysin. PMN and MNC cells were made up to a concentration of 5x106 cells/ml and incubated with PGN at a concentration of 3μg/ml for 30 min at 37°C in ELISA plates. Each well in the ELISA plate had previously been coated with specific antibody mixes. Following incubation, ELISA plates were spun down, supernatant discarded and cells fixed in paraformaldehyde before performing FACS analysis (Chapter 2.18).

The most common event that appeared regularly throughout the screen was the downregulation of a set of receptors on lymphocytes, neutrophils and monocytes in response to PGN digested by Mutanolysin (Table 5.1). However, it was difficult to judge the extent of upregulation as we would expect similar patterns of behaviour in SH1000 unstripped PGN and SH1000*∆tarO* polymeric PGN. However this eludes to the difference in response to soluble ligands. Moreover, as higher concentration of peptidoglycan were needed for a TNF-α response, perhaps in this particular assay 3 μg/ml PGN was not enough to evoke upregulation of receptors and should be repeated with a range of PGN concentrations. All found interactions could be found in (Appendix)

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| Table 5.1 Mean fluorescence intensity of human leukocyte cell surface receptors stimulated by bacterial PGN.  Human lymphocytes, monocytes and neutrophils were stimulated with HF-treated (normal), unstripped or Mutanolysin-digested peptidoglycan from *B. subtilis, M. luteus* and *S. aureus* (SH1000, SH1000Δ*tarO*). Mean fluorescence intensity (MFI) was calculated using FlowJo software Cell surface receptors that were downregulated are marked in green. Non-stimulated cells (buffer) were used as control. |

## Discussion

Initial work on the cytokine and receptor mediated response of human leukocytes to peptidoglycan was carried out. This was essential to inform further *in-vitro* work aimed at deciphering the molecular mechanisms behind augmentation. Since the 1980’s bacterial PGN has been described as one of the main immunostimulatory molecules in humans (Stewart-Tull, 1980), however several of the findings have since been challenged due to contamination of peptidoglycan by lipoproteins (Schmaler *et al.*, 2009). Therefore, it was important to differentiate the effect peptidoglycans that contained lipoproteins and WTA’s to those completely stripped of these molecules. Such as approach allowed to catalogue initial findings and be able identify any associated with an effect of human leukocytes.

### Cytokine and complement response to PGN

The ability of PGN to evoke a cytokine response in cells has been investigated previously (Wang *et al.*, 2000; Kumar, Zhang and Yu, 2004; Cheon *et al.*, 2008), however little is known about the structure of PGN required for activation. What is more, studies have reported that *S. aureus* components are capable of interfering with cytokine and complement function (Lee *et al.*, 2004; Goldmann and Medina, 2017). We therefore aimed to determine if cytokine or complement response to PGN could be influenced by PGN from different species, as well as presence/absence of PGN-associated WTA and LTA.

Data demonstrates that IL-8 upregulation might not depend on particular PGN side chains, as PGN from various bacterial species such as *M. luteus,* *B. subtilis* and *S. aureus* all evoked an IL-8 response. What is more, it appears even fragments of the conserved glycan backbone are enough to cause a response. This is of interest, as some studies suggest that PGN needs to be particulate in order to be recognised and phagocytosed by the cell to cause a cytokine response (Shimada, Park, Andrea J. Wolf, *et al.*, 2010b). Furthermore, digestion of PGN by host lysozyme is deemed to be a defence against the pro-inflammatory capabilities of PGN, therefore activation of IL-8 by soluble PGN may hint at a role of PGN in maintaining cells properly conditioned to a PAMP response. Similar research has shown that soluble PGN is involved in intestinal immune tolerance and the absence of this balance may lead to autoimmune enteritis (Hewitt *et al.*, 2012). However, activation of IL-8 by digested PGN suggests that this particular cytokine response may not be essential for augmentation of *S. aureus.*

Experiments show that similarly to IL-8, the conserved glycan backbone of PGN is required for TNF- ⍺ activation, as peptidoglycan from bacterial species such as *M. luteus* and *B. subtilis,* despite different stem and cross-linking peptides, is capable of TNF- ⍺ activation. What is more, despite previous research suggesting that TNF-⍺ is in fact lipoprotein mediated (Stoll *et al.*, 2005a), experiments clearly show that SH1000 and SH1000*∆lgt* show significant activation of TNF-⍺. Furthermore, the glycan backbone needs to be intact for TNF-⍺ to be activated, and this suggests a potential role in augmentation. Activation of TNF -⍺ in response to PGN is essential in the first instance of disease in order to recruit macrophages and neutrophils to the site of infection (Collart, Baeuerle and Vassalli, 1990). However prolonged activation may lead to sepsis, shock and multiple organ failure and therefore digestion of PGN by host enzymes such as lysozyme and N-acetylmuramyl-L-alanine amidase (NAMLAA) (Hoijer *et al.*, 1997) is likely crucial for avoiding damage to the host. It is therefore unsurprising, that digested peptidoglycan would not activate TNF-⍺. It would be interesting to determine whether PGN digested by NAMLAA would also fail to activate TNF-⍺ under similar experimental procedures.

An unexpected result of TNF- ⍺ upregulation was the ‘bell-curve’ response, seen on all the experiments. This suggests a number of possibilities. First of all, it established an upper and lower limit of monocyte response to PGN, set at approximately 30 and 0.8 µg/ml respectfully. It seems that this is the physiological range in which cells are able to recognise and react appropriately to PGN.However, as we yet do not know how much PGN is released by *S. aureus* during infection, it may be that exceeding concentrations of 30 μg/ml is a defence mechanism to evade host immune responses. It would be of interest to apply intra-vital microscopy methods in order to track real time TNF-⍺ response during *S. aureus* infection, as dampening of TNF-⍺ response may be occurring in the first few min of infection.

TNF-⍺ is not the only cytokine that solely reacts to particulate PGN, as IL-1b is thought to be involved in recruiting neutrophils to the site of infection (Shimada, Park, Andrea J. Wolf, *et al.*, 2010b). For this cytokine response polymeric PGN needs to be internalised by macrophages and digested within the phagosome. It would be of interest to determine if TNF-⍺ upregulation similarly depends on the digestion of PGN within the macrophage. This could be accomplished by introducingN,N′,N′′ -Triacetylchitotriose (triNAG) to macrophages, known for inhibiting the action of lysozyme(Dahlquist, Jao and Raftery, 1966; FUKAMIZO *et al.*, 1992).What is more, latex beads coated with solubilized PGN may give an insight into whether digested PGN is recognised by cells and phagocytosed. To achieve this, solubilised PGN will need to be labelled with a radioactive or fluorescent marker, and attached to latex beads. As solubilised PGN is thought to be poorly recognised by unstimulated immune cells (Iyer and Coggeshall, 2011b), perhaps due to the miniscule size of the monomers, attaching solubilised PGN to a 1μm latex bead (therefore mimicking approximate *S. aureus* size) may lead to the internalisation of solubilized PGN.

### Complement response to PGN

*S. aureus* has been shown to activate the classical, lectin and alternative pathways of the complement system (Wilkinson *et al.*, 1978; Verbrugh *et al.*, 1979; Kawasaki *et al.*, 1987; M Bredius *et al.*, 1992; Neth *et al.*, 2002). All these pathways converge on the opsonin C3, making it a central marker to study complement system activation. Activation of any of the three complement pathways leads to the deposition of C3b on the surface of bacteria and their sequential killing by cells of the immune system. Moreover, studies have shown that suppressing mouse complement C3 convertase enzyme activity by cobra venom factor (CVF) results in an increased severity of septicaemia and septic arthritis in *S. aureus* infected mice (Aikin Charles G Cochrane, 1970; Sakiniene, Bremell and Tarkowski, 1999).

The aim of the experiments was therefore to study whether activation of complement is dependent on specific molecular moieties of PGN including side chains and whether the intact glycan backbone is essential.Data suggests that the activation of C3 and downstream production of C3b is not dependent on PGN being polymeric and neither does it seem to be impacted by differences in peptide side chains. However, it is important to note that only C3b activation was examined and PGN might have had a negative impact on opsonisation downstream of C3b. In order to further investigate this, a similar assay checking for C9b-9 activation could be performed. This would determine the role of PGN in MAC (membrane attack complex) formation.

A general outcome of these sets of experiments may suggest that TNF-⍺ signalling may happen through a different signalling pathway than that of IL-8 and complement, as it does not get activated by PGN solubilized by Mutanolysin.

### Response of cell surface receptors to stimulation with peptidoglycan

Lymphocytes, neutrophils and monocytes contain granules that digest recognised pathogens as well as being involved in production of inflammatory agents.

After performing a screen of more than 54 receptors commonly upregulated during inflammation on monocytes neutrophils and lymphocytes using the Tricolour assay, it was noted that a set of receptors has been downregulated. A group of these, mainly responsible for adhesion have been recognised as downregulated after challenge with PGN. CD162, Siglec-9, CD50, CD321, CD31, CD47, CD44 were identified by flowcytometry as being downregulated. Cell adhesion receptors are necessary for the recruitment of leukocytes from the vasculature for appropriate inflammation response, as they allow for cell-cell and cell-matrix interactions.

Several studies have pointed to the importance of leukocyte migration through the epithelial barrier, resulting in phenotypic changes such as reduced apoptosis in neutrophils (Hu *et al.*, 2005), aiding monocyte into DC differentiation and enhanced migration and proliferation of T-cells (Brezinschek, Oppenheimer-Marks and Lipsky, 1999). Therefore, there is logic behind hindering this process to aid survival by bacteria.

#### CD162

CD162, also known as P-selectin glycoprotein ligand 1 (PSGL-1) is expressed on all leukocytes (Cummings and Cummings, 1999). Its main role has been identified as tethering leukocytes to the vessel wall via endothelial selectins. This has been proven to be essential during inflammation, as mice deficient in PSGL-1 show a 1-2 hr lag in neutrophil recruitment to the site of inflammation, as well as a surprising 2-4 fold increase in overall neutrophil numbers compared to WT mice (Mayadas *et al.*, 1993). Therefore it would be of interest to compare neutrophil count in mice during augmentation, to see if neutrophil numbers have been increased. It is surprising that down regulation of CD162 happens in all three tested leukocytes however mostly only in response to PGN digested with Mutanolysin.

Digested PGN from SH1000 and SH1000∆*tarO* showed downregulation of CD162 in lymphocytes and neutrophils, however it would have also been expected that there would be the same response in SH1000 polymeric (HF- stripped) andSH1000∆*tarO* polymeric peptidoglycan, as both do not have WTA’s. Results demonstrate that in the case ofSH1000∆*tarO*, absence of WTA does not stop downregulation of CD162, however in SH1000 the unstripped version (and not the HF-stripped) downregulates CD162. Therefore, such anomalous results should be re-examined in the future.

#### Siglec-9

Siglec-9 (sialic acid immunoglobulin-like lectin 9) is known to be present on all leukocytes and facilitates binding to inflamed human synovial membranes (Aalto *et al.*, 2011) What is more, it has been found to stimulate production of ROS in neutrophils, triggering cell death though apoptotic and non- apoptotic pathways (von Gunten *et al.*, 2005). Interestingly, it was found that Siglec-9 is downregulated strictly in neutrophils and by digested PGN.This might be linked to the ability of *S. aureus* to survive within neutrophils for prolonged periods.

#### CD50

High expression of CD50, also known as intracellular adhesion protein-3 (ICAM-3) has been linked by several studies to upregulation of leukocyte function (Fawcett, C. L. L. Holness, *et al.*, 1992; Skubitz *et al.*, 1995; Montoya *et al.*, 2002). ICAM-3 has been found to be expressed on leukocytes and counter-receptors on endothelial cells, therefore facilitating adhesion to the endothelium.

Once again, the downregulation of this receptor was caused by digested PGNs on neutrophils, further supporting the idea that digested PGN fragments decrease the ability of leukocytes to arrive at the inflammation site, by decreasing lymphocyte adhesion to extracellular matrix proteins and endothelial cells.

Moreover, high ICAM-3 expression has been found in cells involved in antigen presentation to T- and B- cells, therefore being essential to facilitate the immune response (Fawcett, C. Holness, *et al.*, 1992). Data suggests that perhaps digested PGN in this instance acts as an anti-inflammatory agent.

#### CD87

CD87, also known as urokinase-type plasminogen activator receptor is mainly expressed on the cell surface of leukocytes, and is present in soluble form at a low concentration in healthy human plasma (Guo *et al.*, 2017). The main role of this receptor is to facilitate adhesion of leukocytes to fibronectin and integrins (Trigwell, Wood and Jones, 2000). CD87 was downregulated in response to Mutanolysin-digested peptidoglycan from *M. luteus, B. subtilis* and *S. aureus.* However, the SH1000Δ*tarO* histogram significantly overlapped with buffer control, despite varying MFI and therefore this experiment needs to be repeated.

#### CD47

CD47, also known as integrin associated protein, has been shown to be essential in the arrest of lymphocytes at inflamed epithelium (Ticchioni *et al.*, 2001), as well as enhancing phagocytosis by neutrophils and monocytes through adhesion to endothelial cells (Zhou and Brown, 1993). Moreover, experimental ligation of CD47 has been shown to lead to a decrease in macrophage cytokine synthesis, such as IL-12 (Armant *et al.*, 1999). Results suggest that CD47 is downregulated on lymphocytes and neutrophils by *B. subtilis* PGN, and on all three tested leukocytes by digested *M. luteus* and *S. aureus* PGN. As with previous downregulated receptors, digested PGN seems to downregulate the ability of leukocytes to adhere to epithelial cells near sites of inflammation. Unstripped PGN from SH1000 and SH1000Δ*tarO* show similar results, suggesting that WTA doesn’t play a role in downregulation of CD47.

#### CD44

CD44 is a known leukocyte cell surface adhesion receptor, with a hyaluronate (HA) ligand present on epithelial cells (Aruffo: *et al.*, 1990). Interestingly, lymphocyte CD44 only binds HA in the presence of pro-inflammatory cytokines, such as TNF-⍺.It is also essential for lymphocyte rolling and adhesion to endothelial cells (Degrendele, Kosfiszer and Estess, 2017). Moreover, CD44 has been shown to play an important role in macrophage recruitment and activation during inflammation (Wysoka *et al.*, 1997).

Data suggests that CD44 is downregulated in response to digested PGN from *B. subtilis* on monocytes, from *M. luteus* on all tested leukocytes and from *S. aureus* on lymphocytes. Perhaps CD44 recognises particular PGN side chains, as digested PGN from different bacterial species caused downregulation of CD44 on different leukocytes. The fact that CD44 was downregulated on lymphocytes in response to digested PGN from both SH1000 and SH1000*∆tar0* suggests that WTA is not involved in downregulation of CD44.

#### Conclusion

It is unknown as to how rapidly PGN gets digested by enzymes in serum, but it seems as though the digested products help to keep neutrophils away from the initial site of infection (Atilano *et al.*, 2014). Polymeric PGN may be spread into the bloodstream during infection, digested by enzymes and systematically acting as an anti -inflammatory cue to leukocytes. Therefore, it would be of interest to check the rate of PGN digestion in mouse and human serum. This might give us an idea of how fast adhesion can be impaired during infection. It would be hypothesized, that it should happen within the initial stages of infection, giving *S. aureus* time to spread inside the host.

Due to a technical difficulty, where the wavelengths used where not calibrated, resulting in overlap of the spectrums, and inability to include several repeats for statistical analysis. Therefore the tricolour assay will have to be repeated to obtain statistically significant data for firm conclusions to be made.

# Chapter 6. General discussion

*S. aureus* infections worldwide are a significant healthcare burden in both community and healthcare care settings. Today, our lack of understanding of this pathogen is starkly denoted by the failure of a multitude of vaccine trials while the need for novel antimicrobials is reaching a crucial point. Understanding the stages of bacterial infection is expected to further the development of innovative therapeutic approaches for this pathogen.

## Requirements for infection

Humans are heavily colonised by their microbiome- a collection of microorganisms such as bacteria, co- existing under different conditions (Ehrlich, Hiller and Hu, 2008). The microbiome consists of a wide genetic diversity of varying bacterial species, with certain biological niches such as the nares or gastrointestinal tract harbouring their own, unique microflora (Moeller, 2017; Proctor and Relman, 2017). Importantly, studies have demonstrated that even within a specific microbiome, bacteria such as *H. influenzae,* has a strain turnover rate of approximately once a week (Farjo *et al.*, 2004). This diversity is thought to be linked to transmission rates between the studied populations, highlighting that an individual’s microbiome is highly dependent on their environmental surroundings. Moreover, even specific niche microbiomes differ from individual to individual, significantly varying in the phylogenetic composition of their bacterial population (Ley *et al.*, 2008; Lozupone and Knight, 2008). Despite the evident fluidity of the human microbiome, some similarities can be traced. For instance, nasal decolonization of a *S. aureus* carrier resulted in a 86% chance of re-colonization with the same strain (Mody *et al.*, 2003), whereas a healthcare practitioner had a higher chance of being colonized with a new strain, most likely due to the working environment. Interestingly, when human non-carriers of *S. aureus* were colonized with a *S. aureus* strain, an overwhelming majority reverted back to their original nasal microbiome, free of *S. aureus* (Nouwen *et al.*, 2004)*,* demonstrating that a local established microbiome protects itself from foreign invaders, and that removal of a ‘resident’ bacterium from that microbiome may upset the general balance until successful recolonization with the same strain.

Bacteria colonizing the host can be roughly divided into different categories: mutualistic, where both the host and the bacteria benefit from the interaction (e.g. human intestinal *Bacteroides spp.*); commensal, where one side benefits from the interaction without causing harm to the other (e.g *S. epidermidis* on human skin surfaces) (Nakatsuji *et al.*, 2017) or pathogenic- where the benefit of one of the sides causes a significant decrease of health to the other side (e.g *M. tuberculosis*) (Smith, 2003). However, several bacteria are able to transition between these categories, for example *S. epidermidis,* is thought to largely be a commensal bacterium (Duguid *et al.*, 1992). Being a permanent colonizer of the human skin, *S. epidermidis* has developed several virulence factors allowing for the escape of recognition by the human immune system (Otto, 2009). Therefore, if this bacteria bypasses the skin barrier e.g. through a wound, as it now has a suitable substrate such as an indwelling medical device, it is able to cause severe infection, mediated by characteristics acquired from a successful commensal lifestyle. It is therefore possible to summarize that perhaps the transition of a bacterium from commensal to pathogenic is dependent on the state of the overall microbiome, and perturbations in local and systemic homeostasis may play a role.

This project looked at the importance of common commensal organisms in the initiation of *S. aureus* infection, as often the role of the polymicrobial environment in disease progression is overlooked (Ganesh *et al.*, 2014). For instance, biofilm formation of *S. aureus* in models of chronic wounds have focussed on the interaction between the bacteria and the host immune system (Nakagami *et al.*, 2008; Zhao *et al.*, 2010), however a monospecies model does not represent the polymicrobial nature of a human wound (Dalton *et al.*, 2011). Therefore, the ability of human commensals such as the Gram- positive *M. luteus* and Gram- negative *R. mucosa* to act as ‘pro-infectious agents’ to augment *S. aureus* pathogenesis sheds new light on the possible role the microflora. The breadth of commensal organisms, able to augment *S. aureus* infection, demonstrates that this is potentially a generic mechanism, by which *S. aureus* makes use of any of the wide range of commensals present on the skin surface (Grice and Segre, 2011a). Interestingly, while polymicrobial *S. aureus* bacteraemias (P-SAB) are seen in 20% of patientscompared to those with monomicrobial infections, the severity of disease in patients with multiple recovered species of bacteria is higher (Khatib *et al.*, 2016). What is more, most P-SAB patients have had either an intravascular catheter or urinary tract infections of various severity, known to be colonised by various bacteria including skin commensals such as *P. acnes* and *S. epidermidis* (Pihl *et al.*, 2013), indicating that co-injection of *S. aureus* with microflora in the murine sepsis model allows for the replication of an infectious process more similar to that in humans. What is more, most murine models of *S. aureus* disease require a high inoculum of around 5x107- 5x108CFU (Cheng *et al.*, 2010; McAdow, Kim, Andrea C. DeDent, *et al.*, 2011), and the model proposed in this thesis enables a dose of *S. aureus* as low as 1x105 CFU to form abscesses in the host liver in the presence of commensal organisms. Interestingly, while conventional mouse models observe abscesses in the kidneys ofanimals during *S. aureus* infection (Rauch *et al.*, 2012), the presence of augmenting material seems to influence tissue tropism, with the majority of bacteria now recovered from the liver. Current clinical examinations of patients presenting with *S. aureus* bacteraemiadoes not include organ biopsy to check for bacterial load, and therefore it is unclear to how this reflects human infection progression.

While the *S. aureus* murine sepsis model could be augmented, it was important to test whether other *S. aureus* infection models would behave the same. Therefore our collaborators at the Sahlgrenska University Hospital (Department of Rheumatology, Sweden) showed that septic arthritis severity in the joints of NMRI mice as well as severity of subcutaneous abscesses was increased when *S. aureus* was co-injected with peptidoglycan from *M. luteus.* This not only supports the notion that bacterial debris, previously disregarded when studying pathogenesis, may influence infection, but also demonstrates a wide phenomenon, observed across different infection and mouse models.

## Molecular mechanism of infection

This project has shown that *S. aureus* pathogenesis can not only be augmented by a range of commensal bacteria, but also by a wide range of peptidoglycans. As cell wall material digested with mutanolysin failed to augment, it is possible that this process is mediated through the conserved glycan backbone, and explains why even peptidoglycan from the plant pathogenic bacterium *C. flaccumfaciens* could augment infection. With neutrophils being well-known for playing a role in disseminating *S. aureus* bacteria (Cheng *et al.*, 2011), it was a surprising find by our collaborators at the University of Calgary (Canada) that depletion of neutrophils prior to bacterial challenge did not eradicate augmentation, while the depletion of Kupffer cells with clodronate abolished it. Kupffer cells reside in the sinusoids of the liver and have been recently shown to be the primary cell to capture *S. aureus* in a murine model of infection (Surewaard *et al.*, 2016), and it was therefore hypothesized that during co-infection of *S. aureus* with commensal bacteria or peptidoglycan results in the inability of Kupffer cells to eradicate the pathogen. In an attempt to dissect the molecular mechanism behind augmentation, our collaborator, Dr. Bas Surewaard (University of Calgary, Canada) used knock-out mice to rule out the involvement of major cellular signalling molecules such as Nod1, Nod2, Myd88 and the NLRP3 inflammasome (Takeuchi, Hoshino and Akira, 2000; Chamaillard *et al.*, 2003; McGilligan *et al.*, 2013; Schäffler *et al.*, 2014). However, mice unable to produce reactive oxygen species through the depletion of Cybb-/-, while more susceptible to *S. aureus* infection, showed no signs of augmentation when co-injected with peptidoglycan or commensal bacteria. What is more, it was possible to monitor the chemical composition of the phagolysosome during infection with *S. aureus* alone or co-injected with peptidoglycan. Analysis had shown that the presence of *S. aureus* with augmenting material results in significantly decreased oxidation and acidification of the phagolysosome, possibly allowing *S. aureus* to survive killing.

It has been recently shown that *S. aureus* is capable of launching two distinct genetic programmes, based on the chemical composition of the niche it occupies, to promote either biofilm formation or toxin production (García-Betancur *et al.*, 2017). Possibly, *S. aureus* senses its environment when in the Kupffer cell, and in the presence of bacteria or cell wall material upregulates host catalase production and therefore reducing amount of ROS in the phagolysosome (Das and Bishayi, 2010). Alternatively, *S. aureus* could be expressing factors preventing the docking and/or fusion of lysosomes by interfering with Rab-related mechanisms (Tranchemontagne *et al.*, 2015).

While detailed molecular mechanism of augmentation remain vague, it is evident from clonality experiments that augmentation enhances disease outcome in the favour of the pathogen, as it allows more bacteria to survive the phagocyte-induced bottleneck.

## Further work

Deciphering the molecular mechanisms involved in augmentation is one of the main directions this project could take. Considering the perceived importance of Kupffer cells and release of ROS in augmentation of *S. aureus* pathogenesis it would be worthwhile establishing an *in-vitro* assay of bacterial killing within the macrophage (Kitani *et al.*, 2014). As such, it will allow to test whether Kupffer cell lysis or intracellular pathogen survival dynamics could be altered by the presence of commensal bacteria or cell wall material. It is likely *S. aureus* causes a decrease in phagolysosome maturation through various pathways. Possible perturbation to lysosome fusion could be examined by loading macrophage lysosomes with thorium dioxide and therefore determining if full fusion events occur (Buchmeier and Heffron, 1991). If such is the case, further *in-vitro* tests would allow the determination of Rab and SNARE protein involvement in membrane fusion (Becken *et al.*, 2010). The NADPH oxidase, responsible for ROS production (Segal, 2005), requires assembly at the phagosome membrane and therefore dysregulation of this mechanism could delay production of ROS. NADPH oxidase subunits inside macrophages could be labelled with specific antibodies, allowing to visualise recruitment and assembly of the NADPH oxidase (Smirnov, Daily and Criss, 2014).

Previous work done in our lab (Dr. Emma Boldock) had shown that *S. aureus* pathogenesis could be augmented in the zebrafish model of infection by an attenuated mutant of *S. aureus* (Boldock, 2016)*.* Therefore testing severalmutants of the cell wall pathway such as *fmtA* (Qamar and Golemi-Kotra, 2012), *oatA* (Pushkaran *et al.*, 2015)*,* PBPs (Spratt, 1983), *asp23* (Müller *et al.*, 2014)*, tarO* (Brown, Zhang and Walker, 2008), using the zebrafish embryo model of infection would allow for a relatively quick assessment of the potential mechanisms involved. From a host perspective, knocking out targeted pathways of the zebrafish immune system using the CRISPR/Cas9 method would allow to explore the signalling molecules and pathways possibly involved in augmentation of *S. aureus* (Liu *et al.*, 2017)*.*

Having identified that digestion of the peptidoglycan backbone abolished augmentation of *S. aureus,* it is unclear whether this is due to different signalling in response to digested material or because the digested peptidoglycan is not phagocytosed due to its size. In order to overcome this, peptidoglycan could be digested with various enzymes (lysostaphin, mutanolysin, Atl amidase) and attached to latex beads approximately 1 µm in size (Janusz, Eisenberg and Schwab, 1987; Sugai *et al.*, 1997; Kokai-Kun, Chanturiya and Mond, 2007; Colino, Duke and Snapper, 2013). Thus beads will ensure that digested peptidoglycan is phagocytosed in the same manner as polymeric peptidoglycan, possibly allowing the identification of the active moiety of peptidoglycan.Having identified the active components of peptidoglycan, it would be possible to test using the Tricolour assay various receptors on phagocyte cell surface that respond to stimulation.

In regards to exploration of the breadth of the augmentation phenomenon, several commensal bacteria have been isolated in conjunction with *S. aureus* during infection. For instance, increased *P. acnes* colonization was found in 43% of patients examined (Fanelli *et al.*, 2011), with *S. aureus* making use of the secreted CAMP (Christie, Atkins, Munch-Peterson) by *P. acnes* to enhance its own virulence (Lo *et al.*, 2011). Conversely, commensal bacteria of the *Corynebacterium spp.* co-habit with *S. aureus* in the nares and are thought to promote *S. aureus* commensal-related gene expression programme by repressing *agr* quorum sensing, and therefore it would be of interest if co-infection would result in *S. aureus* augmentation (Fehrmann *et al.*, 2013).

Apart from commensal bacteria, several pathogenic organisms have been described to benefit from the presence of *S. aureus* and the dynamics of these dual infections would be of interest (Nair *et al.*, 2014). *P. aeruginosa* has been shown to lyse *S. aureus* in the rat peritoneum, in order to access iron otherwise unavailable in such an environment (Mashburn *et al.*, 2005). *E. faecalis* and *S. aureus* are frequently isolated from foot ulcers in diabetic patients, though the nature of interaction currently remains unknown (Chellan *et al.*, 2010). Not only bacteria could benefit from a co-infection with *S. aureus,* with the fungus *C. albicans,* normally colonizing human skin, able to degrade the Fc region on IgG with secreted proteinases during infection (Kaminishi *et al.*, 1995). A depletion of opsonisation-capable molecules results in increased immune evasion of *S. aureus,* that in turn impacts formation of fibrin clots through production of coagulase, allowing *C. albicans* to evade phagocytosis (Fehrmann *et al.*, 2013).Finding out whether organisms, known to be either competitive or cooperative with *S. aureus,* could augment infection would shed some onto the possible molecular mechanisms involved. Moreover, in order to expand the concept of augmentation to other bacteria, it would be of interest to see whether pathogenesis of bacteria such as *S. pneumoniae* can also be augmented by commensal bacteria or cell wall material. In an attempt to recapitulate human wound infection with *S. aureus,* it would be of interest to utilize *ex-vivo* grown human skin (MacNeil, 2007; Steinstraesser *et al.*, 2010) to study bacterial dynamics in a polymicrobial environment of a wound infection.

Overall my work has highlighted a novel facet of *S. aureus* pathogenesis. It may be part of a wider phenomenon across pathogenic bacteria with significant translational potential to develop novel central regimes to combat infectious disease.

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# Appendix

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