CHALMERS

# Development of an *in vitro* model for testing novel antimicrobial substances

## Effects of established wound bacterial biofilm on cultured human macrophages

*Master of Science Thesis*

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## Abstract

Chronic or hard to heal wounds are very susceptible to infections. Due to the biofilm mode of growth in these wound, conventional therapies, such as antibiotics, do not eradicate the bacteria. New therapeutics must be developed to be able to combat these infections and one way might be to control or inhibit bacterial communication, so called quorum sensing. Possible quorum sensing inhibitors can be antagonist to the quorum sensing signaling. Developing an *in vitro* model that mimics biofilm infection in chronic wounds will enable new treatment strategies to be evaluated. It will also allow for optimization of the antimicrobial effect and safety of the compound before testing in animal models.

In this *in vitro* model, cultures of monocyte derived macrophages on collagen coated surfaces were exposed to conditioned media from *Pseudomonas aeruginosa*, a well documented biofilm former and present in more than 50% of all chronic wounds. Four different clinically isolated strains from chronic wounds and one laboratory strain, PAO1, were assessed based on their effect on macrophage viability and cytokine response. The different strains were characterized as more virulent strains or less virulent strains. The results from the study showed that a well established biofilm from the less virulent bacteria tend to induce an anti-inflammatory response in the cells while biofilm from the more virulent seemed to induce a pro-inflammatory response.

The model was then evaluated by testing the effect of salicylic acid on bacterial virulence. The results indicated that bacteria cultured together with salicylic acid moved towards a less virulent phenotype. These first studies in the development of an *in vitro* model show promising results, however, more work must be done to further develop the model, before it is fully functional.

Keywords: chronic wounds, biofilm, quorum sensing, AHL, *in vitro* model, macrophage, *Pseudomonas aeruginosa*

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## Abbreviations

| | |
|---|---|
| AHL | N-acyl homoserine lactone |
| APC | Antigen presenting cell |
| BHL | N-butanoyl homoserine lactone |
| CM | Conditioned medium |
| ELISA | Enzyme-linked immunosorbent assay |
| EPS | Extracellular polymeric substances |
| HBSS | Hank's balanced salt solution |
| HHL | N-hexanoyl homoserine lacotone |
| IL | Interleukin |
| INF | Interferon |
| LDH | Lactate dehydrogenase |
| LPS | Lipopolysaccharide |
| MDMΦ | Monocyte derived macrophages |
| OdDHL | N-3-oxododecanoyl homoserine lactone |
| OHHL | N-3-oxohexanoyl homoserine lactone |
| PBS | Phosphate buffered saline |
| PQS | Pseudomonas quinolone signal |
| RPMI | Roswell park memorial institute medium |
| SA | Salicylic acid |
| SEM | Standard error of the mean |
| Th1 | T helper cell 1 |
| Th2 | T helper cell 2 |
| TNF | Tumor necrosis factor |
| QS | Quorum sensing |
| QSI | Quorum sensing inhibitor |
| QSSM | Quorum sensing signaling molecule |

# 1. Introduction

Some wounds do not heal properly, the inflammation phase can be prolonged, the wound matrix might be defected and the reepithalization unsuccessful [1]. In developed countries, 1-2% of the population is experiencing these chronic, or hard to heal wounds [2]. People suffering from diabetes and cardiovascular diseases, often as a result of obesity, are particularly prone to develop these wounds and since obesity is increasing epidemically it is believed that the number of people living with a chronic wound will increase in a similar manner [3]. The health care expenditure is already exceeding $3 billion annually only in the United States, not including cost of rehabilitation, lost work time or disability payments [4].

Chronic wounds are very susceptible to infections. The most common treatment to bacterial infections is antibiotics and acute bacterial infections can in most cases be successfully treated this way. However, the majority of antibiotic treatments available do not eliminate bacteria in a chronic wound [5].

In a chronic wound the bacteria no longer live in a planktonic state but have aggregated and started to produce extracellular polymeric substances (EPS) on the wound surface and in the wound tissue. These aggregates of bacteria are known as biofilm. Dental plaque and the bacterial lining of the intestine are two examples of naturally occurring biofilm in the body. However, biofilm can also form on places where it is undesirable and more difficult to remove, such as on medical implants and catheters, in the lungs of patients with cystic fibrosis, and in chronic wounds, and can cause severe infections [6]. According to the National Institute of Health in the United States, 80% of all difficult bacterial infections involve bacteria colonized in biofilm [7]. Bacteria in a biofilm differs greatly from their planktonic counterparts and within the biofilm the bacteria are resistant to most antibiotics and protected from the body's own defense system, making it very difficult to eradicate the bacteria and treat the infection [8].

One bacterium that commonly colonizes these types of wounds is *Pseudomonas aeruginosa. P. aeruginosa* is an opportunistic pathogen that is a well documented biofilm former and present in more than 50% of all chronic wounds[9]. Wounds infected with this bacterium also appear to be larger than wounds that are not infected with *P. aeruginosa.* It has been shown that the bacterium interferes with the body's own defense system and impairs the wound healing process [9].

Most biofilm infections in wounds are treated with high amounts of antibiotics, even though it is known that this has limited effect. Because of the difficulty to treat bacterial biofilm infections and the increase of antibiotic resistance developed in many bacteria, novel treatment strategies need to be investigated. In order to do this, new *in vitro* models to study biofilm infections must be developed. These models will enable new treatment strategies to be evaluated under simulated wound conditions, as well as optimization of the antimicrobial effect and safety of the compound before testing in animal models. Substances that have potential to treat biofilm infections are such that interfere with the bacterial communication controlling biofilm formation and production of virulence factors, so called quorum sensing, or substances that enhance the innate immune response in such way that it can control an infection.

## 2. Aim

This master thesis aims to study the interactions between cells and bacteria in a wound in order to develop a new *in vitro* model to test novel antimicrobial substances. In the model, human macrophages and *Pseudomonas aeruginosa* will be used. The macrophages will be cultured on collagen coated surfaces and exposed to supernatant from the *P. aeruginosa* biofilm. Soluble factors from the biofilm and their effect on macrophage viability will be evaluated as well as cytokine response from the macrophages. To test whether the *in vitro* model can be used to screen possible new substances, salicylic acid will used and its effect on virulence of *P. aeruginosa* will be assessed.

# 3. Background

The background will describe important areas for understanding the problem concerning biofilm infections and chronic wounds. The concept of biofilm and quorum sensing will be described as well as the basics of the immune system and its response to a pathogen and then finally wound healing.

## 3.1 Biofilm

Microorganisms have throughout history been characterized as planktonic living cells and described by their growth characteristics when cultured in nutrition rich media [10]. However, it has been shown that more than 99.9% of all bacteria in natural ecosystems live in microcolonies, surrounded by extracellular polymeric substances (EPS), called biofilms [11].

The first known observation of biofilm was Antoine van Leeuwenhoek's examination of the plaque on his own teeth in the 17$^{th}$ century. Almost three hundred years later, in 1934, it was concluded that bacteria in marine environments attached to surfaces and form sessile populations, and the first stages of biofilm formation was studied in 1964 [12]. The term biofilm was coined in 1978 by J.W Costerton, a well renowned researcher in the field, after studying bacterial communities in mountain streams [13].

When a biofilm forms, planktonic cells first attach reversible to a biotic or abiotic surface. The bacteria then aggregate and form microcolonies on the surface and start producing EPS, thereby making the attachment irreversible. The EPS mostly consist of polysaccharides, but also contain proteins and nucleic acids [14]. Channels within the biofilm enable nutrients to diffuse in and waste to diffuse out. Depending on the type of bacteria that forms the biofilm and the surrounding environment, the ratio between cells and EPS matrix is 10-25% to 75-90% [12]. The biofilm grows, it can be up to 50µm thick, and matures and the aggregates often get a mushroom-like appearance [15]. The mushroom-shape allow for optimal diffusion of nutrients throughout the entire community [16]. When the biofilm have matured some parts of the biofilm can detach and spread, and cause formation of biofilm at another location. The different stages of biofilm formation are shown in figure 1. For the biofilm to fully mature bacteria utilizes a communication system called quorum sensing to change the phenotype of the bacterial community [17], this is explained further in section 3.2.

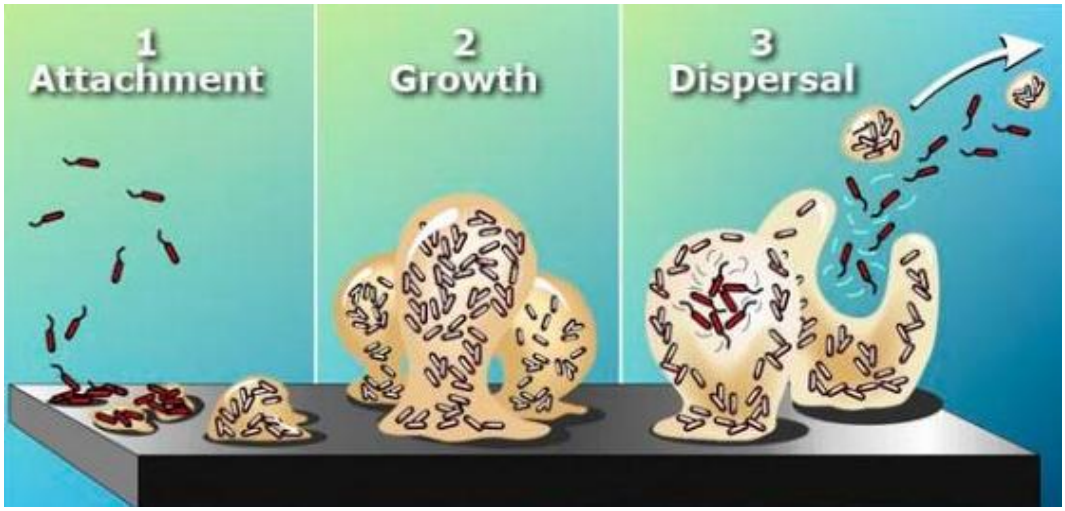



**Figure 1. Biofilm formation. Planktonic cells first attach to the surface reversible. They then form aggregates and start producing EPS making the attachment irreversible. The biofilm grows and matures. Some parts of the biofilm can at this point disperse and form biofilm at another location. Image modified from [18].**

There are several hypotheses of why bacteria form biofilms. One reason might be that the surface provides the bacteria with a stable environment to grow on and it might also have catalytic functions since the biofilm drive the bacteria closer together. The biofilm also protects bacteria from environmental stresses such as toxins, UV and phagocytosis [16]. Furthermore, it is beneficial for the cells to live in this type of community since the biofilm can trap and store nutrients which enables the cells to coordinate their energy resources [16].

For the bacterium to be able to attach to a surface, whether it is biotic or abiotic, it must be able to sense it. Planktonic cells release protons and other signaling molecules that diffuse through the bulk. The surface limits the diffusion and the concentration is therefore higher on that side. The bacterium can because of this difference in concentration “sense” the surface [12].

There are many factors affecting bacterial attachment to a surface. The first factor is surface characteristics. It has been noticed that a rougher surface increase the degree of colonization of bacteria. A rougher surface has a larger surface area and lower shear forces. Hydrophobic, nonpolar surfaces also increase the attachment of bacteria compared to hydrophilic material [10]. Secondly, the hydrodynamics also affects bacterial attachment to a surface. Depending on the velocity of the bulk carrying the bacteria, the settling to a surface will be affected. If the bulk has a very low velocity the association with the surface will mostly be dependent on the cell size and motility, however if the velocity increases it will be more dependent on the velocity characteristics and the bacteria are expected to associate faster to the surface. Of course, at high velocities the bacteria might also be subject to enough shear force to detach from the surface. Biofilm formed under shear stress are more rigid and stronger and have higher densities [10]. Thirdly, it is also believed that composition of the media such as nutrients and ions affects the attachment as well as pH and temperature [10]. Lastly, but very important, is the species-specificity of attachment, since the cell surface, fimbriae and flagella, LPS and EPS production all have an influence on how the bacterium attaches to the surface [10]. Much research has been done on finding a surface that bacteria do not attach to, however this has not been

found yet. It appears that bacteria attaches and form biofilm on any type of surface, it is just a matter of time [16].

Detachment of cells from a biofilm can occur for two reasons. Shear forces can simply cause parts of the biofilm to release. This is what commonly happens with biofilm formed on a rock in a river or biofilms resulting from infection in the heart valve, also known as endocarditis. Single cells can also detach from the matrix, however this is a much more complex situation since the cell must first return to a planktonic phenotype and loosen from the matrix before they can detach [16].

### 3.1.1 Biofilm and chronic infections

Biofilm formation occurs naturally in the body, for example dental plaque and the bacterial lining of the intestine. However, biofilm on medical implants, in lungs of patients with cystic fibrosis, in the heart and in chronic wounds is very difficult to control and can cause severe infections [6].

Bacteria in a biofilm are more resistant to antibiotics than their planktonic counterparts [11]. Antibiotics are therefore often used in combination with topical treatments to enhance the effect, e.g. silver or polyhexamethylene biguanide (PHMB), nevertheless, biofilm related infections is usually very difficult to combat [19]. The antibiotic resistance in biofilms is a result of many factors. Bacteria in a biofilm have an altered growth rate and gene expression compared to the same bacterium in a planktonic state [11]. It is also proposed that the biofilm matrix functions as a diffusion barrier, making it more difficult for the antibiotics to reach the bacteria [11]. Not only do most antibiotic treatments fail to eradicate biofilm infections, but the immune system also has difficulties to eliminate bacteria in a biofilm [11]. Antibodies secreted by immune cells fail to penetrate the biofilm because of the EPS matrix and catalase secreted by the bacteria protects it from hydrogen peroxide penetrating the biofilm. Even if the immune system is fully activated by the presence of a biofilm infection, the phagocytes are ineffective when it comes to ingest sessile bacteria and biofilm [20]. Since neither the host nor antibiotics have an effect on the bacteria, biofilms are extremely difficult to eradicate.

Even though chronic infections in most cases are multispecies, there is a theory that the aggregate forming a biofilm generally consist of only one bacterial strain [6]. In commensal and natural biofilms i.e. aggregation of bacteria in their natural habitat, in contrast to biofilms formed in e.g. wounds, several bacteria live integrated in the same aggregate. Coaggregation in natural biofilms is beneficial for the different bacterial species because they can share compounds for metabolism. The environments where these biofilms form are often limited in nutrients and suitable niches. The bacteria therefore need to adapt to the environment in order to survive resulting in adaptation to niches that are created and/or affected by other bacteria [6]. For bacterial colonization in infections the main challenge is to survive the host defense system. Compared to natural systems, the bacterial diversity in these locations is usually lower since it is a place where the immune system usually maintains a sterile environment. To establish an infection, the right pathogen must be at the right place at the right time and the chance of another bacterium to encounter the same location and also overcome the host response is very low. The environmental conditions in these locations are also different. They are usually high in nutrients due to dead cells and constant blood supply and there is therefore no pressure for the different bacterial species to live in symbiosis [6]. However, even natural biofilms on teeth or intestine can cause

infections. In these cases the diversity of the biofilm has usually been reduced and the believers of single species biofilms in infections conclude that there is a connection between low diversity and high pathogenicity [6].

The biofilm mode of growth in chronic wounds are not yet fully acknowledged, however the clinical signs associated with these types of infections are similar to others caused by biofilm, such as cystic fibrosis [3]. Although it is now recognized that all chronic wounds are infected by multiple pathogens, there still some controversy about their impact on wound healing. A study by Gjødsbøl et al. showed that more than 50% of the investigated wounds contained colonies of *P. aeruginosa* [9]. *P. aeruginosa* is usually found deeper down in the wound, where as other pathogenic bacteria such as *Staphylococcus aureus* are found at the wound surface [6]. Gjødsbøl's study also found that wounds infected with *P. aeruginosa* appeared to be larger than wounds that did not contain the bacteria. This is believed to be due to tissue-destroying enzymes produced by *P. aeruginosa*. Another study has also shown that metalloproteinase produced by *P. aeruginosa* inhibits growth of fibroblasts and degrades wound fluid and human skin proteins [21]. It is therefore likely to believe that the bacteria can alter the wound healing response resulting in enlarged wounds and/or delayed wound healing [9].

### 3.1.2 *Pseudomonas aeruginosa* biofilms

The formation of biofilm is one of the main defense systems of *Pseudomonas aeruginosa* when encountering a hostile environment [3]. The EPS matrix of the *P. aeruginosa* biofilm consists mainly of polysaccharides, proteins and nucleic acids. The exact composition depends on the surrounding environment, how old the biofilm is and which strain of the bacterium forming the biofilm [16]. *P. aeruginosa* attachment to a surface is mediated by type IV pili. Once a bacterium has attached to the surface it moves by twitching the pili towards other cells on the surface in order to form aggregates [16]. Within 15 minutes of contact with the surface, the genes coding for alginate is upregulated and this is believed to initiate biofilm formation [17].

As mentioned earlier, bacteria often form a mushroom shape in the biofilm. When this is formed by *P. aeruginosa*, specific cells at the top of the stalk lyse and release their DNA. Mobile cells, from another clone than the stalk, can then use this DNA as a base for forming the cap of the mushroom. These cells climb the stalk using the same twitching motility as for aggregation [16].

As a biofilm mature it differentiate more and more from the planktonic state. In *P. aeruginosa* around 70% of the phenotype is different between planktonic cells and cells in a biofilm [16]. It has also been shown that over 300 proteins for metabolism, LPS and phospholipid biosynthesis, membrane transport and secretion, are detectable in biofilm that are not detectable in the planktonic bacterium [17].

## 3.2 Quorum sensing

Quorum sensing (QS) is a type of cell-to-cell communication utilized by many bacteria. It is population density dependent and as a population reaches a certain threshold level the concentration of so called autoinducers get high enough to cause a change in gene expression within the bacterial population. Once the right level has been attained the target genes of the QS are induced or repressed depending

on the desired action. In this way, the bacteria can change behavior to fit special environmental needs or defend themselves from threats to the community [22].

Depending on the type of bacteria different QS signals are used. Gram negative bacteria usually use acetylated homoserine lactones (AHL), while Gram positive bacteria often use small peptides. A third QS signaling molecule named autoinducer-2 (AI-2) have been found in both types of bacteria [23].

It its very beneficial for bacteria to use QS. To only produce extracellular factors once a large enough population have established is of economical advantage, since the concentration needs to be high enough when released to the surroundings to be able to affect it. For a pathogen it is also important that it can accumulate at a site and control the release of its virulence factors in order to surprise the host response with an attack [24].

### 3.2.1 N-acylhomoserine lactones

As mentioned above, N-acylhomoserine lactones are quorum sensing molecules produced by Gram negative bacteria. The signaling molecules where first discovered in *Vibrio fischeri* as a regulator for bioluminescence. Since then, autologous systems have been identified in many Gram negative bacteria [25]. AHLs are characterized by a homoserine lactone ring that is N-acetylated with an acyl chain of different lengths (ranging from 4-16 carbons), saturations and oxidation states. It is the acyl chain moiety that specifies the QS signal. The biosythestis of AHL is controlled by the protein family LuxI. As source for the substances to build AHL from, LuxI use S-adenosylmethionine (SAM) and an acyl carrier protein (ACP). The acyl group from the ACP is transferred to the amino group on the SAM, and after lactonization the AHL and mehylthioadenosine is released [26].

### 3.2.2 Quorum sensing in *Pseudomonas aeruginosa*

*P. aeruginosa* is a Gram negative bacterium and therefore utilizes N-acylhomoserine lactone (AHL) as its main quorum sensing signaling molecules (QSSM) [22]. There are two QS systems in *P. aeruginosa* that have been well studied, the *las* and *rhl* systems. These systems are both composed of a transcriptional regulatory protein, either LasR or RhlR, and an autoinducer signal molecule, for the las system it is N-(3-oxododecanoyl)-homoserine lactone (OdDHL) and for the Rhl system N-(butanoyl)-homoserine lactone (BHL). Once the concentration of the AHL reaches a certain threshold level the regulatory protein binds to the autoinducer and forms a complex. The complex then stimulates transcription of virulence factors such as elastase, exotoxin A and alkaline protease for the *las* system and rhamnolipid and pyocyanin for the *rhl* system [27]. Which target genes that are activated is dependent on the concentration of the different AHL [24]. Both systems have positive feedback loops since they regulate the AHL synthase gene, resulting in an autoinduction of the AHL production [26]. The quorum sensing systems in *P. aeruginosa* are arranged in hierarchy with the *las* system on top, meaning that it controls the activation of the *rhl* system [27]. The signaling pathway of these two systems is shown in figure 2, where the triangles represent OdDHL and the pentagons BHL.

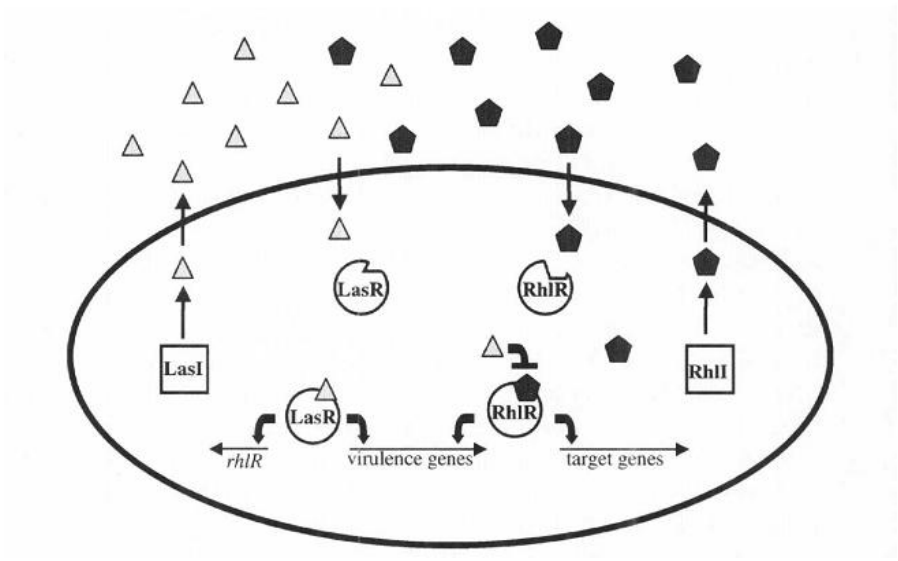



**Figure 2. Signaling pathway for *las* and *rhl* system used for quorum sensing in *P. aeruginosa* [28].**

In addition the two AHLs, OdDHL and BHL, mentioned earlier *P.areuginosa* also produce N-3-oxoheaxanoyl homoserine lactone (OHHL) and N-hexanoyl homoserine lactone (HHL). Their function has not yet been fully characterized but it is believed that they control virulence factors in a similar manner as OdDHL and BHL. OHHL and OdDHL have 3-oxo-substituted side chains while the other two have unsubstituted side chains [29]. The structures of the AHLs are shown in figure 3.

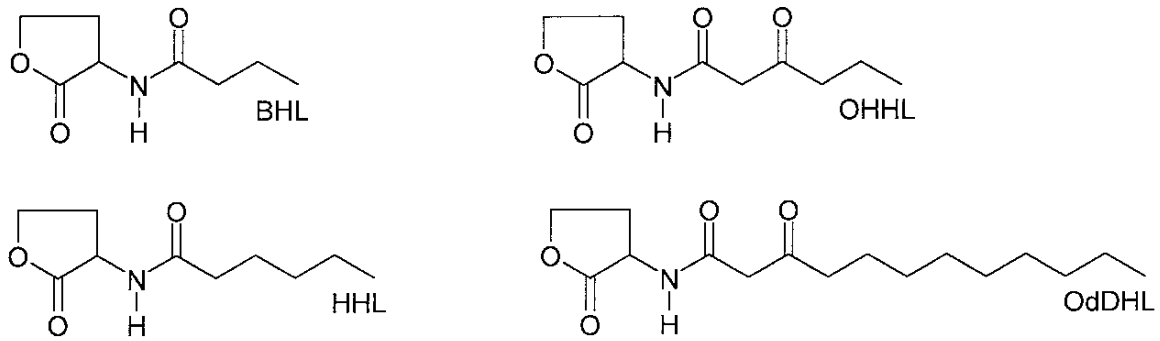



**Figure 3. Chemical structures of the four AHLs produced by *P. aeruginosa*.**

One additional signal molecule, 2-heptyl-3-hydroxy-4-quinolone, have been found important for QS in *P. aeruginosa*. In contrast to the others this is not an AHL but a quinolone and referred to as PQS for pseudomonas quinolone system. The PQS works as a link between the *las*- and the *rhl* system [28].

In *P. aeruginosa*, *QS* regulates genes for metabolism, synthesis of proteins and biofilm maturation. It is believed that OdDHL is required for normal *P. aeruginosa* biofilm formation [17]. One study showed that mutants lacking this signaling molecule produced biofilm that were only 20% as thick as biofilm produced by wild-type bacteria, the biofilm was also flat and undifferentiated [30]. However, other studies have in fact shown that *P. aeruginosa* is capable of producing biofilm even in the absence of

OdDHL [31]. It has also been shown that QS plays an important role in the virulence of *P. aeruginosa*. Using animal models to look at burn wounds, acute pneumonia and chronic lung infections, showed that a mutation in QS genes compared to wild type induced less tissue destruction as well as reduced the size of infection and mortality [32-34]. However, even with mutated QS genes the bacteria still showed virulence supporting the concept of multifactorial virulence in *P. aeruginosa* and that factors other than QS plays a pivotal role in the pathogenesis [23].

### 3.2.3 Immunomodulatory activity of *Pseudomonas aeruginosa* quorum sensing signaling molecules

In addition to regulating production of virulence factors, the QSSMs themselves can also have a direct effect on the host response to a pathogen. Several studies show that OdDHL from *P. aeruginosa* interferes with the host immune response. Since the QSSMs affect the immune response it is also believed that it alters the pathogenesis in the host [35].

One of the first studies in this field was performed in 1998. Telford et al. showed that OdDHL reduced secretion of Interleukin (IL)-12 and tumor necrosis factor α (TNF-α) by macrophages stimulated with LPS, while the shorter OHHL showed no such effects. OdDHL also inhibited T cell proliferation and suppressed antibody production against *P. aeruginosa* virulence factors, at concentrations above 70 µM. However, enhanced production of antibodies was shown at lower concentrations and the authors hypothesized that OdDHL could direct T cell response away from the Th1 phenotype, which is host protective, thus promoting survival of the invading pathogen [36].

Since 1998, several studies have examined the immunomodulatory effects of OdDHL. One study showed that the QSSM induced an increase in the mRNA for IL-1α, IL-6 and macrophage inflammatory protein 2 as well as for the expression of cyclo-oxygenase 2. The secretion of interferon γ (INF-γ) and production of IL-8 by endothelial cells was also induced by OdDHL [37]. These results indicate, in contrast to the results of Telford et al., that OdDHL promotes Th1 phenotype. However, the studies where performed at different concentrations, 100µM and <10µM respectively. Later studies have in fact showed that these high concentrations of OdDHL accelerate apoptosis in eukaryotic cells [38].

It has also been shown that even low concentrations of OdDHL induce apoptosis in macrophages and neutrophils [39]. However, this acceleration of apoptosis has been questioned. One study showed that OdDHL induced chemotaxis and increased phagocytosis of opsonized bacteria by neutrophils [40] and another that macrophages exposed to OdDHL had an increased phagocytic activity and that it stimulated the macrophages through the p38 MAPK pathway [41]. No accelerated apoptosis was shown in either of these studies. The authors question whether low concentrations of OdDHL actually induce apoptosis but explain it by the use of different organisms for the different studies.

Since there are so many contradicting studies concerning the immunomodulatory effect of OdDHL, it has been suggested that the outcome of OdDHL exposure is highly dependent on the immune status of the host [42]. It has also been shown that the effect of OdDHL is highly concentration dependent and it is suggested to be immunosuppressive or anti-inflammatory at concentration <10 µM and proinflammatory or proapoptotic at concentrations > 20 µM [26].

PQS have also been shown to have immunomodulatory effects, however, it does not modulate the immune response in the same way as OdDHL. One study showed that both OdDHL and PQS inhibited T cell proliferation, but only OdDHL affected the release of IL-2. OdDHL also affected the release of TNF-α, which has been shown previously [36], while PQS on the other hand induced the production [43]. Since OdDHL controls the production of PQS through LasR, the two QSSMs are interdependent and it appears that they work in synergy to maximize the immunomodulatory effect [35]. Another study showed that that T cell activity was altered when dendritic cells (DC) were exposed to OdDHL and PQS *in vitro*. The DCs stimulated by LPS showed reduced levels of IL-12 when exposed to OdDHL and PQS. Reduced levels of IL-12 might shift the Th1/Th2 balance since T cells will not get a signal to develop into Th1 cells. The study also showed that OdDHL inhibit surface markers that stimulates T cells and are required for their maturation. Since these QSSMs affect T cell proliferation, the authors postulate that they most likely also have an effect on the level of cytokines produced [44]. For both studies low concentrations, <10 µM, of the QSSM was used. It has been demonstrated that the effect of OdDHL on T cell proliferation is not due to toxicity of the QSSM [45].

It has been shown that the host has its own defense mechanism towards the immunomodulatory effects of OdDHL. In a study by Crebbé et al. a 3D co-culture *in vitro* model with alveolar epithelium and macrophages was developed in order to observe the response in the lung tissue in when subjected to a *P. aeruginosa* infection. The result showed that the alveolar epithelial cells protect the macrophages from the cytotoxic effect of the OdDHL by degrading them before they have a chance to cause any harm [46]. A previous study showed that it was the enzyme paraoxanase, produced by the epithelial cells, which inhibited the effect of OdDHL [47].

Not only do the QSSMs affect the host, and as shown by many studies in a way that is positive for the survival of the pathogen, but It also appears that the bacteria may respond to cytokines produced by the host immune cells and thereafter upregulating the QS response [48, 49].

### 3.2.4 Quorum sensing as antimicrobial target

Since antibiotics have limited effect on bacteria in a biofilm, other methods of treating these infections must be developed. One way of battling the infection is to disturb the quorum sensing thus affecting biofilm formation and production of virulence factors and several compounds have been tested as candidate quorum-sensing inhibitors (QSI). It has been shown that some antibiotics such as azithromycin, ceftazidime and ciprofloxacin actually inhibit QS at concentrations lower than the minimum inhibitory concentration. However, the development of bacterial resistant to antibiotics is still a major threat [15]. Another strategy is to use AHL analogous to block the AHL signal e.g. replacing the carboxaminde bond with a sulfonamide bond [50] or deriving ureas from the AHLs [51].

One of the newest approaches towards finding a potent QSI is to use natural compounds [52] such as halogenated furanones [53], salicylic acid [54], extracts from garlic [55] and polyphenols from cranberries [56]. The furanones have been intensively studied by Giskov et al. They have shown that furanones targeted the quorum sensing system in *P. aeruginosa* and affected the production of virulence factors. It also made the bacteria more sensitive to the antibiotic tobramycin [57]. By studying lung infections in mice, the research group also showed that synthetic furanones inhibited QS in *P.*

*aeruginosa* by interfering with the AHLs and that treatment with furanones enhanced the clearance of bacteria [53].

Salicylic acid (SA) is an interesting substance for possible treatment of biofilm infections. Since it is antiseptic and anti-infective without killing the bacteria there is minimal risk of developing microbial resistance towards SA [58]. SA is produced by plants and is known to play an important part in the plant's defense against an attacking pathogen [59]. In plant systems, SA down regulates production of virulence factors, such as pyocyanin, protease and elastase, from *P. aeruginosa* and one study show that even at concentration low enough not to interfere with the growth of the bacteria, attachment and biofilm formation are still reduced [60]. The study further proved the down regulation of virulence factors by infecting *Caenorhabiditis eleg*ans with *P. aeruginosa* showing that the addition of SA reduced the mortality of the infection in the worms [60]. Poly(anhydride-esters) that degrades into salicylic acid has also been shown to have antibacterial effect. Bacteria grown on polymers secreting SA adhered slower to the surface and produced less biofilm than bacteria grown on plain polymer [54]. Even though SA shows promising result as a QSI, it might affect the immune system response towards a pathogen. SA suppresses the macrophages expression of inflammatory molecules such as cyclo-oxygenase-2, interleukin-4 and induces nitric oxide synthase [61]. However, there are no studies at present that evaluates if the changes in level of these compounds have any effect on how the host responds to a pathogen.

## 3.3 The immune system

The immune system has four main functions that must work properly in order to protect the body from disease. First is the immunological recognition of an infection by leukocytes and lymphocytes. Next the infection needs to be controlled and eliminated by immune effector functions such as the complement system, antibodies and phagocytes. It is also important that the immune system is controlled and regulated so it does not damage the host, so called immune regulation. Allergy and autoimmune diseases, e.g. diabetes, are examples of what happens if the immune regulation fails. Finally, the immune system can create an immunological memory, so that if a person is exposed to the same infectious agent a second time the response will be stronger and faster [62].

The immune system can be divided into two parts, the innate or non-specific immune system and the adaptive or specific immune system. The first line of defense is the innate immune system, consisting of anatomical, humoral and cellular barriers such as the skin, signaling molecules and immune cells e.g. macrophages. To be infected by a pathogen the host must first be exposed to it, usually via an epithelial surface (skin or mucosal surface). The pathogen then adheres to the surface or penetrates the epithelial barrier and reaches the tissue, where it establishes a focus of infection. At this point the infection is processed only by the innate immunity [62].

If the innate immunity fails to clear the pathogen, the adaptive immune system will be activated. The activation is mediated by antigen presenting cells (APC) from the innate immunity, i.e. macrophages and dendritic cells, which recognize bacterial endotoxins such as lipopolysaccharides (LPS). When specific receptors on the APC bind to these microbial components, the pathogen is engulfed and degraded and parts of the pathogen are then displayed on the cell surface. Development of the adaptive system takes

a few days, and is mediated by B and T cells as a response to antigens from the invading pathogen presented on the surface of the APCs [62]. Immune regulation, as mentioned above, is an important part of the adaptive immune system and the immune response can either be stimulated or inhibited depending on the received signals. One theory of how the immune system is controlled involves the balance between two distinct subsets of T cells, namely T-helper 1 (Th1) and T-helper 2 (Th2). Th1 is responsible for the cell-mediated immunity, thus recruits and activates macrophages and neutrophils to eradicate the invading pathogen. If this subset is dominant, the response is often associated with inflammation. Th2 is responsible for the humoral immunity and stimulates production of antibodies from B cells. Most of the cytokines produced by Th2 are anti-inflammatory and a dominant Th2 subset inhibits both acute and chronic inflammation [63].

For this study macrophages have been chosen to represent the immune system in an *in vitro* model. Macrophages will therefore be the only cell type of the immune system described in more detail.

### 3.3.1 Monocytes/ macrophages

Macrophages are phagocytosing leukocytes and are the first line of defense in the innate immunity together with neutrophils. Macrophages can be found in almost all tissues. In the blood the cells are present as monocytes, but as they migrate into tissues they differentiate into macrophages. The cell changes quite radically when it differentiates, it grows to more than twice its size, increases the number of mitochondria and its phagocytic function is enhanced. Macrophages are amongst the first cells to reach the infected area where they eliminate invading microorganisms and other foreign particles by phagocytosis. They also induce inflammation, secrete cytokines to activate other parts of the immune system and presents antigens to the adaptive immune system as a response to inflammation [62]. Finally they can also shift the Th1/Th2 balance by suppressing Th1 and promoting Th2 and they are a major source of transforming growth factor β (TGF-β) which promotes many factors in the wound healing process [64].

Monocytes are derived from hemapoetic stem cells in the bone marrow. These hemapoetic stem cells can be divided into two lineages, the lymphoid and myeloid. The monocytes belong to the myeloid linage together with granulocytes, megakaryocytes, erythroblast and mast cells [62]. The monocytes circulate in the blood to be able to reach the site of inflammation quickly and can either differentiate to macrophages or dendritic cells, which is another important APC in the immune system, depending on the signals received. A portion of the monocytes is stored in the spleen and released as the amount of monocytes in the blood decreases [64].

In the wound macrophages can be divided into two different phenotypes depending on the mode of activation. The macrophage maybe classically activated, referred to as M1, if it is activated by, for example, LPS from bacteria or the cytokine INF-γ. These macrophages release inflammatory mediators such as TNF-α and IL-6. Macrophages that are activated by for example IL-4 or IL-13 develop the other phenotype, M2, and works to suppress the inflammatory reaction and promote tissue repair. Both types are present in a wound, however M1 is predominate in the early inflammatory phase while M2 is important once the wound has started to heal [64].

### 3.3.2 Cytokines

Cytokines are small signaling proteins secreted by cells that have been exposed to some kind of stimulus. The cytokines bind to specific receptors on the target cell and induces a response. When macrophages are activated by pathogens they secrete IL-1β, IL-6, IL-8, IL-12 and TNF-α, causing different local and systemic effects and initiate inflammation [62]. Once the invading pathogen has been cleared the inflammatory response needs to retract. Now macrophages release cytokines such as IL-1ra, IL-4 and IL-10 and, if they are present in a wound, different growth factors to promote healing such as TGF-β and vascular endothelial growth factor (VEGF) [64].

Four different cytokines will be evaluated in this study, IL-1ra, IL-8, IL-10 and IL-12. These are described in more detail below.

#### *3.3.2.1 Interleukin 1RA*

The interleukin 1 receptor antagonist is a member of the IL-1 family of cytokines. When macrophages are stimulated they start producing IL-1β. IL-1β then binds to specific receptors on different cells causing pro-inflammatory reactions, such as release of IL-6 and recruitment of neutrophils. Through competitive binding to the receptors, IL-1ra blocks the action of IL-1 β and thus works as an anti-inflammatory mediator [65, 66].

#### *3.3.2.2 Interleukin 8*

Chemotactic cytokines, or chemokines, are a special class of cytokines that induces directed chemotaxis on the target cells. IL-8 is a chemokine for neutrophils, basophils and T cells produced by many cells of the immune system including macrophages. It is released as an early response to infection. IL-8 also goes under the name CXCL8. IL-8 causes neutrophils to leave the blood stream and migrate into the tissue. There are two ways in which IL-8 acts. First it causes a conformational change in the integrins on the leukocyte resulting in attachment of the cell to the blood vessel wall. This action makes it possible for the leukocyte to migrate across the wall by squeezing between the endothelial cells. Second, in the tissue there is a concentration gradient of IL-8 that increases towards the site of infection. This gradient helps the cells to migrate to the correct location [62]. IL-8 also activates leukocytes in other ways, for example it causes neutrophils to release lysosomal enzymes, generate superoxide and hydrogen peroxide and induce respiratory burst [67].

#### *3.3.2.3 Interleukin 10*

Interleukin 10 was first known by the name cytokine synthesis inhibition factor due to its inhibition of INF-γ produced by Th1 cells causing a shift in the balance towards Th2 [68]. IL-10 is a homodimer with a molecular weight of 38 kDa and it is produced by many cells of the immune system, however, macrophages are the major source. IL-10 is anti-inflammatory and thus inhibits factors that induce the inflammatory response, such as IL-1β, IL-6, IL-8, IL-12 and TNF-α, and stimulate those associated with tolerance of the adaptive immunity, such as IL-1RA and soluble TNF-α receptors. The cytokine also inhibit the antigen presenting capacity of the macrophages by reducing the expression of the antigen binding receptor major histocompatibity complex (MHC) II. In addition, undifferentiated monocytes in the blood cannot differentiate into type 1 dendritic cells in the presence of IL-10 [69].

#### *3.3.2.4 Interleukin 12*

Interleukin 12 is a heterodimeric cytokine that is produced in response to bacteria, mostly by macrophages and B cells. It also goes under the name natural killer cell stimulating factor (NKSF). IL-12 induces proliferation and cytokine production in T- and NK-cells, in particular it induces the production of INF-γ, and increases the cytotoxic effect of T cells and NK cells. It is also an important factor for development of Th1 cells from naïve T-cells [70, 71].

## 3.4 Wound healing

Wound healing can be divided into four phases, hemostasis, inflammation, proliferation and remodeling. However, the phases are not distinguished but rather overlap in time. Immediately after injury, the platelets in the blood aggregate and form a blood clot to prevent blood loss. The clot provides incoming inflammatory cells with a temporary matrix for cell migration and a source of cytokines and growth factors. It also works as a temporary cover to protect the wounded area from further stress from the outside [64].

Inflammation is triggered by hemostasis and the release of chemo-attractants. Neutrophils are the first cells to arrive and start clearing the area from foreign particles and bacteria within hours. The monocytes then arrive and start differentiating into macrophages. Endothelial cells in the area are activated by thrombin from the platelets to produce IL-6 and IL-8, two pro-inflammatory cytokines that drives the differentiation of monocytes to type M1 macrophages. Neutrophils and monocytes also produce pro-inflammatory cytokines such as IL-1β, IL-6, INF-γ and TNF-α [64, 72]. The neutrophils are short lived and within three to five days macrophages are the most prominent cell type in the wound tissue [64]. The inflammation also recruits other cells of the innate immunity from the blood to the infected tissue as well as increasing the flow of lymph containing microbes and antigen-bearing cells towards the local lymphoid tissues. This will activate the adaptive immune response and recruit antibody producing B cells and effector T cells to the area [62].

The visible effect around the wound of the inflammatory response to cytokines and the cells is often described by the Latin words *calor*, *dolor*, *rubor* and *tumor*, which means heat, pain, redness and swelling. This is caused by the dilation and increased permeability of the blood vessels which leads to increased blood flow and leakage of fluid and migration of cells from the blood to the tissue [62].

Macrophages that have phagocytosed dead cells and tissue undergo apoptosis. The ones that survive undergo a phenotypic change from M1- to M2 macrophages and is believed to play an important role in the transition between inflammation and proliferation. Proliferation is characterized by the formation of granulation tissue, which starts filling the wound space a few days after injury [72]. At this point, the M2 macrophages are dominating at the site and provide the area with growth factors important for formation of fibrous tissue and new blood vessels, such as TGF-β, VEGF and TNF-α [64]. Another part of the proliferation phase is reepithalization. The clotted blood and damaged stroma is removed by adjacent epidermal cells. The epidermal cells undergo phenotypic alteration and start migrating across the wound while they dissect it and separates the eschar from the viable tissue underneath. After one or two days, the epidermal cells start to proliferate and as the reepithalization continues, the epidermal cells regain their normal phenotype [72].

The final phase of wound healing is remodeling, which can continue for months. This phase is characterized by low cell proliferation and protein synthesis. The newly formed collagen is remodeled into fibrils that are larger and more organized. The capillaries formed during proliferation phase will retract due to lower demand of nutrients and many cells will undergo apoptosis or exit the wound leaving an almost acellular scar where the injury was [64].

### 3.4.1 Chronic wounds

In a healthy person an acute wound will heal relatively fast. However, for persons with an impeded immune response due to other diseases, i.e. diabetes or vascular disease, normal wound healing may be disturbed and the wound will heal extremely slowly. Hard to heal wounds, or chronic wounds, are characterized by a prolonged inflammation. Macrophages and neutrophils are continuously recruited to the wound, often due to heavy bacterial load or necrotic tissue. These wounds also have lower levels of growth factors and higher concentration of proteases. The cellular response to the growth factors is usually lower and the cells do not proliferate normally [73].

# 4. Materials and Method

This chapter describes how the different analyses were executed. All cell study experiments were performed in a LAF-hood. The cells are referred to monocytes in the cell adhesion study and as monocyte derived macrophages (MDMΦ) for the cell viability and cytokine study because of different culture conditions resulting in different phenotypes. Viable cells are defined as cells that are attached to the collagen coated surface after exposure to conditioned medium from the bacterial cultures.

## 4.1 Bacterial strains and AHL detection

Five different strains of *Pseudomonas aeruginosa* were used in this study, PAO1 wild-type laboratory strain (a gift from Dr Bengt Wretlind, Karolinska Institutet, Stockholm, Sweden) and four strains isolated from chronic wounds, here named Pa1, Pa2, Pa3 and Pa4 (isolated by Arthur Schmidtchen, BMC, Lund, former names 10.5, 11.2, 11.3 and 13.1 respectively). Pa1 and Pa4 are known to produce BHL, OdDHL, pyocyanin, rhamnolipid and elastase while Pa2 and Pa3 do not produce any of these factors. All of the strains form biofilm when cultured in vitro [74]. With respect to their production of virulence factors PAO1, Pa1 and Pa4 will be referred to as “more virulent strains” and Pa2 and Pa3 as “less virulent strains”.

To examine whether the strains produce the $C_6$ AHLs, HHL and OHHL, *Chromobacterium violaceum* CV026 was used as a reporter strain. *C.violaceum* produces the purple pigment violacein when it comes in contact with AHLs with N-acyl side chains varying between 4 to 8 carbons [75]. Colonies of the different strains of *P. aeruginosa* was stroked on tryptic soy agar (TSA) plates (Department of Bacteriology, Sahlgrenska university hospital, Gothenburg) and placed in incubator at 37°C over night. *C.violaceum* was then stroked parallel to *P. aeruginosa* and the plate was placed in room temperature for 24 h.

## 4.2 Growth of biofilm and collection of supernatant

Single colonies from each of the five strains were grown in 3 ml of tryptic soy broth (TSB) (Department of Bacteriology, Sahlgrenska university hospital, Gothenburg) at 37°C for 72 h in order for the biofilm to form.

After incubation, the culture containing the biofilm was vigorously vortexed to release virulence factors from the biofilm and then centrifuged for 5 min at 10 000g before collection of the supernatant. The supernatant were sterile filtered (Acrodisc® syringe filers 0.2 µm, Pall corporation, Cornwall, UK) before added to the cell culture plate.

For some of the experiments the bacteria were cultured in the presence of Salicylic acid (Merck Schuchardt OHG, Hohenbrunn, Germany,). SA was dissolved in TSB to obtain a stock solution with the concentration of 0.2M. This was added to the culture, for final concentrations of 1mM and 2mM, before incubation of the bacteria.

## 4.3 Isolation of human monocytes

Human monocytes were isolated by a two-step isolation method using the separation gradient Percoll™ (Pharmacia, Sweden) [76]. Buffy coat, e.g. concentrate of white blood cells after centrifugation of whole blood, were received from Blodcentralen (Sahlgrenska university hospital, Gothenburg). 3 ml of 1.076kg/l Percoll were placed in 15 ml centrifugation tubes and then 5 ml of the buffy coat were carefully placed on top, without mixing the gradient and the blood. The tubes were centrifuged at 800g in room temperature for 30 min. To prevent cell activation the cells was hereafter kept on ice.

After centrifugation the layer containing mononuclear cells were transferred by a Pasteur pipette to phosphate buffered saline (PBS) without Ca and Mg (PAA, Pasching, Austria ) and the cell suspension was washed twice under centrifugation at 150 g and 4°C for 5 min. The pellet was then resuspended in 20 ml of PBS and placed on top of 1.064 kg/l Percoll in new 15 ml centrifugation tubes. Again, the cells were carefully placed on top of the gradient not to mix the two. The tubes were centrifuged at 800g at 4°C for 60 min.

The visible band of cells were collected and this time transferred to Hank's balanced salt solution (HBSS) without Ca and Mg (Invitrogen, Paisley, UK) and washed at 150g for 5 min at least two times or until the cell suspension no longer contained any thrombocytes. The cell were then counted in a Bürker chamber and thereafter diluted in cell culture media to the desired concentration. The cell culture media contained RPMI 1640 with L-glutamine and HEPES, 5% fetal bovine serum and 1% antibiotic/antimycotic (all from PAA, Pasching, Austria).

## 4.4 Culture of human monocytes

Two different sets of experiments were conducted. The first experiment assessed influence of bacterial biofilm on cell adhesion. 500 µl of the cell suspension, resulting in a concentration of $3x10^5$ cells/well, were seeded to a 48 well plate and immediately exposed to 100µl/ml of conditioned media (CM) from the biofilm cultures. The cells were placed in an incubator for 24 h (37°C, 5% $CO_2$) before analysis.

For the second experiment, the 48 well plates were coated with 0.5 ml collagen type I (BD Bioscience, Bedford, USA) at a concentration of 82,5 µg/ml. The collagen where left to adhere to the well over night and the wells were then washed twice with PBS before seeding the cells. The monocytes were seeded at a concentration of 3x10^5 cells per well and let to adhere to the surface for 24 h incubated at 37°C (5% $CO_2$). The media were then changed and the adhered cells, from now on referred to as MDMΦ, were exposed to CM from the biofilm cultures at a concentration of 100µl/ml. The cells were exposed to the different bacteria for 24 h (37°C, 5% $CO_2$) after which the samples were analyzed.

After 24 h, the supernatant from each well were collected and centrifuged at 400g for 10 min and 200 µl was used for the LDH assay (described in 4.6). The rest was stored at -18°C until further analysis.

## 4.5 Cell quantification

The number of viable cells in the wells after 24 h exposure to conditioned media from the biofilm cultures was analyzed using a NucleoCounter (ChemoMetec, Allerod, Denmark). The NucleoCounter counts the number of nuclei in the sample by staining with propidium iodide. The cells in the wells were

first lysed using 250 µl of the lysis reagent A for approximately 10 min and then stabilized by 250 µl of the stabilizing reagent B. The solution in the well was carefully, but well stirred, before loading the NucleoCassette for analysis. The analysis was executed according to the manufacturer's protocol.

## 4.6 Cell viability

The viability of the cells was measured by analyzing the levels of lactate dehydrogenas (LDH) in the supernatant. LDH is an intracellular enzyme and released from cells when the cell membrane ruptures indicating cell death [77]. 200 µl of the cell supernatant were transferred to microtubes and sent to Provtagningsenheten (Sahlgrenska university hospital, Göteborg) for analysis.

## 4.7 Cytokine analysis

The levels of cytokines released by the MDMΦ were quantified using enzyme-linked immunosorbent assay (ELISA) (Quantikine® Colorimetric Sandwich ELISA, RnD systems, Abingdon, United Kingdom). The samples were removed from the freezer and thawed in room temperature. Four different cytokines, IL-1ra, IL-8, Il-10 and IL-12, were analyzed according to manufacturer's protocol. The color intensity in the sample was measured at 450 nm with correction at 540 nm, using a plate reader (SPECTRA max, Molecular devices, UK) and the data were analyzed using SoftPro software (Molecular devices, UK).

## 4.8 Statistical analysis

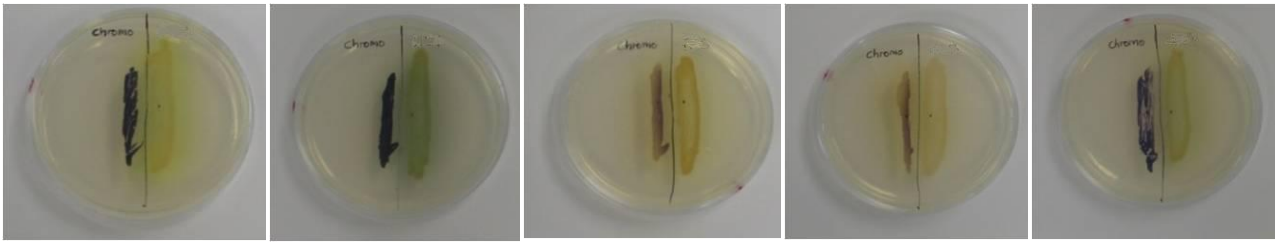
To analyze possible significant differences between samples with consideration to donor variation a two way analysis of variance (ANOVA) combined with Fisher's least significant difference was used. In the charts, standard error of the mean visualize the deviation between samples. P-value $<0.05$ was considered significant.

# 5. Results

To investigate the effect of bacterial biofilm on the human immune system, monocyte derived macrophages (MDMΦ) were cultured together with conditioned medium from 3 day old *P. aeruginosa* cultures. Cytokine response and number of viable cells were analyzed in this *in vitro* model to study inflammatory response. In addition, salicylic acid was tested as a possible quorum sensing inhibitor in order to evaluate the functionality of the model. The clinical wound isolated bacteria used in this study had previously been characterized regarding QS-signaling and a few virulence factors [74], however, characterization of other possible AHLs released from *P. aeruginosa* was needed.

## 5.1 AHL detection assay

Five different bacterial strains of *P. aeruginosa* were used in this study, four clinically isolated strains and the well defined laboratory strain PAO1. QSSM production of the four clinical isolates had previous been studied, showing that OdDHL and BHL were produced only by Pa1 and Pa4. To test whether the two other AHLs, OHHL and HHL, were produced *C.violaceum* CV026 was used as a reporter strain. The results from the detection assay are shown in figure 4. The reporter strain have been colored violet for all five strains as shown in image 4a-e, implicating that all five strains tested produce AHL with chains ranging between 4 to 8 carbons. Since Pa2 and Pa3 do not produce BHL the coloring must be caused by OHHL, HHL or both. The darker stain for PAO1, Pa1 and Pa4 is probably due to the additional detection of BHL.



a) PAO1 b) Pa1 c) Pa2 d) Pa3 e) Pa4

**Figure 4. Detection assay for short AHLs. Image c and d shows light violet coloring, indicating that both Pa2 and Pa3 produce OHHL, HHL or both. PAO1, Pa1 and Pa4 all produce BHL resulting in a darker violet coloring for these three.**

## 5.2 Cell adhesion and cell viability

The number of adhered monocytes after exposure to CM from the bacteria immediately after seeding the cells was evaluated after 24 h using a NucleoCounter. All five strains caused a reduction of adhered cells compared to the untreated control, see figure 5. However, the reduction was only significant for the cells exposed to CM from PAO1 and Pa4. The results from the LDH analysis of the supernatant from the cell adhesion experiment are shown in figure 6, with significantly increased LDH values for PAO1 and Pa4.

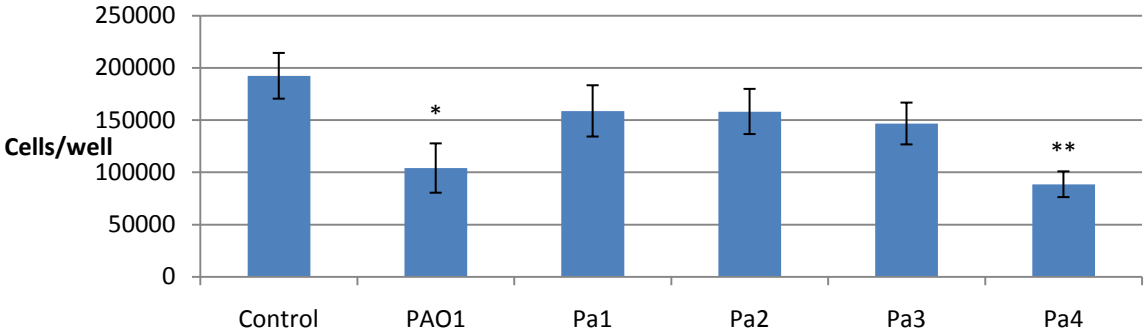



**Figure 5. Cell adhesion after 24h exposure to CM from *P. aeruginosa* biofilm cultures. Data shown as mean ± SEM. n=9 for control, PAO1, Pa1 and Pa2 (3 donors), n=6 for Pa3 and Pa4, (two donor). * P<0.05 ** P<0.01.**

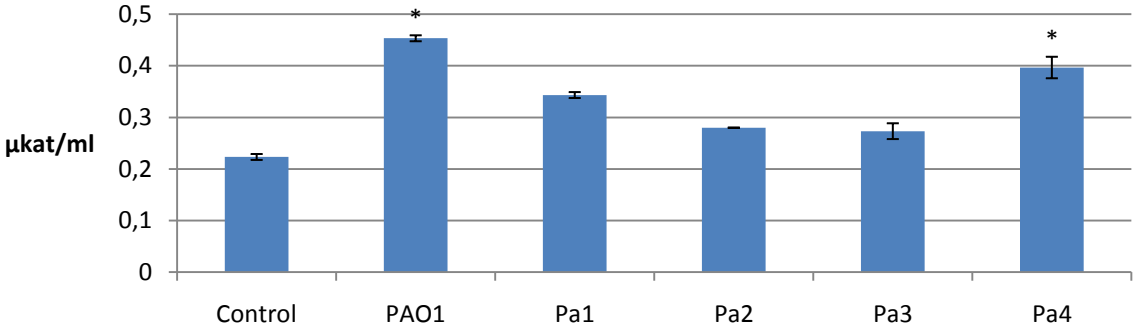



**Figure 6. LDH values for the cell adhesion study in figure 6. Data shown as mean ± SEM. n=3 (1 donor). * P<0.05 ** P<0.01.**

The cell viability of the MDMΦ cultured on collagen coated surfaces after 24 h of bacterial treatment was assessed by counting adhered cells using a NucleoCounter and the result is shown in figure 7. Pa3 shows the only significant reduction of cell number. LDH values corresponding to the cell viability study are shown in figure 8. A significant increase in level of LDH is shown for PAO1 and Pa4.

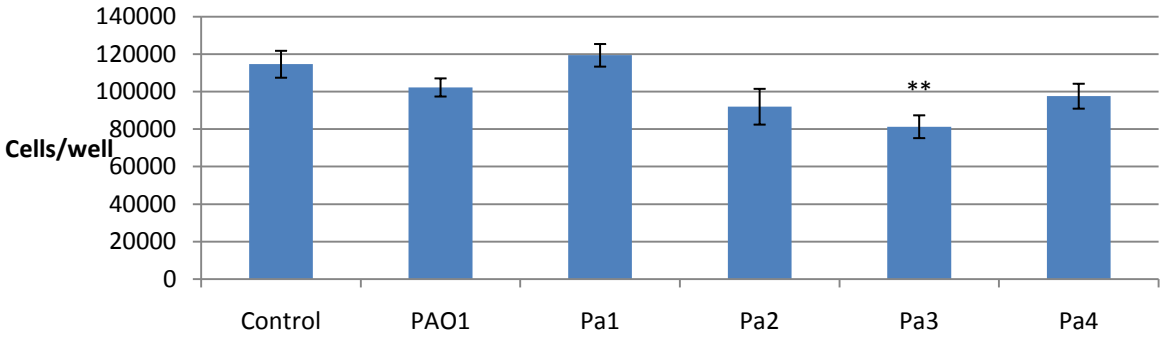



**Figure 7. Viable MDMΦ on collagen surface after 24 h exposure to CM from *P. aeruginosa* biofilm cultures. Data shown as mean ± SEM. n=15 for control, PAO1, Pa1 and Pa4, n=12 for Pa2 and Pa3 (4 donors). * P<0.05 ** P<0.01.**

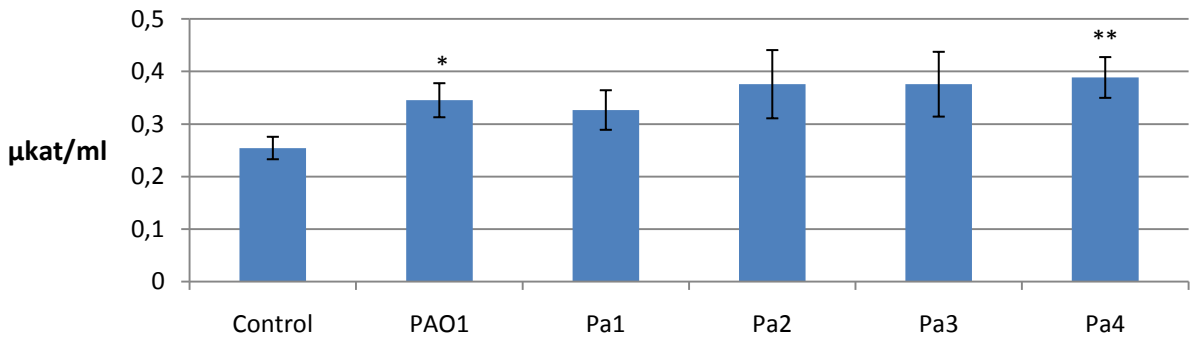



**Figure 8. LDH values corresponding to the cell viability study, see figure 7. Data shown as mean ± SEM. n=15 for control, PAO1, Pa1 and P8, n=12 for Pa2 and Pa3 (4 donors). * P<0.05 ** P<0.01.**

## 5.3 Cytokine response to bacterial exposure

The release of four different cytokines from the MDMΦ was quantified using ELISA assays. IL-10 and IL-1ra where chosen to describe the anti-inflammatory activation and IL-8 and IL-12 to describe pro-inflammatory activation. Before analysis, the supernatant from the cell culture wells were pooled giving one sample per treatment and donor.

The results for the IL-10 assay are shown in figure 9. CM from Pa2 and Pa3 significantly induced IL-10 production by MDMΦ whereas the other three strains did not affect the IL-10 levels. All of the *P. aeruginosa* strains induced IL-1ra production in MDMΦ, however the increase was only significant for PAO1, Pa1 and Pa4. The result can be seen in figure 10.

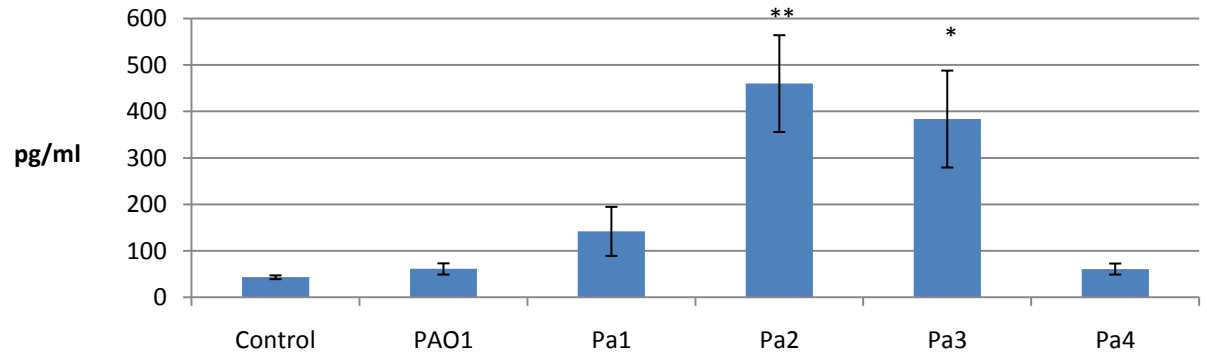



**Figure 9. IL-10 released by MDMΦ exposed to *P. aeruginosa*. Data shown as mean ± SEM. n=4 (4 donors). * P<0.05, ** P<0.01.**

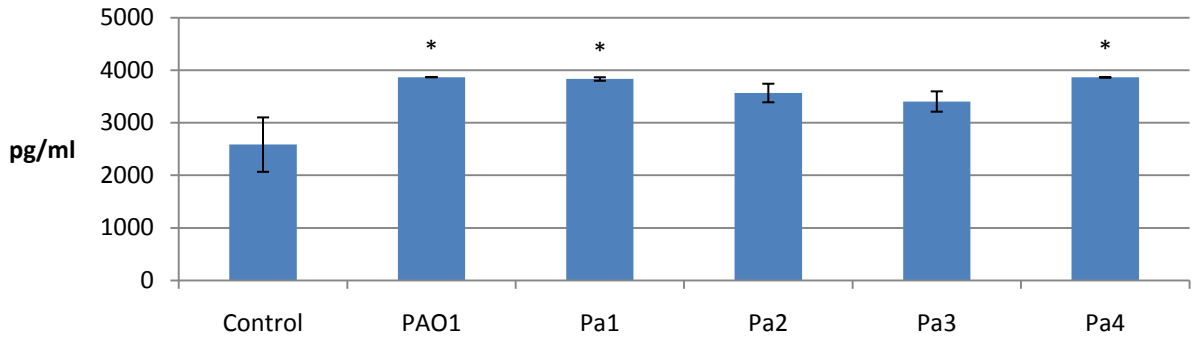



**Figure 10. IL-1ra released by MDMΦ exposed to CM from *P. aeruginosa* biofilm cultures. Data shown as mean ± SEM. n=4 (4 donors). * P<0.05, ** P<0.01.**

Figure 11 shows the results from the IL-8 ELISA. MDMΦ release of IL-8 was strongly induced and significantly higher than the untreated control for all five strains. However, MDMΦ exposed to CM from PAO1, Pa1 and Pa4 had also significantly higher values than both Pa2 and Pa3 (significance not shown in figure). The results are shown in figure 11.

No results are available for the IL-12 assay because of undetectable concentrations in the sample.

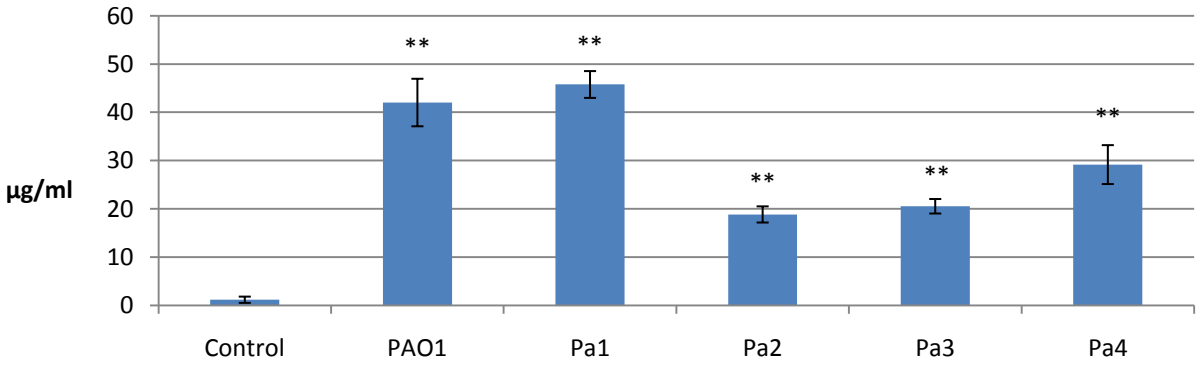



**Figure 11. IL-8 released by MDMΦ exposed to CM from *P. aeruginosa* biofilm cultures. Data shown as mean ± SEM. n=4 (4 donors). * P<0.05, ** P<0.01.**

## 5.4 Effect of salicylic acid on bacterial virulence

Salicylic acid was used to test the potential of the model to screen for new substances that might be used as QSIs. The three strains of *P. aeruginosa,* PAO1, Pa1 and Pa4, were chosen because of their quorum sensing regulated virulence, and cultured for 3 days in the presence of 1mM or 2mM SA. As shown in figure 12, the PAO1 culture no longer have the characteristic green color coming from pyocyanin, indicating that the level of pyocyanin was reduced in the presence of SA.

**Figure 12. Effect of salicylic acid on PAO1 production of the virulence factor pyocyanin. PAO1 cultured in the presence of SA. Left: 0 mM, middle: 1mM, right: 2mM. The cultures with SA no longer have the characteristic green color from pyocyanin.**

Figure 13 shows how the MDMΦ cell viability is affected when the cells are cultured together with CM from *P. aeruginosa* containing different concentrations of SA. No significant difference can be seen between the control and the different strains or SA concentrations, however, there is a tendency of decrease in cell number for MDMΦ exposed to CM containing SA from Pa1. LDH values for the same experiment are shown in figure 14. There is a trend of increasing values after treatment with PAO1 and Pa1 containing SA, although not significant.

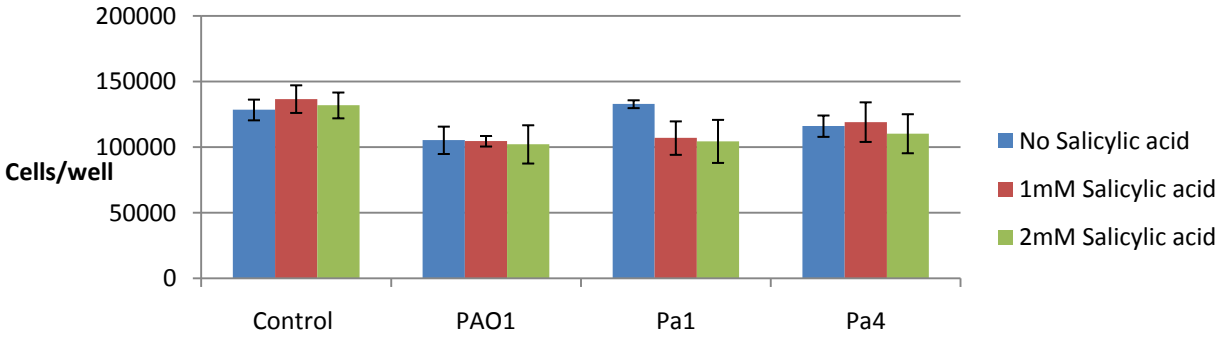



**Figure 13. Cell viability for MDMΦ exposed to CM from *P. aeruginosa* biofilm cultured in the presence of SA. Data shown as mean ± SEM. n=9 for control, n=6 for PAO1, Pa1 and Pa4 (2 donors). * P<0.05, ** P<0.01.**

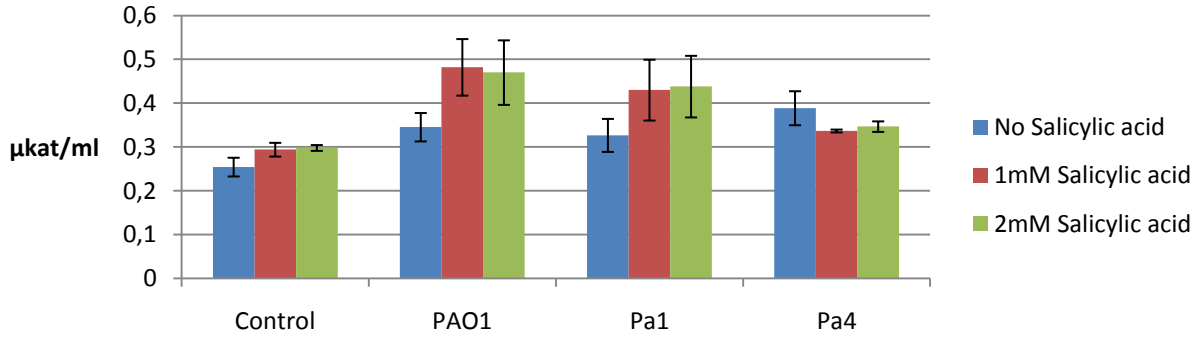



**Figure 14. LDH values for MDMΦ exposed to CM from *P. aeruginosa* biofilm cultured in the presence of SA. Data shown as mean ± SEM. n=9 for control, n=6 for PAO1, Pa1 and Pa4 (2 donors). * P<0.05, ** P<0.01.**

The results from the IL-10 ELISA indicate that bacteria cultured in the presence of SA induce a higher response of IL-10 in the MDMΦ for all three bacteria, shown in figure 15, although only significant for MDMΦ exposed to Pa4. SA alone has no effect on IL-10 levels as shown by the similar values in the control group.

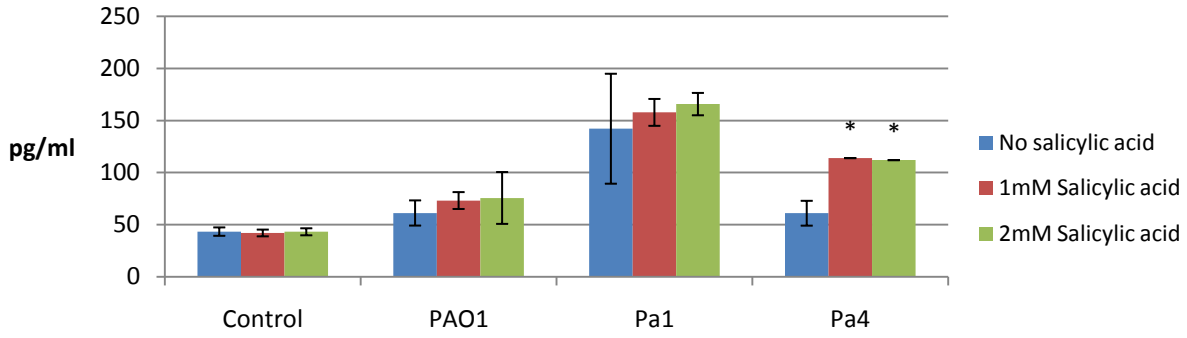



**Figure 15. IL-10 released by MDMΦ exposed to CM from *P. aeruginosa* biofilm cultured in the presence of SA. Data shown as mean ± SEM. n=2 (2 donors). * P<0.05, ** P<0.01.**

Figure 16 shows the results from the IL-1ra ELISA. Although there is no significant difference between the samples, there is a trend of lower levels of IL-1ra for MDMΦ exposed to CM containing SA from PAO1.

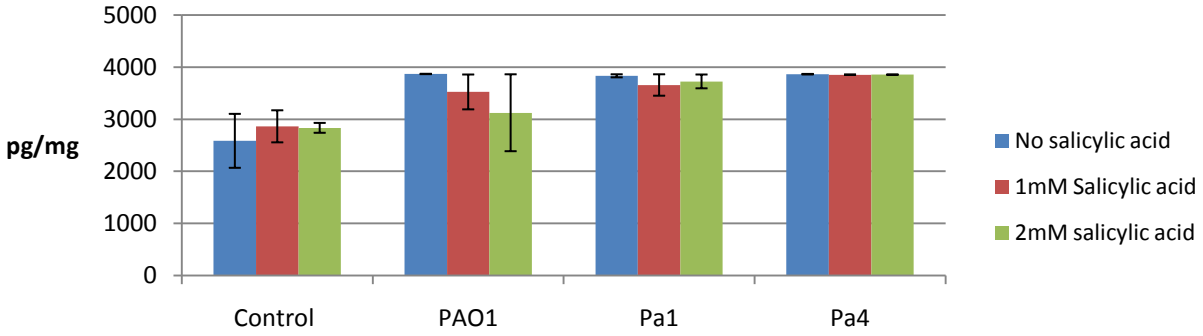



**Figure 16. IL-1ra released by MDMΦ exposed to CM from *P. aeruginosa* biofilm cultured in the presence of SA. Data shown as mean ± SEM. n=2 (2 donors). * P<0.05, ** P<0.01.**

The induction of IL-8 in response to different concentrations of SA gave a very diverse result for the three bacterial strains. For MDMΦ exposed to CM from PAO1 it seems that a higher concentration of SA results in a lower induction of IL-8 and treatment with CM from Pa4 shows the opposite response, giving higher concentrations for the bacterial cultures with SA. For Pa1 the value first decreases significantly for 1mM salicylic acid and then increases again for 2mM salicylic acid. The results are shown in figure 17.

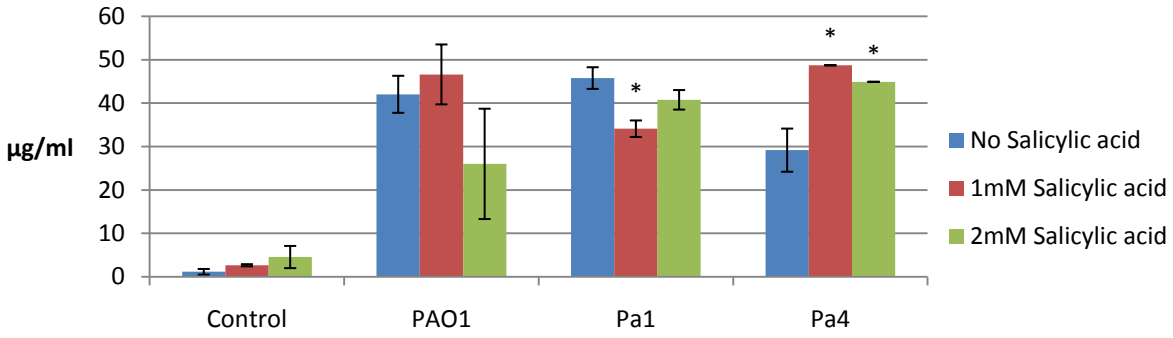



**Figure 17. IL-8 released by MDMΦ exposed to CM from *P. aeruginosa* biofilm cultured in the presence of SA. Data shown as mean ± SEM. n=2 (2 donors). * P<0.05, ** P<0.01.**

# 6. Discussion

Chronic wounds are easy targets for bacterial infections. With a damaged epidermal cover and an immune system that is out of control, the invading pathogen can relatively easy colonize the wound bed [73]. Most antibiotics are not effective enough against wounds infections due to the biofilm mode of growth, and are therefore not an optimal treatment alternative [15]. Topical treatment such as silver or PHMB are nowadays often used in combination with antibiotics, however, there is currently no treatment available to fully eradicate the bacteria [19]. The number of people experiencing chronic wounds is increasing, both as a result of increases in well fare diseases such as obesity and because of a growing elderly population, and new treatment possibilities are necessary [3].

Even though it is now recognized that bacteria in a chronic wound live in biofilm [78], rather than as planktonic cells, few studies have investigated the effect on mammalian cells when exposed to bacteria in a biofilm [79]. This study is the first step in the development of a wound infection model that makes it possible to study cell-bacterial interactions in biofilm infections from an inflammatory point of view. This type of model would enable testing of new antimicrobial substances and their effect on both the pathogen and the host.

*P. aeruginosa* is present in more than 50 % of all chronic wounds [9]. Studies have also shown that this pathogen cause more severe damage in a wound than other wound bacteria [21]. It is therefore of relevance to examine how *P. aeruginosa* interferes with the inflammatory response and wound healing. Since focus in this project was to mimic biofilm infections in wounds *in vitro*, macrophages was chosen to represent the inflammatory response towards a pathogen, here *P. aeruginosa*. *P. aeruginosa* was not only chosen due to its occurrence in chronic wounds, but it is also a well documented biofilm former with a well studied quorum sensing system. QS plays an important role in the virulence of *P. aeruginosa* and one potential way of battling biofilm infections is to disturb or inhibit quorum sensing signaling. If the pathogen no longer can produce the virulence factors regulated by QS nor produce a fully functional biofilm it will be easier to eradicate.

## 6.1 Influence of bacterial biofilm on human monocytes and monocyte derived macrophages *in vitro*

Before starting the work on the model, the wild-type laboratory strain PAO1 and four different clinically isolated strains of *P. aeruginosa* from chronic wounds were analyzed to see whether they produce the *P. aeruginosa* $C_6$ AHLs, namely OHHL and HHL. The five strains are for the simplicity of this discussion divided into two groups based on previous studies on their production on OdDHL, BHL and the virulence factors pyocyanin, rhamnolipid and elastase. The three more virulent strains, PAO1, Pa1 and Pa4 produce all of these while the two less virulent strains, Pa2 and Pa3, produce none [74].

The AHL analysis of the five different strains using *C.violaceum* as a reporter strain showed that all produce AHL with chains varying between 4 and 8 carbons (figure 4). Since Pa2 and Pa3 do not produce BHL, the detection of short AHLs for these two strains must therefore be HHL or OHHL. The darker coloring seen for PAO1, Pa1 and Pa4 is due to presence of high concentration of BHL as well as possibly OHHL and HHL.

Attachment of monocytes to a surface is in this study seen as a sign of inflammation [81]. The decrease in number of attached monocytes on the surface when exposed to CM from the different bacterial biofilms (figure 5) could therefore be seen as a lowering of the inflammatory response in the cells. All five bacterial treatments caused a reduction of cells on the surface, although only significant for PAO1 and Pa4. However, the results from the LDH assay (figure 6) show that the cells exposed to the more virulent strains also have significantly higher values of LDH. LDH is released from cells independently of whether the cells die from apoptosis or necrosis, however, the amount released from an apoptotic cell is much lower than what is released from a necrotic cell [82]. It is therefore probable that the increase in LDH value for PAO1 and Pa4 is mainly caused by release of LDH by necrotic cells. With this assumption, the more virulent strains probably do not cause a lower inflammatory response, but rather a cytotoxic response causing some of the monocytes to undergo necrosis and inducing inflammation. The trend of decreasing numbers of adhered cells, although not significant, for the monocytes exposed to the less virulent strains, Pa2 and Pa3, are not followed by an increase in LDH value, and may therefore be seen as a non-inflammatory response.

The cell viability was assessed by allowing the cells to adhere to a collagen coated surface for 24 h before exposing them to CM from the bacterial biofilm. The results from counting the number of MDMΦ left on the surface showed that the cells exposed to CM from the less virulent strains of *P. aeruginosa* had the lowest number of adhered cells left, although only significant for Pa3 (figure 7). PAO1 and Pa4 also show a trend of reduced cell number, even though not significant. The release of IL-10 from the MDMΦ (figure 9) show a clear anti-inflammatory response towards CM from Pa2 and Pa3 and this can be one reason why there are fewer cells attached to the surface. The significant decrease in number of viable MDMΦ exposed to CM from Pa3 are not met by an increase in the LDH value (figure 8), which further suggest a non-inflammatory reaction to the less virulent strain. Based on these data it can be speculated that the cells that have detached from the surface, due to the anti-inflammatory signal, might have undergone apoptosis and are therefore not detected in the LDH assay. The more virulent strains, PAO1 and Pa4, had LDH values that were significantly different from the untreated control. Since these two strains also showed a trend of decreasing cell number, this might be interpreted as a sign of necrosis for the released MDMΦ.

Four different cytokine responses in the MDMΦ were evaluated in this study, however, only three assays were successful. The significant increase in levels of the anti-inflammatory cytokine IL-10, mentioned above, for MDMΦ exposed to CM from biofilm formed by the less virulent strains can be interpreted as an anti-inflammatory response from the MDMΦ towards these two strains. This is further supported by the significantly lower levels of the pro-inflammatory cytokine IL-8 for MDMΦ exposed to CM from Pa2 and Pa3 compared to MDMΦ exposed to the more virulent strains, PAO1, Pa1 and Pa4 (figure 11). The induction of IL-8 release by the MDMΦ as a response the more virulent strains might, besides QS regulated virulence factors, be due to OdDHL. This AHL previously has been shown to induce IL-8 in epithelial cells [83].

IL-1ra, on the other hand, shows the opposite response in the release of cytokines from the MDMΦ (figure 10), however, there is no significant difference between the IL-1ra levels for the five different bacterial treatments. The results from this IL-1ra assay should be analyzed with care, since the values

were very high. The similar values for PAO1, Pa1 and Pa4 indicate that the highest detection value for the assay was close. Even still, PAO1, Pa1 and Pa4 are significantly higher than the control and there is a trend of higher values for the MDMΦ exposed to more virulent strains compared to the less virulent.

Unfortunately no results could be obtained from the IL-12 ELISA. Since this cytokine are important for the development of Th1 cells [70], it would have given additional information on how the different bacterial strains affected the pro- or anti-inflammatory response in MDMΦ. Cytokine responses are often dependent on each other, for example IL-10 is known to inhibit production of both IL-8 and IL-12 and stimulate IL-1ra [69]. To be able to make a correct evaluation of the inflammatory response several different cytokine must be evaluated and compared.

It can be speculated that this anti-inflammatory response in the cells exposed to the less virulent strains, seen both in cell adhesion, cell viability and ELISA assay, might be caused by an immunomodulatory action from the AHLs. It has been shown previously that the AHLs produced by *P. aeruginosa* not only regulate production of virulence factors, but can also modulate the host's immune response [36, 40-45]. When studying the immunomodulatory effects of the AHLs, OdDHL is most often the only one described. Not much interest has yet been paid to the short AHLs and only one study that includes any other AHL than OdDHL can be found. This study was published in 1998 and showed that OHHL have no effect on the levels of TNF-α or IL-12 produced by human monocytes [36]. Conversely, unpublished data shows that both OHHL and HHL can induce IL-10 in unstimulated macrophages [80], speaking for a possible hypothesis of an anti-inflammatory immune modulation by OHHL and HHL. This study is in line with the results from the present study, where MDMΦ exposed to CM from Pa2 and Pa3 showed significantly increased levels of IL-10 (figure 9). Since these two less virulent strains only produce OHHL and/or HHL, the induction of IL-10 might be seen as a response towards these AHLs. It is possible to speculate that the main induction of IL-10 was due to the AHLs in the sample. However, because supernatant from the bacterial culture was used in this study, other factors might also have affected the IL-10 induction in the MDMΦ.

From the *C.violaceum* staining no distinction between BHL, OHHL or HHL detection can be made, and since previous characterization of the strains showed production of BHL, there is no way to say for certain if Pa1 and Pa4 produce the $C_6$ AHLs. PAO1, on the other hand, is known to produce all four of the AHLs [29]. Supposedly, if all three strains produce the $C_6$ AHLs and no production of IL-10 is induced in the MDMΦ then something might interfere with this signal, for example the other AHLs produced or the QS regulated virulence factors, making it less potent. It might also be speculated that when a bacterium produces the two main AHLs, OdDHL and BHL, the other AHLs are produced at much lower concentrations not giving the same response as for bacteria where OHHL and HHL are only ones produced.

The effect of the AHL on the immune response is highly dependent on concentration [26]. One can speculate on the reason behind the concentration dependent immunomodulatory effect of the AHLs. It might be possible that low concentrations of the AHL, allow the bacteria to hide behind the AHL signal not to evoke an inflammatory response. The AHL thus promotes an anti-inflammatory reaction. Once the number of bacteria increases and the concentration of the AHL are high enough autoinduction is

initiated. The bacteria no longer can, nor need, to hide from the immune system and the higher concentration of AHLs cause pro-inflammatory responses instead.

The study of bacterial influence on MDMΦ *in vitro* showed that biofilm from the two less virulent strains, Pa2 and Pa3, seemed to caused a clear anti-inflammatory response in the MDMΦ based on the results from both IL-8 and IL-10 assays. In addition, the lower number of viable cells adhered to the surface after exposure to CM from the biofilm cultures did not correspond to an increase in the LDH value. This further suggests an anti-inflammatory reaction of the MDMΦ. This can be compared to biofilm from the more virulent strains, PAO1, Pa1 and Pa4 where the opposite cytokine response were seen in the MDMΦ. They also showed a reduction of viable cells adhered to the surface which corresponded to a significant increase in LDH value, indicating a pro-inflammatory reaction of the MDMΦ.

## 6.2 Effect of salicylic acid on bacterial virulence as evaluation of the *in vitro* model

Developing an *in vitro* model that mimics biofilm infection in chronic wounds will enable new treatment strategies to be evaluated. It will also allow for optimization of the antimicrobial effect and safety of the compound before testing in animal models. In this study a model consisting of human monocyte derived macrophages cultured on collagen coated surfaces exposed to conditioned medium from *P. aeruginosa* biofilm cultures was developed.

To test whether this kind of model could be used for screening possible new substances for treating biofilm infections, salicylic acids was used. Salicylic acid has been shown to down-regulate the production of *P. aeruginosa* virulence factors in plant systems [60] and studies have shown that SA has potential as a QS inhibitor for biomaterial related biofilm infections [54]. SA was only tested together with the three most virulent strains, PAO1, Pa1 and Pa4, to study whether SA could have any effect on their virulence.

The three days old cultures of PAO1 showed evidence that a lower amount of the virulence factor pyocyanin had been produced when the bacteria was cultured in the presence of SA, since these cultures no longer had the characteristic green color coming from the virulence factor (figure 4). This indicates that SA has an effect on the QS regulated virulence factors produced by *P. aeruginosa,* which is consistent with previous studies [60].

Measuring the viability of the MDMΦ exposed to CM from *P. aeruginosa* cultured in the presence of SA did not show any significant change in cell numbers compared to cells exposed to CM from *P. aeruginosa* without SA (figure 13), indicating that these concentrations of SA have no effect on the viability of the MDMΦ. It has also previously been shown that bacterial growth is not affected by SA in these concentrations [6].

The LDH values, corresponding to the cell viability study, increased although not significant, for PAO1 and Pa1 (figure 14). With the same reasoning as before, that an increase in LDH is a sign of necrosis and subsequently inflammation, these two strains induce a higher inflammatory response when cultured in

the presence of SA. Based on these results, it can be speculated that the presence of SA in the bacterial culture causes alteration in the secretion of soluble factors from the bacteria. These alterations might, in turn, change the cellular response towards these bacteria and causes the MDMΦ to undergo necrosis and thus promote inflammation.

MDMΦ exposed to CM from bacterial biofilm cultured in the presence of SA show a trend of increased IL-10 concentration, however only significant for Pa4 (figure 15). Seeing an induction of this anti-inflammatory cytokine could be interpreted as a change towards decreasing virulence in bacteria, since this cytokine was highly induced for the two less virulent strains in the absence of SA (figure 9). There is also a trend, although not significant, of decreasing amount of IL-1ra released by MDMΦ exposed to CM from PAO1 when cultured in the presence of SA (figure 16) which is also in line with the lower value of IL-1ra for MDMΦ exposed to the less virulent strains.

The levels of IL-8 (figure 17) also indicate that the production is reduced for MDMΦ exposed to CM treated SA from PAO1 and Pa1, although only significant for Pa1. This can also be seen as a move towards a less virulent state, since the more virulent strains show significantly higher values of IL-8 than the less virulent (figure 11). MDMΦ exposed to CM from Pa4 did not show the same response when SA was present in the culture as the other two bacteria, instead of decreasing values there was a significant increase in the level of IL-8. Why this response was seen for Pa4 is difficult to speculate on with these limited data.

Since the bacterial growth are not affected by these concentrations of SA, the altered response from the MDMΦ when cultured together with CM from *P. aeruginosa* cultures containing SA could possibly be due to inhibition of the QS system in the bacteria, making them less virulent. Most of the cytokine analyses indicate that the bacteria might be moving towards a less virulent phenotype. These results show that the model has potential to work as a screening tool for antimicrobial substances.

# 7. Conclusion

This study is a first step in developing a new *in vitro* model that makes it possible to study cell-bacterial interactions in biofilm infections and enables testing of new antimicrobial substances and its affect on both the pathogen and the host. The analyses of cell viability and cytokine response show promising results that this type of model can illustrate the immune response towards a pathogen. A well established biofilm from the more virulent strains seemed to cause a pro-inflammatory response in the MDMΦ, while biofilm from the less virulent strains appeared to cause an anti-inflammatory response. Testing of salicylic acid also showed potential of the *in vitro* model since bacteria treated with salicylic acid showed indications of moving towards a less virulent phenotype.

## 8. Future works

This master thesis is only the beginning in the development of an *in vitro* model to study biofilm infections and it must be further developed to be fully functional model. The parameters analyzed here are good indications on how the cells are affected by the bacteria, however, it would be good to involve more factors to better understand the inflammatory response. The first thing that should be added is to look at apoptosis as a complement to the LDH assay to better be able to determine how the cell has died, since this in a way can indicate how the inflammatory response are turning. It could also be of interest to analyze other cytokines, such as IL-4 or IL-6, or perhaps growth factors, for example TNF-α. To ensure if it is the AHLs secreted from the bacteria that is causing the immunomodulatory effect, purified AHLs should be tested in parallel with the bacteria.

For the statistical relevance of the model, more donors should be used in order to minimize the effect of difference in the results due to donor samples. Moreover, it would be interesting to evaluate the model for other cells important for infection or wound healing such as T cells or fibroblasts. The model can also be further developed by growing the biofilm in tissue culture insert and expose the cell culture to the entire biofilm not only the supernatant. This would be one step in the right direction towards a more wound like model.

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