LEONARDO CECOTTO

Saving Private Macrophage

Alternative therapeutic options for
Staphylococcus aureus implant-associated infections

Leonardo Cecotto

Saving Private Macrophage
PhD Thesis, Utrecht University, The Netherlands

ISBN: 978-90-393-7576-1
doi: https://doi.org/10.33540/1899

Author: Leonardo Cecotto
Cover design: Leonardo Cecotto, Marco Cecotto
Layout: Eleonora Leghissa
Printing: Ridderprint | www.ridderprint.nl.

©Copyright Leonardo Cecotto, 2023, Utrecht, The Netherlands. All rights reserved. No part of this publication may be reproduced, stored, in a retrieval system or transmitted, in any form of by any means, electronic, mechanical, photocopying, recording, or otherwise, without prior permission of the author or the copyright-owning journals for previously published chapters.

The printing of this thesis was kindly partly financially supported by EU's H2020 research and innovation program under Marie S. Curie cofund RESCUE grant agreement.

Saving Private Macrophage

Alternative therapeutic options for *Staphylococcus aureus* implant-associated infections

Saving Private Macrophage

Alternatieve therapeutische opties voor *Staphylococcus aureus* implantaat-geassocieerde infecties

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de
Universiteit Utrecht
op gezag van de
rector magnificus, prof.dr. H.R.B.M. Kummeling,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op

maandag 11 september 2023 des ochtends te 10.15 uur

door

Leonardo Cecotto

geboren op 17 november 1993
te San Dona' di Piave, Italië

Promotoren:
Prof. dr. J.A.G. van Strijp
Prof. dr. ir. H. Weinans

Copromotoren:
Dr. S. Amin Yavari
Dr. B.C.H. van der Wal

Beoordelingscommissie:
Prof. dr. P.W.M. Hermans
Prof. dr. H.P. Haagsman
Prof. dr. S.H.M. Rooijakkers
Prof. dr. H.C. van der Mei
Dr. D.A.C. Stapels

Paranimfen:
L. Costa
J. Egido

Table of contents

Chapter 1……………………………………………………………….page 4
General introduction. The conflict: *S. aureus* orthopedic implant-associated infections

Chapter 2……………………………………………………………….page 30
Evaluation of silver bio-functionality in a multicellular in vitro model: towards reduced animal usage in implant-associated infection research
Published in: Frontiers in Cellular and Infection Microbiology. 2023. 13:1186936

Chapter 3……………………………………………………………….page 58
Antibacterial and anti-inflammatory properties of host defense peptides against *Staphylococcus aureus*
Published in: iScience. 2022. 25(10):105211

Chapter 4……………………………………………………………….page 86
The relative role of human neutrophils and macrophages in phagocytosis and killing of Staphylococcus aureus
Manuscript submitted

Chapter 5……………………………………………………………….page 114
Unraveling the therapeutic potential of monoclonal antibodies: comparing neutrophils and macrophages in the context of *Staphylococcus aureus* infections
Manuscript in preparation

Chapter 6……………………………………………………………….page 140
General discussion. “Someday we might look back on this and decide that saving Private [Macrophage] was the one decent thing we were able to pull out of this whole [...] mess”

Closing pages…………………………………………………………page 154
Summary in English
Samenvatting in het Nederlands
Riassunto in Italiano
About the author
List of publications

CHAPTER
1

General introduction

The conflict:
S. aureus orthopedic implant-associated infections

The conflict: *S. aureus* orthopedic implant-associated infections

Orthopedic surgery can be a challenging experience from a patient's point of view. It entails enduring a lengthy operation, having foreign materials inserted into the body to hold bones together, and undergoing extensive physiotherapy to regain mobility aiming to be comparable to pre-surgery functions. On the other hand, from a biological point of view, the procedure places a significant burden on our cells as they work tirelessly to restore tissue functionality. The ultimate goal is for both the patient and host cells to return to their normal routine. Nevertheless, surgery is inherently risky, and unforeseen complications can arise.

In this thesis, we focus on the threat posed by the invasion of *Staphylococcus aureus* in our body and the defenses raised against it. As with any conflict, it is critical to comprehend the battlefield, the adversary, and the available resources to develop novel strategies to overcome this threat.

The battlefield: the surface of biomaterials

Biomaterial use for medical purposes have been employed by *Homo sapiens* for thousands of years (1). Almost 4000 years ago, the first human societies in Egypt, China, Africa, and India used linen threads to facilitate wound healing, built dental implants from bamboo sticks or precious metals, and applied the heads of ants as stitches. Less than 1000 years ago, Inca surgeons used golden plates to repair cranial fractures. The first successful reconstructive surgeries were operated by Gaspare Tagliacozzi in the 16th century, when autogenous skin flaps were used to replace missing noses. Many more examples of medical devices can be found throughout history; however, until 150 years ago, none of these surgical procedures were performed considering the organism's reaction towards the introduction of exogenous materials. Concepts as sterilization, immune reaction, or tissue integration were still missing. For example, it was a common practice in war hospitals of the XIX century to amputate legs or arms even after superficial wounds to avoid the risk of infections or gangrene (2). Only in the 1960s Per-Ingvar Brånemark described the process of osseointegration as the functional and structural connection between bone tissue and implant, while less than 40 years ago, scientists agreed on the definition of biocompatibility, as the ability of a biomaterial to promote an adequate response from the host (3).

Nowadays, tens of millions of medical devices are successfully used every year for all kinds of applications. Due to the ageing of the world's population, the demand for

orthopedic implants is constantly increasing. In the United States alone in 2014, more than 1 million primary hip and knee arthroplasties were performed each year (4).

Regardless of the progress in materials science and knowledge about the host reaction against exogenous elements, any manufactured implant will always be recognized as a foreign body by our organism, and therefore attacked by our immune system. The intensity of the immune response correlates with the anatomical location and the time the biomaterial would reside in our body. For instance, most orthopedic surgeries employ non-biodegradable implants designed to stay in our body for the long-term or permanently, resulting in a stronger and prolonged activation of the immune system.

Over time, small fragments may detach from the implant and be phagocytosed by circulating immune cells. This uptake of wear particles leads to the onset of a significant aseptic inflammation around the implantation site, eventually leading to joint loosening and bone loss (5).

As soon as a biomaterial enters our body, the innate immune system coordinates the activity of serum proteins, neutrophils, and macrophages into an inflammatory response aimed at removing the implant (**Figure 1**). However, in the presence of permanent implants, this acute inflammatory reaction evolves into a fibrotic process where macrophages and fibroblasts cooperate to cover the implant surface with a thick extracellular matrix capsule (6). Use of different biomaterials (inert, bioactive, or biodegradable) or functionalization of the implant surface have been shown to modulate and reduce the severity of the host response (3). For instance, titanium and its alloys have emerged as the preferred materials for orthopedic implants thanks to their favorable biocompatibility and limited fibrous encapsulation (7).

Nonetheless, improvements in the implant's biocompatibility do not compensate for the impact of the surgical procedure. Patient's age and clinical history, magnitude of the surgical insult, use of anesthetic compounds, and post-operative anxiety, are all factors that contribute to creating a generalized state of immunosuppression (8–11). The sum of these events makes the environment surrounding the implant surface a locus minoris resistentiae (12) where the immune response is compromised and therefore less responsive to external threats, such as bacterial infections. It is possible that pathogen invasion may go unnoticed since the surgical procedure and implant take most of our immune system's energy. Consequently, the environment surrounding the implant surface becomes an ideal hub for bacterial undisturbed proliferation. In such conditions, just 100 colony forming units (CFU) of *S. aureus* are enough to establish clinically relevant infections in an animal model, numbers that are 100000 times lower than in absence of an implant (13,14).

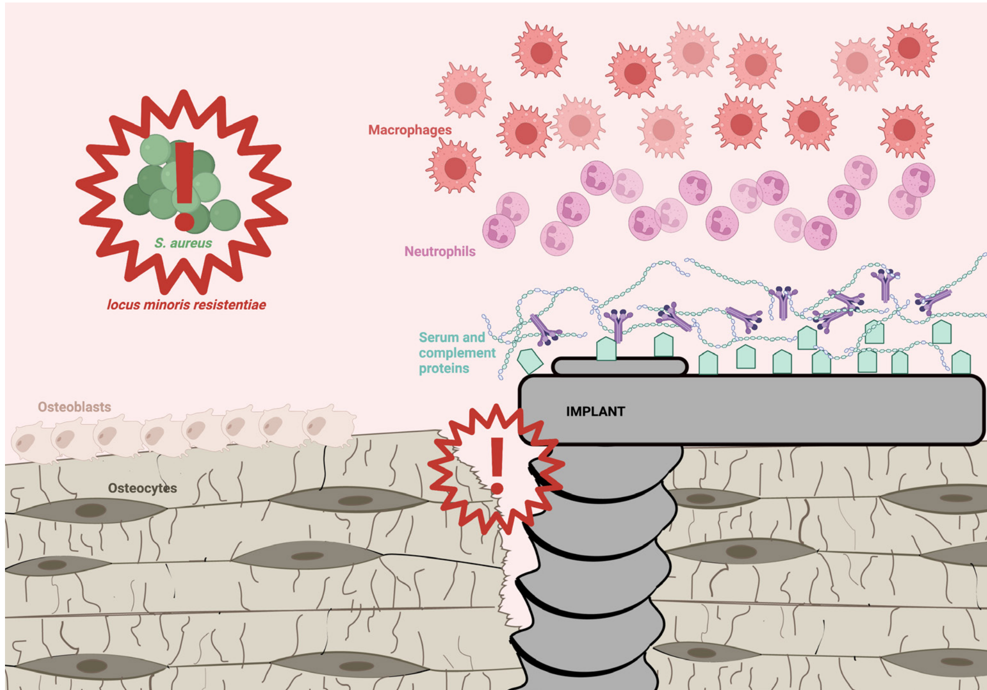


Figure 1. Events following implant insertion in the body. First, complement and serum proteins coat the surface of the implant to favor adhesion of host cells. Neutrophils and macrophages are recruited at the implantation site to coordinate the host response against the foreign biomaterial. As the immune response is mainly focused on the implant, other external threats such as bacterial infections, might go unnoticed by the immune system. Therefore, surgery contributes to establish a locus minoris resistentiae around the surface of the implant, where the immune response is weakened while bacterial invasion and proliferation is favored.

During the hospitalization period or the surgical procedure, pathogens present in the environment, on medical staff, or patients themselves, can find their way through the open wound towards the surface of the implant (15). This is the case in 1 - 5% of patients undergoing surgeries for joint replacement (16). The numbers further increase when looking at revision surgeries and even higher when these are caused by implant-associated infections (IAI) that lead to 14% reinfections of total hip arthroplasties and 25% reinfections of total knee arthroplasties (17). Besides an excruciating pain for the patients and risk for their health, repeated surgeries and treatment following IAI also represent an economical burden for the health care system.

The enemy: *Staphylococcus aureus*

The common clinical practice to treat orthopedic IAI involves two-stage revision surgery where the infected prosthesis is removed, antibiotics are systemically and locally delivered for a prolonged period, and finally a new implant is placed. According to clinical case and hospital regulations, it is possible to shorten the procedure by opting for one-stage revision surgery (18). However, the success of these procedures depends on the pathogens causing the infection.

The majority of IAI cases are caused by Gram-positive bacteria, particularly Staphylococcal species, with *Staphylococcus aureus* being the most prevalent. *S. aureus* belongs to the ESKAPE pathogens group together with *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species. All these pathogens represent a worldwide threat to human health due to antimicrobial resistance (19).

Bacterial ability to survive antibiotic treatment has been detected since the very beginning of the antibiotic era in the 1940s with the identification of penicillin-resistant strains. This trend continued over the years, with antibitioc resistance being detected shortly after the introduction of new drugs in the clinics, ultimately leading to a steep decline in the discovery and development of new antibiotics today (20,21). The fast development of resistance against new drugs could be ascribed to horizontal transfer via mobile genetic elements or mutations in chromosomal genes among the bacterial population (22). As the use of single compounds failed to eradicate bacterial infections, different strategies have been employed in the clinics to improve treatment efficacy: switch in administration of antibiotics with different killing mechanisms, such as beta-lactams and quinolones; simultaneous administration of different antibiotics with a synergistic effect; use of antibiotic-loaded implants to deliver locally higher drug dosages compared to systemic administration (23,24).

However, evolution of resistance genes is not the only mechanism adopted by *S. aureus* to survive antibiotic treatment. *S. aureus* can switch from a single free-floating cells lifestyle to growing into multicellular biofilms. In such a state, bacteria cover themselves with a thick extracellular matrix consisting of proteins, polysaccharides, and extracellular DNA that confers additional protection from antibiotics and host immune clearance (25). Within a biofilm or after exposure to external environment stresses, *S. aureus* can switch into a slow-growing or growth-arrested phenotype, also referred as persisters or small colony variants (SCV). In this state, by shutting down almost all the metabolic and cellular activities, bacteria become temporarily drug tolerant, as antibiotic targets are hidden or not active (26,27). Once the environmental pressure disappears, these bacteria can switch back to their original phenotype and re-establish growth, although becoming once again antibiotic susceptible (28). Besides antibiotic treatment, prolonged exposure to low temperatures, very acidic environments such as those of immune cells lysosomes, and osmotic stress can be considered additional factors contributing to *S. aureus* phenotype switch (27,29). The mechanisms behind induction into a persisters, or SCV, or growth-arrested state

and re-start of growth are still under debate, together with a unanimous terminology and definition for this phenotype.

During revision surgery, in combination with antibiotic administration, the infection site is thoroughly cleaned by surgeons to remove all dead and infected tissues and bone surrounding the area. Even so, *S. aureus* can survive this debridement practice by hiding inside bone tissue. Although always considered a sessile organism, *S. aureus* can actively infiltrate the osteocyte's canaliculi network, both in healthy and devitalized bone, adapting its morphology to penetrate the narrowest and deepest spaces (30,31). Hidden in such remote locations, pathogens can survive for years using the bone matrix as a nutritional source (32).

Besides the remarkable efficacy on surviving human drugs and clinical practice from one side, *S. aureus* evolved multiple strategies to sustain the attacks from our immune system (**Figure 2**).

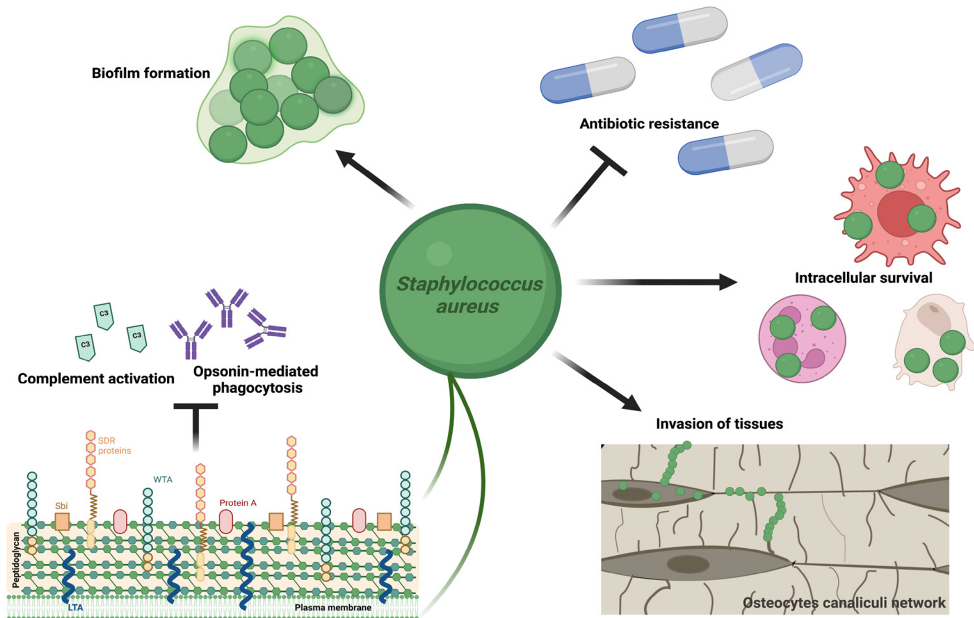


Figure 2. *S. aureus* evolved multiple strategies to extend its stay in our organism. These include resistance to antibiotics acquired by horizontal transfer via mobile genetic elements or mutations in chromosomal genes; switch from free floating single-cells to biofilm formation; invasion and intracellular survival in several host cell types, and tissues, such as osteocytes canaliculi network; expression of virulence factors to evade the innate immune system by inhibiting complement activation or opsonin-mediated phagocytosis.

Circulating antibodies (or immunoglobulins, Ig) and complement proteins are the first alarm siren of our body against invading pathogens. Binding of these molecules to the bacterial membrane leads to the activation of the complement cascade and the start of the innate immune response. There are three different mechanisms leading to complement activation: the classical pathway, via interaction of C1q and IgG-bound to the bacterial membrane; the lectin pathway, via recognition of bacterial carbohydrate patterns by lectins; the alternative pathway, via spontaneous hydrolysis and deposition of C3 on bacterial surfaces. All three pathways converge towards the same outcome. In the presence of Gram-negative bacteria, deposition on the bacterial surface of complement proteins leads to direct killing of the pathogen via the formation of pore-forming complexes known as membrane attack complex (MAC). However, due to their thick cell wall, Gram-positive bacteria are naturally resistant to MAC-mediated lysis (33). Therefore, in the presence of pathogens like *S. aureus*, complement activation derived products, C3a and C5a, guide immune cells towards the infection site. Plus, coating of the bacterial surface by antibodies and complement proteins, an event named opsonization, improve the recognition, ingestion, and therefore killing of the pathogens by phagocytes.

However, *S. aureus* evolved a wide range of proteins and virulence factors to counteract all the above-mentioned activation mechanisms of the innate immune system, from recognition to elimination of invading pathogens (34,35). For example, *S. aureus* secretes staphylococcal complement inhibitor (SCIN) to inhibit all tree complement activation pathways leading to a decline in C3b deposition on the bacterial membrane and release of chemoattractant C5a. Moreover, *S. aureus* expresses on its surface two proteins, staphylococcal protein A (SpA) and *S. aureus* binder of IgG (Sbi), that interfere with complement activation and opsonin-dependent phagocytosis. Both proteins bind to the Fc region of IgGs inhibiting Fcγ receptors (FcγR)-mediated phagocytosis by neutrophils and macrophages (36). Plus, SpA inhibits the classical pathway by interfering with IgG hexamerization (37), while Sbi obstructs the alternative pathway by binding to complement components factor H and C3 (38). Alternatively, *S. aureus* can kill host cells by secreting toxins such as luekocidins (Luk) or pore forming and membrane damaging toxins, like α-toxin (or α-hemolysin, Hla) used to escape from the phagosome (34,39).

Finally, *S. aureus* can evade immune system recognition and find protection from antibiotic exposure by invading, surviving, and proliferating within several types of host cells, such as endothelial, epithelial, bone, and immune cells (40). The intracellular survival of *S. aureus* within host cells has been identified as a cause of recurrent and metastatic infections. This means a secondary infection is anatomically unrelated to the primary site of contact with the pathogen. Once gained access to our body via orthopedic surgeries, *S. aureus* might reside in our body for days, months, or even years before spreading throughout the organism leading to bacteremia and eventually cause infective endocarditis, septic arthritis, or vertebral osteomyelitis (41).

The front line: bone cells, neutrophils, and macrophages

Once adapted to an intracellular lifestyle, *S. aureus* can hijack the functions of the targeted cell to its own advantage. On the one side, this affects new bone deposition on the implant surface and therefore functional implant osseointegration; on the other hand, immune cells fail to completely eradicate pathogens from our body.

Following implant insertion, new bone is deposited on the implant surface by osteoblasts. The osteoblasts progenitors derive from mesenchymal stem cells (MSCs) and differentiate towards the osteogenic lineage into osteoblast, which builds new bone tissue around the implant (42). The first building blocks towards new bone formation are represented by the deposition of an extracellular matrix (ECM) rich in collagen type I, with a small amount of non-collagenous proteins such as bone sialoprotein, fibronectin, and osteopontin. *S. aureus* expresses on its surface several adhesins named microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) that bind to the core components of the bone ECM, such as collagen adhesins (Cna), bone sialoprotein binding protein (bbp), and fibronectin binding proteins A and B (FnBP A, B) (43). Connection between ECM proteins and staphylococcal adhesins favors bacterial attachment and invasion of bone cells. Nonetheless, pathogen internalization in bone cells is not only a passive mechanism. Osteoblasts actively uptake bacteria via rearrangements of actin microfilaments, microtubules, and receptor-mediated endocytosis (44).

Following internalization, *S. aureus* can survive and proliferate within osteoblasts for up to 5-7 days (45) (**Figure 3**). During this coexistence, intracellular pathogens hijack cell functions and contrast wound healing by slowing down new bone deposition while favoring bone resorption. Infected osteoblasts showed lower mineralization capacity, lower expression of osteoprotegerin (OPG), and higher production of receptor activator of NF-κB ligand (RANK-L). By disrupting the balance of the OPG/RANK/RANK-L signaling system (46), intracellular *S. aureus* favors the recruitment at the infection site of a higher number of differentiated osteoclasts. Furthermore, *S. aureus* can infect osteoclasts as well, increasing their bone resorption activity (47,48). The sum of these events results in a strong negative correlation between bacterial infection and bone regeneration. Nonetheless, it has been shown that mild inflammatory conditions, low bacterial numbers, or bacteria-derived antigens, might even promote new bone deposition (49,50).

Osteoblasts are not completely defenseless against *S. aureus*. After detection of pathogens, osteoblasts contribute to the onset of the inflammatory response by releasing several cytokines, such as interleukin 6 and 8 (IL-6, 8), and chemokines, such as monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1α and 2α (MIP-1α, 2α), to activate and recruit neutrophils and macrophages (44). Cytokines and chemokines secretion by osteoblasts is further enhanced by the detection of circulating complement proteins C3a and C5a (51). Although the increase in mRNA production coding for pro-inflammatory cytokines starts few hours

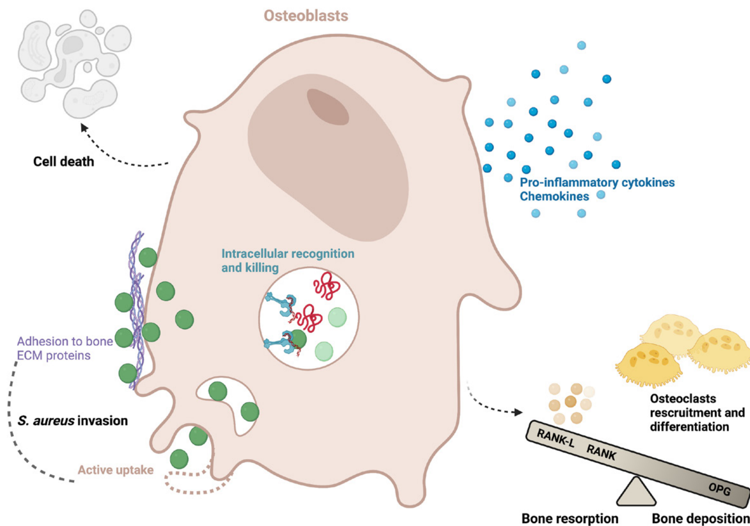


Figure 3. Invasion, survival, and proliferation mechanisms of *S. aureus* within osteoblasts. Internalization of *S. aureus* is driven by both passive mechanisms, adhesion to bone ECM proteins, and active uptake mediated by osteoblasts. Following internalization, *S. aureus* disrupts the OPG/RANK/RANK-L signaling system in favor of RANK-L production causing an increase in bone resorption by osteoclasts. Infected osteoblasts contribute to the onset of the inflammatory response by secreting pro-inflammatory cytokines and chemokines to recruit neutrophils and macrophages. Moreover, osteoblasts retain bactericidal capacity against intracellular pathogens.

after the encounter with pathogens, the actual proteins are released only after few days, therefore scaling down the contribution of osteoblasts in the fight against infection (44). Moreover, osteoblasts retain bactericidal capacity via induction of oxidative stress following toll-like receptor 9 (TRL9) stimulation (52), or production of host defense peptides such as human β-defensin 3 (HBD-3) (53). Similarly, MSCs have been shown to exert antibacterial effects via secretion of extracellular vesicles, production of host defense peptides, and modulation of immune cells response (54–57).

Osseointegration is not exclusively driven by bone cells. Recently, a new theory stressed out the role of immune cells in driving bone formation around the implant surface (58). This idea emerged after the identification of bone tissue-resident macrophages, called osteomacs, involved in bone homeostasis (59–62). Presence of tissue-resident macrophages has been described in several tissues, placing this cell type in a pivotal role in the regulation of tissue homeostasis, response to local damage or presence of invading pathogens, and resolution of inflammation (63).

However, tissue-resident macrophages can be used by *S. aureus* as a safe harbor to survive intracellularly, replicate, and finally evade towards neighboring tissues and organs or the bloodstream (64). Once in the bloodstream, *S. aureus* can reach different locations of the organism as a free-floating cell or carried by circulating immune cells. The first observation of *S. aureus* dissemination was made by Rogers in 1956. After the injection of *S. aureus* in rabbits, most bacteria were captured by the liver, but a small proportion could still be detected in the bloodstream causing bacteremia. Rogers concluded that the survival of those bacteria was not due to saturation of the clearance mechanisms within the liver but to the hiding of pathogens within circulating phagocytes (65). This may be considered the cornerstone of the "Trojan horse" theory, suggesting that *S. aureus* intracellular survival in phagocytes is associated with the persistence of the infection. This may not be limited to spreading the infection within the same organism but to others as well. For instance, Gresham *et al.* showed that the injection of neutrophils carrying intracellular viable *S. aureus* was sufficient to establish infection in naïve animals (66). To date, whether neutrophils, or macrophages, or both cell types can be used as a Trojan horse by *S. aureus* is still debated. However, it is well-established that *S. aureus* can survive intracellularly and affect the functions of both phagocytes.

Historically, the antibacterial functions of macrophages and neutrophils were first described by Elie Metchnikoff in 1883 (67,68). At the time, size was the only difference between the two cell types, as the smaller neutrophils were named "microphages". Nowadays, several differences such as number of circulating cells, life span, and antibacterial functions have been defined for neutrophils and macrophages.

Neutrophils are the most abundant leukocytes present in the bloodstream, with around 10^{11} new cells produced daily from the bone marrow (69), while monocytes/macrophages represent around 10% of the circulating immune cells (70). This imbalance in numbers is compensated by their different life span. While neutrophils survive in the bloodstream from few hours up to 5 days, although the exact range is still under debate (71), macrophages live from a few days to even years, *i.e.* tissue-resident macrophages (72). Differences in life span also correlate to different strategies adopted by the two phagocytes in killing invading pathogens. Surviving for a shorter time at the infection site, neutrophils have a higher microbicidal activity via rapid generation of reactive oxygen species (ROS) and release of granule-derived mediators, like proteases and antimicrobial peptides, eventually leading to the death of the cell itself with the formation of neutrophils extracellular traps (NETs) (73). On the other hand, persisting for a longer time around the infection site, macrophages have a slower bactericidal activity, and their killing mostly relies on the acidification of the phagolysosome, following activation of several proteases, enzymes, and production of reactive nitrogen species (RNS) (70). Moreover, macrophages can increase their bactericidal capacity by acquiring neutrophils-derived microbicidal proteins and enzymes (74). In fact, a cooperative interaction between neutrophils and macrophages is essential in clearing both intracellular and extracellular pathogens (75,76), as the absence of one or the other cell type would negatively

impact infection resolution (64,77,78).

S. aureus has evolved several strategies to survive the above-mentioned killing mechanisms of our phagocytes (70,79,80) (**Figure 4**). For example, staphylococcal peroxidase inhibitor (SPIN) binds and inhibits the activity of myeloperoxidase (MPO), a key enzyme in the oxidative response of neutrophils (81). Alternatively, *S. aureus* can endure the acidic environment of the phagolysosome and even start proliferating inside it (39,82). By contrasting the killing mechanisms, *S. aureus* can extend its intracellular survival within phagocytes. It has been shown that *S. aureus* can survive up to 3-4 days inside macrophages before lysing the host cell from within (83,84). Notably, intracellular pathogens extract the nutrients necessary for their growth from the same metabolites ingested and used by macrophages for their own sustenance (85). On the other hand, as neutrophils have a shorter life span than macrophages, according to the number of pathogens ingested, *S. aureus* can manipulate the fate of the phagocyte. High number of intracellular bacteria triggers a rapid apoptosis or necroptosis (86,87), while a lower number of intracellular pathogens might extend the life span of neutrophils via inhibition of apoptotic genes expression (40). Similarly, intracellular *S. aureus* forces neutrophils to increase their

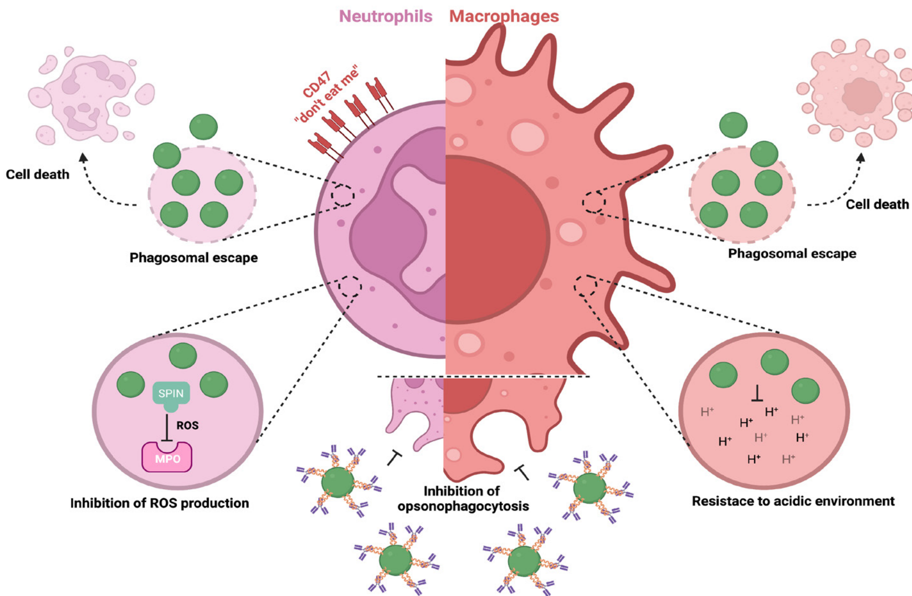


Figure 4. Invasion, survival, and proliferation mechanisms of *S. aureus* within neutrophils and macrophages. *S. aureus* can inhibit opsonin-mediated uptake by phagocytes. By contrasting the killing mechanisms employed by the phagocytes, such as inhibition of ROS production or resistance to the acidic environment of the phagosome, *S. aureus* can survive and proliferate within phagocytes, before escaping to the cytoplasm and eventually causing cell death. Moreover, intracellular *S. aureus* can extend the life span and circulation of neutrophils by increasing the membrane expression of CD47 coding for the “don’t eat me” signal.

surface membrane expression of CD47, protein coding for the “don’t eat me” signal that prevents macrophages from removing neutrophils from the infection site by efferocytosis (88).

In summary, orthopedic surgeries that involve implants carry a significant risk of introducing pathogens into our bodies. Once inside, *S. aureus* evolved several strategies to prolong, almost indefinitely, its stay within the host. Once inside, traditional antibacterial treatments and host defense mechanisms fail to solve the infection. As such, it is crucial to develop effective strategies that target pathogens immediately upon entry into the body. This will prevent their survival and proliferation in the area surrounding the implant.

The supply weapons: alternative to antibiotics

According to the National Cancer Institute (NCI) dictionary, prevention in medicine describes “an action taken to decrease the chance of getting a disease or condition. For example, cancer prevention includes avoiding risk factors (such as smoking, obesity, lack of exercise, and radiation exposure) and increasing protective factors (such as getting regular physical activity, staying at a healthy weight, and having a healthy diet)” (89). Translating the definition to the prevention of IAI, starting from the consideration of patient-related risk factors predisposing to infection (90), therapeutic strategies should aim to protect newly inserted implants from bacterial colonization, survival, and proliferation to guarantee safe implant-tissue integration.

To date, surface modification of the implant remains one of the most investigated approaches to preventing IAI (**Figure 5**). According to the “race for the surface” theory coined by Gristina, the first cell type – host or bacterial – to reach the surface of the implant will prevent the other to adhere (91). However, this theory might oversimplify reality. Chu *et al.*, demonstrated that *S. aureus* always reached the surface of the implant even when previously covered by host cells (92). Similarly, Subbiahdoss *et al.*, showed that osteoblasts were able to spread and proliferate on a glass surface colonized by *S. epidermidis* (93). In fact, the co-existence of pathogens and host cells interacting at the same time with the surface of the implant is more likely. Therefore, several designs have been developed to make the biomaterials surface able to control the host reaction (94), promote new bone deposition (95,96), and prevent adhesion or directly kill pathogens (97–99).

Notably, the concept of developing local drug delivery systems on titanium implants has gradually become one of the most promising approaches according to orthopedic surgeons (100). This might overcome several limitations linked to systemic drug delivery systems, such as high administration doses to reach an optimal concentration locally, tissue toxicity, difficult pharmacodynamics, and low drug solubility and selectivity. For example, use of antibiotic-loaded implants may create at the surgical site an inhospitable environment for bacterial growth

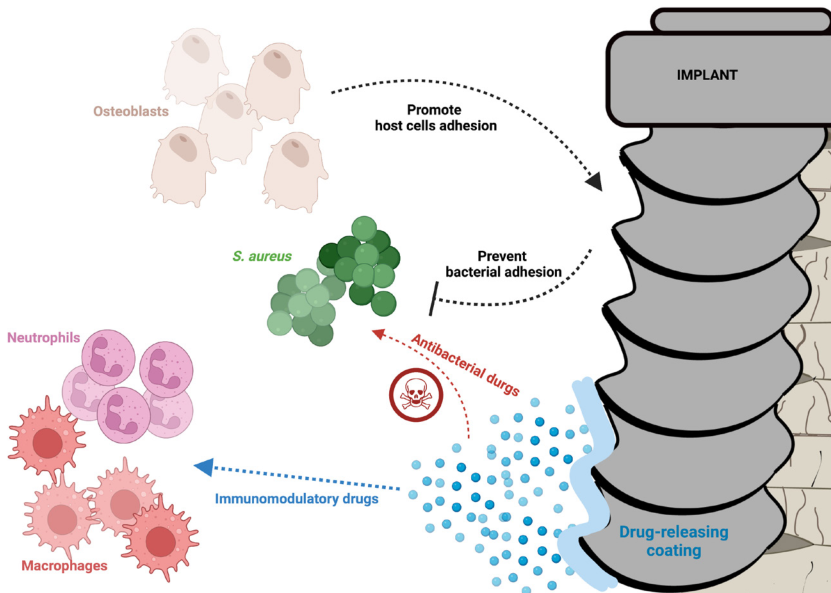


Figure 5. Strategies to prevent implant-associated infections aim to modify the surface of the implant to enhance host cells adhesion and new bone deposition, and/or prevent bacterial adhesion, or directly kill approaching pathogens. Alternatively, drug-loaded coatings can be applied to the surface of the implant, releasing compounds that directly kill pathogens and/or exert an immunomodulatory effect on phagocytes, enhancing the intrinsic antibacterial functions of immune cells

or replication, so that the immune system can efficiently clear the infection while coordinating the wound healing process. However, this raises some challenges in the design of implants as the addition of drugs to implants might affect their stability and biomechanical properties (101). Plus, host cells covering the surface of the implant may interfere with drug release properties and the antibacterial efficiency of the implant (102). On the other hand, higher local concentrations of antibiotics might be effective only against extracellular bacteria. Due to the low permeability of drugs into the membrane of host cells, antibiotic efficacy is reduced against intracellular bacteria (103). Differently, use of antibiotics with high permeability for host cells membrane, are often associated with rapid emergence of bacterial resistance (104,105). To address this challenge, antibiotics might be encapsulated into nanocarriers synthetized with polymers that favor uptake by host cells and release of their content once in contact with the acidic environment of the phagosomes where also bacteria are located (106,107). However, this strategy poses few limitations. First, it is crucial for drug-loaded nanocarriers to be uniformly internalized by all host cells susceptible to infection and intracellular survival of pathogens, without any discrimination between professional and non-professional phagocytes. Then,

the nanocarriers must be able to release the encapsulated antibiotic and reach antibacterial concentrations intracellularly. Finally, the released drug should be able to diffuse from the intracellular vesicles to the cytoplasm to prevent bacterial escape from the phagosomes (108).

Despite the progress in treatment efficiency by releasing drugs locally (109,110), yet therapeutic compounds able to completely overcome pathogens defense mechanisms and intracellular survival are still missing. To enhance the therapeutic outcome, antibacterial strategies should shift focus from antibiotics that directly target bacteria to compounds that enhance host cells response (111). This might prevent the emergence of resistance mechanisms in the bacterial population, while improving the intrinsic antibacterial functions of immune cells. Macrophages represent an ideal target for such therapeutic approach aimed at preventing IAI due to their crucial role in coordinating the host response against the implant, regulating new bone formation, protecting the organism from invading pathogens, and being susceptible to long-term intracellular survival of *S. aureus*.

The aim of the thesis

In this thesis we aim to characterize the therapeutic potential of alternative compounds to antibiotics for preventing IAI. As *S. aureus* survival within host cells, especially macrophages, has been identified as a risk factor for recalcitrant infections, we have investigated the efficacy of each therapeutic compound in reducing the number of intracellular pathogens, either by direct killing or modulation of immune cells' functions. We examined several therapeutic alternatives:

- Silver ions and nanoparticles (**chapter 2**) are known for their broad-spectrum antibacterial activity, although counterbalanced by equally high toxicity to host cells.
- Host defense peptides (**chapter 3**) are known for their direct antibacterial activity together with immunomodulatory properties such as increased bactericidal capacity and anti-inflammatory effects.
- Pathogen-specific monoclonal antibodies (**chapter 4 and 5**) as a basis for the development of a vaccine therapy to enhance recognition, uptake, and killing of pathogens by innate immune cells.

Moreover, in chapter 2 we designed an *in vitro* model mimicking an IAI-like scenario where bacteria-host and host-host cells interactions were studied. To support the design of a vaccine, in chapter 4 we compared the contribution of neutrophils and macrophages to the elimination of *S. aureus* under the same conditions *in vitro*.

The ultimate goal of this thesis is to identify the most effective strategy for preventing *S. aureus* infections and to integrate, for instance, coatings for orthopedic implants.

References

1. Todros S, Todesco M, Bagno A. Biomaterials and their biomedical applications: From replacement to regeneration. Processes (2021) 9: doi: 10.3390/pr9111949
2. Harari YN. Sapiens. A Brief History of Humankind. VINT (2015).
3. Festas AJ, Ramos A, Davim JP. Medical devices biomaterials – A review. Proceedings of the Institution of Mechanical Engineers, Part L: Journal of Materials: Design and Applications (2020) 234:218–228. doi: 10.1177/1464420719882458
4. Zimmerli W. Clinical presentation and treatment of orthopaedic implant-associated infection. J Intern Med (2014) 276:111–119. doi: 10.1111/joim.12233
5. St. Pierre CA, Chan M, Iwakura Y, Ayers DC, Kurt-Jones EA, Finberg RW. Periprosthetic osteolysis: Characterizing the innate immune response to titanium wear-particles. Journal of Orthopaedic Research (2010) 28:1418–1424. doi: 10.1002/jor.21149
6. Carnicer-Lombarte A, Chen ST, Malliaras GG, Barone DG. Foreign Body Reaction to Implanted Biomaterials and Its Impact in Nerve Neuroprosthetics. Front Bioeng Biotechnol (2021) 9: doi: 10.3389/fbioe.2021.622524
7. Kaur M, Singh K. Review on titanium and titanium based alloys as biomaterials for orthopaedic applications. Materials Science and Engineering: C (2019) 102:844–862. doi: 10.1016/J.MSEC.2019.04.064
8. Hogan B V., Peter MB, Shenoy HG, Horgan K, Hughes TA. Surgery induced immunosuppression. Surgeon (2011) 9:38–43. doi: 10.1016/j.surge.2010.07.011
9. Gaston MS, Simpson AHRW. Inhibition of fracture healing. Journal of Bone and Joint Surgery - Series B (2007) 89:1553–1560. doi: 10.1302/0301-620X.89B12.19671
10. Chen RM, Wu CH, Chang HC, Wu GJ, Lin YL, Sheu JR, Chen TL. Propofol suppresses macrophage functions and modulates mitochondrial membrane potential and cellular adenosine triphosphate synthesis. Anesthesiology (2003) 98:1178–1185. doi: 10.1097/00000542-200305000-00021
11. Moudgil GC, Pandya AR, Ludlow DJ. Influence of anaesthesia and surgery on neutrophil chemotaxis. Can Anaesth Soc J (1981) 28:232–238. doi: 10.1007/BF03005506
12. Lo Schiavo A, Ruocco E, Russo T, Brancaccio G. Locus minoris resistentiae: An old but still valid way of thinking in medicine. Clin Dermatol (2014) 32:553–556. doi: https://doi.org/10.1016/j.clindermatol.2014.04.001
13. Elek SD, Conen PE. The virulence of staphylococcus pyogenes for man. Br J Exp Pathol (1957) 38:573–586.
14. Zimmerli W, Waldvogel FA, Vaudaux P, Nydegger UE. Pathogenesis of Foreign Body Infection: Description and Characteristics of an Animal Model. J Infect Dis (1982) 146:487–497. doi: 10.1093/infdis/146.4.487
15. Busscher HJ, Van Der Mei HC, Subbiahdoss G, Jutte PC, Van Den Dungen JJAM, Zaat SAJ, Schultz MJ, Grainger DW. Biomaterial-associated infection: Locating the finish line in the race for the surface. Sci Transl Med (2012) 4: doi: 10.1126/scitranslmed.3004528
16. Van Epps JS, Younger JG. Implantable device-related infection. Shock (2016) 46:597–608. doi: 10.1097/SHK.0000000000000692
17. Kaufman MG, Meaike JD, Izaddoost SA. Orthopedic Prosthetic Infections: Diagnosis and Orthopedic Salvage. Semin Plast Surg (2016) 30:66–72. doi: 10.1055/s-0036-1580730
18. Lindberg-Larsen M, Odgaard A, Fredborg C, Schrøder HM, Bagger J, Lind-Hansen TB, Kunov A, Hansen P, Lange J, Kappel A, *et al.* One-stage versus two-stage revision of the

infected knee arthroplasty - a randomized multicenter clinical trial study protocol. BMC Musculoskelet Disord (2021) 22: doi: 10.1186/s12891-021-04044-8

19. Murray CJ, Ikuta KS, Sharara F, Swetschinski L, Robles Aguilar G, Gray A, Han C, Bisignano C, Rao P, Wool E, *et al.* Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. The Lancet (2022) 399:629–655. doi: 10.1016/S0140-6736(21)02724-0
20. Swartz MN. Hospital-acquired infections: Diseases with increasingly limited therapies. Proc Natl Acad Sci U S A (1994) 91:2420–2427. doi: 10.1073/pnas.91.7.2420
21. Li B, Webster TJ. Bacteria antibiotic resistance: New challenges and opportunities for implant-associated orthopedic infections. Journal of Orthopaedic Research (2018) 36:22–32. doi: 10.1002/jor.23656
22. Mlynarczyk-Bonikowska B, Kowalewski C, Krolak-Ulinska A, Marusza W. Molecular Mechanisms of Drug Resistance in *Staphylococcus aureus*. Int J Mol Sci (2022) 23: doi: 10.3390/ijms23158088
23. Moriarty TF, Kuehl R, Coenye T, Metsemakers WJ, Morgenstern M, Schwarz EM, Riool M, Zaat SAJ, Khana N, Kates SL, *et al.* Orthopaedic device-related infection: Current and future interventions for improved prevention and treatment. EFORT Open Rev (2016) 1:89–99. doi: 10.1302/2058-5241.1.000037
24. Taha M, Abdelbary H, Ross FP, Carli A V. New Innovations in the Treatment of PJI and Biofilms—Clinical and Preclinical Topics. Curr Rev Musculoskelet Med (2018) 11:380–388. doi: 10.1007/s12178-018-9500-5
25. Schilcher K, Horswill AR. Staphylococcal Biofilm Development: Structure, Regulation, and Treatment Strategies. (2020). https://doi.org/10
26. Maisonneuve E, Gerdes K. Molecular mechanisms underlying bacterial persisters. Cell (2014) 157:539–548. doi: 10.1016/j.cell.2014.02.050
27. Leimer N, Rachmühl C, Palheiros Marques M, Bahlmann AS, Furrer A, Eichenseher F, Seidl K, Matt U, Loessner MJ, Schuepbach RA, *et al.* Nonstable staphylococcus aureus small-colony variants are induced by low pH and sensitized to antimicrobial therapy by phagolysosomal alkalinization. Journal of Infectious Diseases (2016) 213:305–313. doi: 10.1093/infdis/jiv388
28. Fisher RA, Gollan B, Helaine S. Persistent bacterial infections and persister cells. Nat Rev Microbiol (2017) 15:453–464. doi: 10.1038/nrmicro.2017.42
29. Onyango LA, Hugh Dunstan R, Roberts TK, Macdonald MM, Gottfries J. Phenotypic Variants of Staphylococci and Their Underlying Population Distributions Following Exposure to Stress. PLoS One (2013) 8:1–9. doi: 10.1371/journal.pone.0077614
30. de Mesy Bentley KL, Trombetta R, Nishitani K, Bello-Irizarry SN, Ninomiya M, Zhang L, Chung HL, McGrath JL, Daiss JL, Awad HA, *et al.* Evidence of Staphylococcus Aureus Deformation, Proliferation, and Migration in Canaliculi of Live Cortical Bone in Murine Models of Osteomyelitis. Journal of Bone and Mineral Research (2017) 32:985–990. doi: 10.1002/jbmr.3055
31. Masters EA, Muthukrishnan G, Ho L, Gill AL, de Mesy Bentley KL, Galloway CA, McGrath JL, Awad HA, Gill SR, Schwarz EM. *Staphylococcus aureus* Cell Wall Biosynthesis Modulates Bone Invasion and Osteomyelitis Pathogenesis. Front Microbiol (2021) 12: doi: 10.3389/fmicb.2021.723498
32. Muthukrishnan G, Masters EA, Daiss JL, Schwarz EM. Mechanisms of Immune Evasion and Bone Tissue Colonization That Make *Staphylococcus aureus* the Primary Pathogen in Osteomyelitis. Curr Osteoporos Rep (2019) 17:395–404. doi: 10.1007/s11914-019-00548-4

33. Berends ETM, Dekkers JF, Nijland R, Kuipers A, Soppe JA, van Strijp JAG, Rooijakkers SHM. Distinct localization of the complement C5b-9 complex on Gram-positive bacteria. Cell Microbiol (2013) 15:1955–1968. doi: 10.1111/cmi.12170
34. Pietrocola G, Nobile G, Rindi S, Speziale P. *Staphylococcus aureus* manipulates innate immunity through own and host-expressed proteases. Front Cell Infect Microbiol (2017) 7:1–15. doi: 10.3389/fcimb.2017.00166
35. de Jong NWM, van Kessel KPM, van Strijp JAG. Immune Evasion by *Staphylococcus aureus*. Microbiol Spectr (2019) 7:1–27. doi: 10.1128/microbiolspec.gpp3-0061-2019
36. Cruz AR, Bentlage AEH, Blonk R, de Haas CJC, Aerts PC, Scheepmaker LM, Bouwmeester IG, Lux A, van Strijp JAG, Nimmerjahn F, *et al.* Toward Understanding How Staphylococcal Protein A Inhibits IgG-Mediated Phagocytosis. The Journal of Immunology (2022) 209:1146–1155. doi: 10.4049/jimmunol.2200080
37. Cruz AR, den Boer MA, Strasser J, Zwarthoff SA, Beurskens FJ, de Haas CJC, Aerts PC, Wang G, de Jong RN, Bagnoli F, *et al.* Staphylococcal protein A inhibits complement activation by interfering with IgG hexamer formation. Proc Natl Acad Sci U S A (2021) doi: 10.1073/pnas.2016772118/-/DCSupplemental
38. Dasari P, Nordengrün M, Vilhena C, Steil L, Abdurrahman G, Surmann K, Dhople V, Lahrberg J, Bachert C, Skerka C, *et al.* The Protease SplB of *Staphylococcus aureus* Targets Host Complement Components and Inhibits Complement-Mediated Bacterial Opsonophagocytosis. J Bacteriol (2022) 204: doi: 10.1128/JB.00184-21
39. Flannagan RS, Heit B, Heinrichs DE. Antimicrobial mechanisms of macrophages and the immune evasion strategies of *Staphylococcus aureus*. (2015). 826–868 p. doi: 10.3390/pathogens4040826
40. Vozza EG, Mulcahy ME, McLoughlin RM. Making the Most of the Host; Targeting the Autophagy Pathway Facilitates *Staphylococcus aureus* Intracellular Survival in Neutrophils. Front Immunol (2021) 12:1–16. doi: 10.3389/fimmu.2021.667387
41. Horino T, Hori S. Metastatic infection during *Staphylococcus aureus* bacteremia. Journal of Infection and Chemotherapy (2020) 26:162–169. doi: 10.1016/j.jiac.2019.10.003
42. Albrektsson T, Johansson C. Osteoinduction, osteoconduction and osseointegration. European Spine Journal (2001) 10:S96–S101. doi: 10.1007/s005860100282
43. Claro T, Widaa A, O'Seaghdha M, Miajlovic H, Foster TJ, O'Brien FJ, Kerrigan SW. *Staphylococcus aureus* protein a binds to osteoblasts and triggers signals that weaken bone in osteomyelitis. PLoS One (2011) 6: doi: 10.1371/journal.pone.0018748
44. Josse J, Velard F, Gangloff SC. *Staphylococcus aureus* vs. osteoblast: Relationship and consequences in osteomyelitis. Front Cell Infect Microbiol (2015) 5: doi: 10.3389/fcimb.2015.00085
45. Hamza T, Li B. Differential responses of osteoblasts and macrophages upon *Staphylococcus aureus* infection. BMC Microbiol (2014) 14:1–11. doi: 10.1186/s12866-014-0207-5
46. Boyce BF, Xing L. Functions of RANKL/RANK/OPG in bone modeling and remodeling. Arch Biochem Biophys (2008) 473:139–146. doi: 10.1016/J.ABB.2008.03.018
47. Raynaud-Messina B, Verollet C, Maridonneau-Parini I. The osteoclast, a target cell for microorganisms. Bone (2019) 127:315–323. doi: 10.1016/j.bone.2019.06.023
48. Trouillet-Assant S, Gallet M, Nauroy P, Rasigade JP, Flammier S, Parroche P, Marvel J, Ferry T, Vandenesch F, Jurdic P, *et al.* Dual impact of live staphylococcus aureus on the osteoclast lineage, leading to increased bone resorption. Journal of Infectious Diseases (2015) 211:571–581. doi: 10.1093/infdis/jiu386
49. Croes M, van der Wal BCH, Vogely HC. Impact of Bacterial Infections on Osteogenesis:

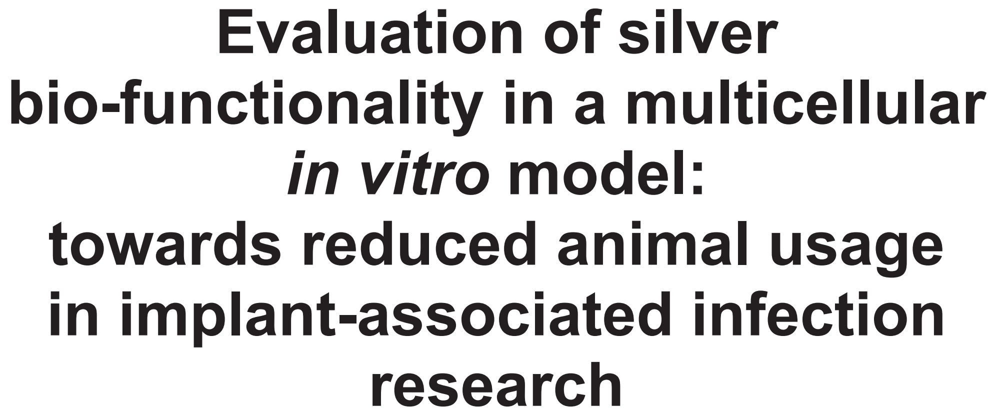
Evidence From In Vivo Studies. Journal of Orthopaedic Research (2019) 37:2067–2076. doi: 10.1002/jor.24422
50. Khokhani P, Rahmani NR, Kok A, Öner FC, Alblas J, Weinans H, Kruyt MC, Croes M. Use of therapeutic pathogen recognition receptor ligands for osteo-immunomodulation. Materials (2021) 14:1–15. doi: 10.3390/ma14051119
51. Mödinger Y, Löffler B, Huber-Lang M, Ignatius A. Complement involvement in bone homeostasis and bone disorders. Semin Immunol (2018) 37:53–65. doi: 10.1016/j.smim.2018.01.001
52. Mohamed W, Domann E, Chakraborty T, Mannala G, Lips KS, Heiss C, Schnettler R, Alt V. TLR9 mediates *S. aureus* killing inside osteoblasts via induction of oxidative stress. BMC Microbiol (2016) 16:1–8. doi: 10.1186/s12866-016-0855-8
53. Varoga D, Wruck CJ, Tohidnezhad M, Brandenburg L, Paulsen F, Mentlein R, Seekamp A, Besch L, Pufe T. Osteoblasts participate in the innate immunity of the bone by producing human beta defensin-3. Histochem Cell Biol (2009) 131:207–218. doi: 10.1007/s00418-008-0522-8
54. Chow L, Johnson V, Impastato R, Coy J, Strumpf A, Dow S. Antibacterial activity of human mesenchymal stem cells mediated directly by constitutively secreted factors and indirectly by activation of innate immune effector cells. Stem Cells Transl Med (2020) 9:235–249. doi: 10.1002/sctm.19-0092
55. Jafari N, Khoradmehr A, Moghiminasr R, Seyed Habashi M. Mesenchymal Stromal/Stem Cells-Derived Exosomes as an Antimicrobial Weapon for Orodental Infections. Front Microbiol (2022) 12: doi: 10.3389/fmicb.2021.795682
56. Marrazzo P, Crupi AN, Alviano F, Teodori L, Bonsi L. Exploring the roles of MSCs in infections: focus on bacterial diseases. J Mol Med (2019) 97:437–450. doi: 10.1007/s00109-019-01752-6
57. Yamamuro Y, Kabata T, Nojima T, Hayashi K, Tokoro M, Kajino Y, Inoue D, Ohmori T, Yoshitani J, Ueno T, *et al.* Combined adipose-derived mesenchymal stem cell and antibiotic therapy can effectively treat periprosthetic joint infection in rats. Sci Rep (2023) 13:3949. doi: 10.1038/s41598-023-30087-z
58. Amengual-Peñafiel L, Córdova LA, Constanza Jara-Sepúlveda M, Brañes-Aroca M, Marchesani-Carrasco F, Cartes-Velásquez R. Osteoimmunology drives dental implant osseointegration: A new paradigm for implant dentistry. Japanese Dental Science Review (2021) 57:12–19. doi: 10.1016/j.jdsr.2021.01.001
59. Pettit AR, Chang MK, Hume DA, Raggatt LJ. Osteal macrophages: A new twist on coupling during bone dynamics. Bone (2008) 43:976–982. doi: 10.1016/j.bone.2008.08.128
60. Alexander KA, Chang MK, Maylin ER, Kohler T, Müller R, Wu AC, Van Rooijen N, Sweet MJ, Hume DA, Raggatt LJ, *et al.* Osteal macrophages promote *in vivo* intramembranous bone healing in a mouse tibial injury model. Journal of Bone and Mineral Research (2011) 26:1517–1532. doi: 10.1002/jbmr.354
61. Chang MK, Raggatt L-J, Alexander KA, Kuliwaba JS, Fazzalari NL, Schroder K, Maylin ER, Ripoll VM, Hume DA, Pettit AR. Osteal Tissue Macrophages Are Intercalated throughout Human and Mouse Bone Lining Tissues and Regulate Osteoblast Function In Vitro and In Vivo. The Journal of Immunology (2008) 181:1232–1244. doi: 10.4049/jimmunol.181.2.1232
62. Miron RJ, Bosshardt DD. OsteoMacs: Key players around bone biomaterials. Biomaterials (2016) 82:1–19. doi: 10.1016/j.biomaterials.2015.12.017
63. Mu X, Li Y, Fan GC. Tissue-resident macrophages in the control of infection and resolution of inflammation. Shock (2021) 55:14–23. doi: 10.1097/SHK.0000000000001601

64. Jorch SK, Surewaard BGJ, Hossain M, Peiseler M, Deppermann C, Deng J, Bogoslowski A, van der Wal F, Omri A, Hickey MJ, *et al.* Peritoneal GATA6+ macrophages function as a portal for *Staphylococcus aureus* dissemination. Journal of Clinical Investigation (2019) 129:4643–4656. doi: 10.1172/JCI127286
65. Rogers DE. Studies on bacteriemia. I. Mechanisms relating to the persistence of bacteriemia in rabbits following the intravenous injection of staphylococci. J Exp Med (1956)713. http://rupress.org/jem/article-pdf/103/6/713/1078802/713.pdf
66. Gresham HD, Lowrance JH, Caver TE, Wilson BS, Cheung AL, Lindberg FP. Survival of *Staphylococcus aureus* Inside Neutrophils Contributes to Infection. The Journal of Immunology (2000) 164:3713–3722. doi: 10.4049/jimmunol.164.7.3713
67. Cavaillon J-M. The historical milestones in the understanding of leukocyte biology initiated by Elie Metchnikoff. J Leukoc Biol (2011) 90:413–424. doi: 10.1189/jlb.0211094
68. Silva MT, Correia-Neves M. Neutrophils and macrophages: The main partners of phagocyte cell systems. Front Immunol (2012) 3:2008–2013. doi: 10.3389/fimmu.2012.00174
69. Rosales C. Neutrophil: A cell with many roles in inflammation or several cell types? Front Physiol (2018) 9:1–17. doi: 10.3389/fphys.2018.00113
70. Pidwill GR, Gibson JF, Cole J, Renshaw SA, Foster SJ. The Role of Macrophages in *Staphylococcus aureus* Infection. Front Immunol (2021) 11:1–30. doi: 10.3389/fimmu.2020.620339
71. Koenderman L, Tesselaar K, Vrisekoop N. Human neutrophil kinetics: a call to revisit old evidence. Trends Immunol (2022) 43:868–876. doi: 10.1016/J.IT.2022.09.008
72. Patel AA, Ginhoux F, Yona S. Monocytes, macrophages, dendritic cells and neutrophils: an update on lifespan kinetics in health and disease. Immunology (2021) 163:250–261. doi: 10.1111/imm.13320
73. Amulic B, Cazalet C, Hayes GL, Metzler KD, Zychlinsky A. Neutrophil function: From mechanisms to disease. Annu Rev Immunol (2012) 30:459–489. doi: 10.1146/annurev-immunol-020711-074942
74. Silva MT. Macrophage phagocytosis of neutrophils at inflammatory/infectious foci: a cooperative mechanism in the control of infection and infectious inflammation. J Leukoc Biol (2011) 89:675–683. doi: 10.1189/jlb.0910536
75. Silva MT. When two is better than one: macrophages and neutrophils work in concert in innate immunity as complementary and cooperative partners of a myeloid phagocyte system. J Leukoc Biol (2010) 87:93–106. doi: 10.1189/jlb.0809549
76. Rosowski EE. Determining macrophage versus neutrophil contributions to innate immunity using larval zebrafish. DMM Disease Models and Mechanisms (2020) 13: doi: 10.1242/dmm.041889
77. Verma AK, Bansal S, Bauer C, Muralidharan A, Sun K. Influenza Infection Induces Alveolar Macrophage Dysfunction and Thereby Enables Noninvasive Streptococcus pneumoniae to Cause Deadly Pneumonia . The Journal of Immunology (2020) 205:1601–1607. doi: 10.4049/jimmunol.2000094
78. Ridder MJ, McReynolds AKG, Dai H, Pritchard MT, Markiewicz MA, Bose JL. Kinetic Characterization of the Immune Response to Methicillin-Resistant *Staphylococcus aureus* Subcutaneous Skin Infection. Infect Immun (2022) doi: 10.1128/iai.00065-22
79. van Kessel KPM, Bestebroer J, van Strijp JAG. Neutrophil-mediated phagocytosis of *Staphylococcus aureus*. Front Immunol (2014) 5: doi: 10.3389/fimmu.2014.00467
80. Moldovan A, Fraunholz MJ. In or out: Phagosomal escape of *Staphylococcus aureus*. Cell Microbiol (2019) 21:1–9. doi: 10.1111/cmi.12997
81. De Jong NWM, Ramyar KX, Guerra FE, Nijland R, Fevre C, Voyich JM, McCarthy AJ,

Garcia BL, Van Kessel KPM, Van Strijp JAG, *et al.* Immune evasion by a staphylococcal inhibitor of myeloperoxidase. Proc Natl Acad Sci U S A (2017) 114:9439–9444. doi: 10.1073/pnas.1707032114
82. Flannagan RS, Heit B, Heinrichs DE. Intracellular replication of *Staphylococcus aureus* in mature phagolysosomes in macrophages precedes host cell death, and bacterial escape and dissemination. Cell Microbiol (2016) 18:514–535. doi: 10.1111/cmi.12527
83. Koziel J, Maciag-Gudowska A, Mikolajczyk T, Bzowska M, Sturdevant DE, Whitney AR, Shaw LN, DeLeo FR, Potempa J. Phagocytosis of *Staphylococcus aureus* by macrophages exerts cytoprotective effects manifested by the upregulation of antiapoptotic factors. PLoS One (2009) 4: doi: 10.1371/journal.pone.0005210
84. Kubica M, Guzik K, Koziel J, Zarebski M, Richter W, Gajkowska B, Golda A, Maciag-Gudowska A, Brix K, Shaw L, *et al.* A potential new pathway for *Staphylococcus aureus* dissemination: The silent survival of *S. aureus* phagocytosed by human monocyte-derived macrophages. PLoS One (2008) 3: doi: 10.1371/journal.pone.0001409
85. Flannagan RS, Heinrichs DE. Macrophage□driven nutrient delivery to phagosomal *Staphylococcus aureus* supports bacterial growth. EMBO Rep (2020) 21:1–14. doi: 10.15252/embr.202050348
86. McCracken JM, Allen LAH. Regulation of human neutrophil apoptosis and lifespan in health and disease. J Cell Death (2014) 7:15–23. doi: 10.4137/JCD.S11038
87. Kobayashi SD, Braughton KR, Palazzolo-Ballance AM, Kennedy AD, Sampaio E, Kristosturyan E, Whitney AR, Sturdevant DE, Dorward DW, Holland SM, *et al.* Rapid neutrophil destruction following phagocytosis of *Staphylococcus aureus*. J Innate Immun (2010) 2:560–575. doi: 10.1159/000317134
88. Greenlee-Wacker MC, Rigby KM, Kobayashi SD, Porter AR, DeLeo FR, Nauseef WM. Phagocytosis of *Staphylococcus aureus* by Human Neutrophils Prevents Macrophage Efferocytosis and Induces Programmed Necrosis. The Journal of Immunology (2014) 192:4709–4717. doi: 10.4049/jimmunol.1302692
89. NCI. Definition of prevention - NCI Dictionary of Cancer Terms - NCI. (2023) https://www.cancer.gov/publications/dictionaries/cancer-terms/def/prevention [Accessed April 2, 2023]
90. Eka A, Chen AF. Patient-related medical risk factors for periprosthetic joint infection of the hip and knee. Ann Transl Med (2015) 3: doi: 10.3978/j.issn.2305-5839.2015.09.26
91. Gristina AG, Naylor P, Myrvik Q. Infections from biomaterials and implants: a race for the surface. Med Prog Technol (1989) 14:205–224.
92. Chu L, Yang Y, Yang S, Fan Q, Yu Z, Hu X Le, James TD, He XP, Tang T. Preferential colonization of osteoblasts over co-cultured bacteria on a bifunctional biomaterial surface. Front Microbiol (2018) 9:1–13. doi: 10.3389/fmicb.2018.02219
93. Subbiahdoss G, Kuijer R, Grijpma DW, van der Mei HC, Busscher HJ. Microbial biofilm growth vs. tissue integration: “The race for the surface” experimentally studied. Acta Biomater (2009) 5:1399–1404. doi: 10.1016/j.actbio.2008.12.011
94. Zhao T, Chu Z, Ma J, Ouyang L. Immunomodulation Effect of Biomaterials on Bone Formation. J Funct Biomater (2022) 13: doi: 10.3390/jfb13030103
95. Tang G, Liu Z, Liu Y, Yu J, Wang X, Tan Z, Ye X. Recent Trends in the Development of Bone Regenerative Biomaterials. Front Cell Dev Biol (2021) 9: doi: 10.3389/fcell.2021.665813
96. Montoya C, Du Y, Gianforcaro AL, Orrego S, Yang M, Lelkes PI. On the road to smart biomaterials for bone research: definitions, concepts, advances, and outlook. Bone Res (2021) 9: doi: 10.1038/s41413-020-00131-z
97. Vallet-Regí M, Lozano D, González B, Izquierdo-Barba I. Biomaterials against Bone

Infection. Adv Healthc Mater (2020) 9: doi: 10.1002/adhm.202000310
98. Campoccia D, Montanaro L, Arciola CR. A review of the biomaterials technologies for infection-resistant surfaces. Biomaterials (2013) 34:8533–8554. doi: 10.1016/j.biomaterials.2013.07.089
99. Tiwari A, Sharma P, Vishwamitra B, Singh G. Review on surface treatment for implant infection via gentamicin and antibiotic releasing coatings. Coatings (2021) 11: doi: 10.3390/coatings11081006
100. Ma X, Gao Y, Zhao D, Zhang W, Zhao W, Wu M, Cui Y, Li Q, Zhang Z, Ma C. Titanium implants and local drug delivery systems become mutual promoters in orthopedic clinics. Nanomaterials (2022) 12: doi: 10.3390/nano12010047
101. Kreis CA, Raschke MJ, Roßlenbroich SB, Tholema-Hans N, Löffler B, Fuchs T. Therapy of intracellular *Staphylococcus aureus* by tigecyclin. BMC Infect Dis (2013) 13:2–7. doi: 10.1186/1471-2334-13-267
102. Zaatreh S, Wegner K, Strauß M, Pasold J, Mittelmeier W, Podbielski A, Kreikemeyer B, Bader R. Co-culture of S. epidermidis and human osteoblasts on implant surfaces: An advanced *in vivo* model for implant-associated infections. PLoS One (2016) 11:1–16. doi: 10.1371/journal.pone.0151534
103. Rowe SE, Beam JE, Conlon BP. Recalcitrant *Staphylococcus aureus* infections: Obstacles and solutions. Infect Immun (2021) 89:1–14. doi: 10.1128/IAI.00694-20
104. Goldstein BP. Resistance to rifampicin: A review. Journal of Antibiotics (2014) 67:625–630. doi: 10.1038/ja.2014.107
105. Izadi E, Afshan G, Patel RP, Rao VM, Liew K Bin, Affandi MMRMM, Kifli N, Suleiman A, Lee KS, Sarker MMR, *et al.* Levofloxacin: Insights into antibiotic resistance and product quality. Front Pharmacol (2019) 10:1–7. doi: 10.3389/fphar.2019.00881
106. Maghrebi S, Joyce P, Jambhrunkar M, Thomas N, Prestidge CA. Poly(lactic-co-glycolic) Acid-Lipid Hybrid Microparticles Enhance the Intracellular Uptake and Antibacterial Activity of Rifampicin. ACS Appl Mater Interfaces (2020) 12:8030–8039. doi: 10.1021/acsami.9b22991
107. Smitha KT, Nisha N, Maya S, Biswas R, Jayakumar R. Delivery of rifampicin-chitin nanoparticles into the intracellular compartment of polymorphonuclear leukocytes. Int J Biol Macromol (2015) 74:36–43. doi: 10.1016/j.ijbiomac.2014.11.006
108. Anversa Dimer F, de Souza Carvalho-Wodarz C, Goes A, Cirnski K, Herrmann J, Schmitt V, Pätzold L, Abed N, De Rossi C, Bischoff M, *et al.* PLGA nanocapsules improve the delivery of clarithromycin to kill intracellular *Staphylococcus aureus* and Mycobacterium abscessus. Nanomedicine (2020) 24: doi: 10.1016/j.nano.2019.102125
109. Wassif RK, Elkayal M, Shamma RN, Elkheshen SA. Recent advances in the local antibiotics delivery systems for management of osteomyelitis. Drug Deliv (2021) 28:2392–2414. doi: 10.1080/10717544.2021.1998246
110. Souza JGS, Bertolini MM, Costa RC, Nagay BE, Dongari-Bagtzoglou A, Barão VAR. Targeting implant-associated infections: titanium surface loaded with antimicrobial. iScience (2021) 24: doi: 10.1016/j.isci.2020.102008
111. Amin Yavari S, Castenmiller SM, van Strijp JAG, Croes M. Combating Implant Infections: Shifting Focus from Bacteria to Host. Advanced Materials (2020) 32:1–25. doi: 10.1002/adma.202002962

CHAPTER
2



Evaluation of silver bio-functionality in a multicellular *in vitro* model: towards reduced animal usage in implant-associated infection research

Leonardo Cecotto[1,2], Daphne A.C. Stapels[2,3], Kok P.M. van Kessel[2], Michiel Croes[1], Zeldali Lourens[2], H. Charles Vogely[1], Bart C.H. van der Wal[1], Jos A.G. van Strijp[2], Harrie Weinans[1,4], Saber Amin Yavari[1,5]

[1] Department of Orthopedics, University Medical Center Utrecht, Utrecht, The Netherlands
[2] Department of Medical Microbiology, University Medical Center Utrecht, Utrecht, The Netherlands
[3] Infection Biology group, Department of Biomolecular Health Sciences, Utrecht University, Utrecht, The Netherlands
[4] Department of Biomechanical Engineering, Delft University of Technology, Delft, The Netherlands
[5] Regenerative Medicine Centre Utrecht, Utrecht University, Utrecht, The Netherlands

Published in: *Frontiers in Cellular and Infection Microbiology.* 2023. 13:1186936

Abstract

Despite the extensive use of silver ions or nanoparticles in research related to preventing implant-associated infections (IAI), their use in clinical practice has been debated. This is because the strong antibacterial properties of silver are counterbalanced by adverse effects on host cells. One of the reasons for this may be the lack of comprehensive *in vitro* models that are capable of analyzing host-bacteria and host-host interactions. In this study, we tested silver efficacy through multicellular *in vitro* models involving macrophages (immune system), mesenchymal stem cells (MSCs, bone cells), and *S. aureus* (pathogen). Our model showed to be capable of identifying each element of culture as well as tracking the intracellular survival of bacteria. Furthermore, the model enabled to find a therapeutic window for silver ions ($AgNO_3$) and silver nanoparticles (AgNPs) where the viability of host cells was not compromised, and the antibacterial properties of silver were maintained. While $AgNO_3$ between 0.00017 and 0.017 µg/mL retained antibacterial properties, host cell viability was not affected. The multicellular model, however, demonstrated that those concentrations had no effect on the survival of *S. aureus*, inside or outside host cells. Similarly, treatment with 20 nm AgNPs did not influence the phagocytic and killing capacity of macrophages or prevent *S. aureus* from invading MSCs. Moreover, exposure to 100 nm AgNPs elicited an inflammatory response by host cells as detected by the increased production of TNF-α and IL-6. This was visible only when macrophages and MSCs were cultured together. Multicellular *in vitro* models such as the one used here that simulate complex *in vivo* scenarios can be used to screen other therapeutic compounds or antibacterial biomaterials without the need to use animals.

Introduction

Implant-related infections are one of the most frequent and severe complications associated with the use of biomaterials (1). Despite best practice in medical and surgical management, infection occurs in approximately 5% of all operated orthopedic patients (2). The majority of orthopedic implant-associated infections (IAI) are caused by *Staphylococci*, in particular by *Staphyloccocus aureus* (3,4). This pathogen evolved multiple strategies to evade recognition and killing by our immune system and has developed resistance to commonly used antibiotics (5–8). Moreover, its ability to invade and survive within different host cells and tissues (9–11) and infiltrate within the osteocyte canaliculi network (12) further complicates treatment. Altogether, these features make *S. aureus* one of the most challenging causes of IAI to treat with traditional antibacterial therapies.

Metallic silver (Ag) is a known, broad-spectrum antibacterial agent that was already used to treat infections before the introduction of antibiotics (13). Over the years, numerous coating techniques have been developed to couple the antibacterial properties of silver to orthopedic implants (14,15). Such Ag-coated implants have already been implemented in the clinic to decrease the incidence of infection after primary and revision surgeries, especially in oncologic patients who are susceptible to infections (16–20). However, the favorable antibacterial properties of silver are counterbalanced by adverse effects such as the skin blue coloring called argyria (16). Although most clinical studies do not report significant side effects, there is no consensus around the concentration at which silver may cause serious local or systemic damage (16,17,21).

At a cellular level, many *in vitro* studies showed that silver exposure, both in free-ion ($AgNO_3$) and nanoparticle (AgNP) form, correlates with DNA damage, increased production of inflammatory stimuli and reactive oxygen species, eventually leading to cell death (22,23). However, whether AgNP effects are derived by the physical interaction with the nanoparticles, or the ions released from it is still under debate (24–26). Nonetheless, nanoparticles might release lower concentrations of silver ions compared to $AgNO_3$ (27–29), therefore reducing silver toxicity. Moreover, as nanoparticles efficacy is shape-, size-, charge-, dose-, and time-dependent (26,30), multiple studies tried to identify a therapeutic window where silver could retain its antibacterial activity while losing its toxicity to the host. Specifically, the optimal condition should not affect bone tissue and immune cells, which are key players involved in the post-operative (bone) healing process and control of infection around the implant.

Although the only place to start, simple *in vitro* studies are limited in the scope of their findings. For example, previous studies have shown that osteoblast viability, mesenchymal stem cell (MSC) proliferation and osteogenic differentiation were not negatively affected after prolonged incubation with silver at concentrations that still

retain antibacterial properties (31–33). However, some signs of cell activation and toxicity were described at high silver concentrations (34–36), and other studies showed the negative impact of silver on osteoblast survival and MSCs osteogenic differentiation (37,38). In contrast to bone tissue, the viability of cells from the innate immune system is highly affected by silver. Micromolar concentrations of $AgNO_3$ are already toxic to neutrophils (39), and macrophages could withstand exposure to silver for up to 24 h only at sub-antibacterial concentrations (40). However, even such short exposure to silver negatively affected the metabolic activity of macrophages (41) and their phagocytotic and bacterial killing properties did not improve (42,43). Although these studies provide useful insights into silver action against single types of host cells, they were incapable of verifying interactions between host cells and bacteria, and between host cell types that occur in the complex *in vivo* situation. Consequently, silver-coated implants with promising cytotoxicity and antibacterial properties *in vitro* generated contrasting results when tested in animal models (39,44).

Since the current *in vitro* models are not able to mimic a physiological environment, they consequently, cannot predict *in vivo* behavior very well (45). Therefore, forced by regulatory authorities, medical device companies tend to undertake a vast amount of animal testing (46). Not surprisingly, many of these animal trials are not conclusive due to variability between animals, highly scattered read-outs, and dead animals pre-/post-operation (47).

In order to better predict effectivity of antibacterial coating of implants and limit animal testing, we designed and built a multifaceted *in vitro* setup, primarily composed of a combination of host bone cells, bacteria, and immune cells. This model can precisely mimic the *in vivo* arena, test antibacterial properties and identify undesired foreign body responses to the developed implants. In our model, we chose to test the effectivity of two different forms of silver – $AgNO_3$ or AgNP – due to their debatable effects on host-cell viability and bacterial survival. In our model, we co-cultured human bone marrow-derived MSCs, human monocyte-derived macrophages, and *S. aureus* in search of a therapeutic window for $AgNO_3$ or AgNP that are used in implant coating, where neither the viability of bone nor immune cells was compromised. We here show that silver has its limitations as a therapeutic agent. This *in vitro* multicellular culture system represents a valid screening tool to predict the effects of various antibacterial compounds in an environment similar to the *in vivo* scenario without involving any animal testing.

Materials and Methods

Human monocyte-derive macrophage culture

Blood from healthy human donors was supplied by the Dutch blood bank (Sanquin, Amsterdam, The Netherlands). Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats using Ficoll-Paque (Pharmacia, Uppsala, Sweden) density centrifugation. Monocytes were positively selected by magnetic-activated cell sorting

(MACS) with anti-CD14 labelled microbeads (Miltenyi Biotec, 130050201) according to the manufacturer's instructions.

Isolated monocytes were seeded in a 24-wells plate at a density of 3 x 10^5 cells/well, except when stated otherwise. When monocytes were seeded on top of 13-mm diameter titanium disks (Alfa Aesar, 10385-HP), these were placed in the 24-wells plate before seeding. Monocytes were differentiated into macrophages by culturing for 7 days at 37°C, 5% CO_2 in α-Minimum Essential Medium (α-MEM, Gibco Paisley, 22561021) supplemented with 10% (v/v) hyFBSclone fetal bovine serum (hyFBS, Biowest, HYCLSV30160), 100 U/mL penicillin- streptomycin (1% p/s, Gibco, 15140122), and 40 ng/mL human recombinant M-CSF (Peprotech, 300-25). Culture media was refreshed after 3-4 days.

Human mesenchymal stem cell culture

Mesenchymal stem cells (MSCs) were isolated from human bone marrow aspirates upon informed consent. The aspiration procedure was approved by the local medical research ethics committee, University Medical Center Utrecht, under the protocols METC 08-001/K and METC 07-125/C.

Aspirates were diluted in PBS, filtered through a 100 μm cell strainer and the mononuclear cell layer was collected after Ficoll-Paque density centrifugation. Approximately 2.5 x 10^5 mononuclear cells were plated per cm² in MSC expansion medium consisting of α-MEM supplemented with 10% (v/v) heat-inactivated FBS (FBS, Biowest, S181H), 1% p/s, 0,2 mM L-ascorbic acid-2-phosphate (ASAP, Sigma-Aldrich, A8960) and incubated at 37°C, 5% CO_2. Cells starting from passage 3 were used in the experimental setups.

Macrophage-MSC co-culture

To build the macrophage-MSC co-culture the following steps were adopted. Monocytes were seeded in 24-wells plates at a density of 3 x 10^5 cells/well and differentiated into macrophages by culturing for 7 days. Meanwhile, MSCs were cultured to reach ~70% confluency until monocytes fully differentiated into macrophages. Then, MSCs were fluorescently labelled with celltrace violet (Invitrogen, C34557) diluted in Hanks balanced salt solution (HBSS) according to the manufacturer's instructions for labelling adherent cells. After staining, MSCs were detached with 0,25% trypsin/EDTA (Gibco, 25200056) and re-seeded together with macrophages at a density of 1 x 10^5 cells/well according to the experimental setup. Cells were allowed to adhere for about ~24 h before readouts started or bacteria were added, as explained below in the section "Multicellular infection model to study intracellular survival of bacteria".

Cell seeding densities and culture plate format were adjusted according to the experimental setup. For instance, when measuring cytokine production, monocytes and MSCs were combined in a 96-well plate at a density of 1.5 and 0.5 x 10^5 cells/well, respectively. Moreover, when the identification of single cell types was not relevant for the experimental setup, fluorescence labelling was omitted.

Bacterial culture

All experiments used GFP-labelled *Staphylococcus aureus* (kind gift from Prof. Simon Foster) and *Staphylococcus epidermidis* (kind gift from Prof. Leo Koenderman) were transformed with a GFP-expressing plasmid pCM29 to constitutively express GFP, as previously described (48). Bacteria were grown overnight in Todd-Hewitt broth (THB) with 10 ng/mL chloramphenicol to reach stationary phase.

Silver ions and nanoparticles

A solution of silver ions ($AgNO_3$) was prepared by dissolving silver nitrate (Sigma-Aldrich, S6506) in ultrapure water. Commercially available 20 nm and 100 nm silver nanoparticles (AgNP) (Alfa Aesar, J67067 and J67099), were used. Before each experiment, both sizes of nanoparticles were pelleted by centrifugation for 30 min at 4°C with 17000 x g (20 nm AgNP) or 300 x g (100 nm AgNP). A stock solution of 80 µg/mL was prepared for each type of nanoparticle in ultrapure water. AgNP and $AgNO_3$ were diluted to various concentrations in α-MEM with 10% FBS or in THB to test their effects on host or bacterial cells.

Effect of silver on cell viability and cytokine production

Monocytes and MSCs were seeded in a 96-wells plate at a density of 1.5 and 0.5 x 10^5 cells/well, respectively. The same numbers of cells were combined in the co-culture, where macrophages were combined with MSCs as previously described.

Macrophages, MSCs, and the co-culture were incubated with fresh media containing 10 ng/mL LPS O111:B4 from *Escherichia coli* (Sigma-Aldrich), or various concentration of $AgNO_3$, 20 nm AgNP, and 100 nm AgNP. After 24 h stimulation, the culture medium was harvested to measure cytokine production and cells were processed for viability evaluation. When measuring cell viability, the culture medium was replaced by α-MEM with 10% FBS and 10% Alamar Blue solution prepared by dissolving Resazurin sodium salt (Sigma-Aldrich, R7017) in PBS. Then cells were incubated at 37°C for 2-3 h, in the dark. Next, the supernatant was transferred to a new plate and fluorescence was measured at 530-10/580-10 nm (Ex/Em) with a Clariostar plate reader (BMG labtech). Background fluorescence values were subtracted, and metabolic activity was normalized to the control sample. Production of cytokine TNF-α and IL-6 was measured in the collected supernatant by ELISA (Duoset, R&D Systems, DY210 and DY217B), according to the manufacturer's instructions. ELISA values are expressed as fold-change over non-stimulated controls. Samples were analyzed in triplicates and experiments repeated three times with different monocytes and MSCs donors.

Direct antimicrobial properties of silver

The direct antimicrobial properties of silver were determined by measuring OD (600nm), combined with the broth microdilution method. Overnight bacterial culture was diluted in THB to reach a final inoculum of 5 x 10^5 colony-forming units per mL (CFU/mL). In a flat-bottom 96-well plate, the bacterial suspension was mixed with

$AgNO_3$, 20 nm AgNPs, or 100 nm AgNP in equal parts in triplicates, with a final volume of 200 µL. Bacterial growth was monitored at 37°C by measuring OD (600nm) every 5 min, for a total time of 12 h, on a Clariostar plate reader with gentle shaking before each measurement. Then, bacterial suspensions were serially diluted and plated on Todd-Hewitt agar (THA), and colonies counted after overnight incubation at 37°C. Samples were analyzed in triplicates.

Multicellular infection model to study intracellular survival of bacteria

An overnight bacterial culture was diluted in α-MEM to reach a final concentration of 1 x 10^7 CFU/mL. To mimic the *in vivo* situation, bacteria were incubated with 5% normal human serum (NHS) for 15 min at 37°C, which coats them with antibodies and complement (opsonization) to enable their uptake by immune cells. Serum was collected from blood obtained from healthy donors after informed consent, as previously described (48). Approval from the Medical Ethics Committee of the University Medical Center Utrecht was obtained (METC protocol 07-125/C approved on March 1, 2010).

Opsonized bacteria were added to the co-culture at various multiplicity of infection (MOI). To synchronize bacterial uptake, plates were centrifuged for 5 min with 110 x g at RT, and then incubated at 37°C, 5% CO_2. To study intracellular bacterial survival, the cells were washed twice after 30 min of infection to remove free bacteria and cultured in media supplemented with 100 µg/mL gentamicin (Serva, 22185.02, to kill the bacteria) and 20 µg/mL lysostaphin (Bioconnect, MBS635842, to lyse the bacteria and lose the GFP signal) for 1 h. Afterwards, cells were washed twice and incubated in media with only 5 µg/mL gentamicin. All washing steps were performed with warm α-MEM. This treatment allows only intracellular bacteria to survive, as both gentamicin and lysostaphin are unable to penetrate mammalian cell membranes within short time periods (49,50). To verify treatment efficacy in lysing bacteria, serum-opsonized *S. aureus* was incubated for 1 h at 37°C in α-MEM in presence of 100 µg/mL gentamicin and 20 µg/mL lysostaphin. Then, GFP expression was measured with a MACSquant VYB (Miltenyi Biotech) flow cytometer and data were analyzed with FlowJo (v.10.1., FlowJo LLC).

At the desired time points, co-culture samples were processed for flow cytometry analysis, microscopy observation, or quantification of intracellular bacteria via CFU count.

For flow cytometry, cells were detached by a combination of trypsin and eventually gentle scraping in 1 mM DPBS/EDTA if cells were still attached to the bottom of the culture plate. Cells were transferred to a 96-wells plate and stained with sytox orange dead cell stain for flow cytometry (Invitrogen, S34861) according to the manufacturer's instructions. Samples were measured with a MACSquant VYB flow cytometer and data were analyzed with FlowJo. The gating strategy is summarized in **Supplementary Figure 1**. Briefly, a total of 10,000 events were collected for each sample gated based on forward scatter (FSC) and side scatter (SSC) parameters. The two cell types were selected based on the signal of CellTrace violet. Non-infected

samples from the sytox negative population were used to set GFP fluorescence baseline and define the proportion of infected, GFP-positive cells.

For confocal imaging, cells were collected as described for flow cytometry analysis and fixed in 1.5% paraformaldehyde. Cell membranes were stained with 3 μg/mL Alexa Fluor 647-conjugated Wheat Germ Agglutinin (WGA, Invitrogen, W32466) for 10 min at RT, on a shaking plate. Then, samples were transferred to CELLview slide (Greiner Bio-One, 543079) previously coated with poly-L-lysine (Sigma-Aldrich, P4707), and imaged on a Leica TCS SP5 microscope with a HCX PL AP CS 63x/1.40-0.60 OIL objective (Leica Microsystems). Images were adjusted for publication using Image J Fiji.

Finally, to quantify the number of intracellular bacteria, cells were lysed with 0.1% Triton X-100 and plated on THA plates in serial dilutions. Plates were incubated overnight at 37°C after which colonies were counted.

Multicellular infection model to study the effects of silver

A variation of this model was used to study silver effects. Briefly, cells were incubated for 24 h with different concentrations of $AgNO_3$ and 20 nm AgNP before adding *S. aureus* at a MOI=10. Samples were analyzed by flow cytometry and CFU counting at 30 min and 4 h after infection. In this setup, when cells were incubated with silver, gentamicin and lysostaphin treatment was not employed for the 4 h time point. Samples were analyzed in triplicates and experiments repeated three times with different monocytes and MSCs donors.

Statistical analysis and graphics

GraphPad Prism 9 (version 9.3) was used to create the graphs and determine statistical significance via a two-way or one-way ANOVA, or t-test. $p<0.05$ was considered statistically significant. Illustrations were created with BioRender.com.

Results

Establishing a tunable, multicellular, *in vitro* model to study IAI

To build a comprehensive, multicellular *in vitro* model that mimics the main players in IAI, we co-cultured primary MSC and monocyte-derived human macrophages as summarized in **Figure 1**.

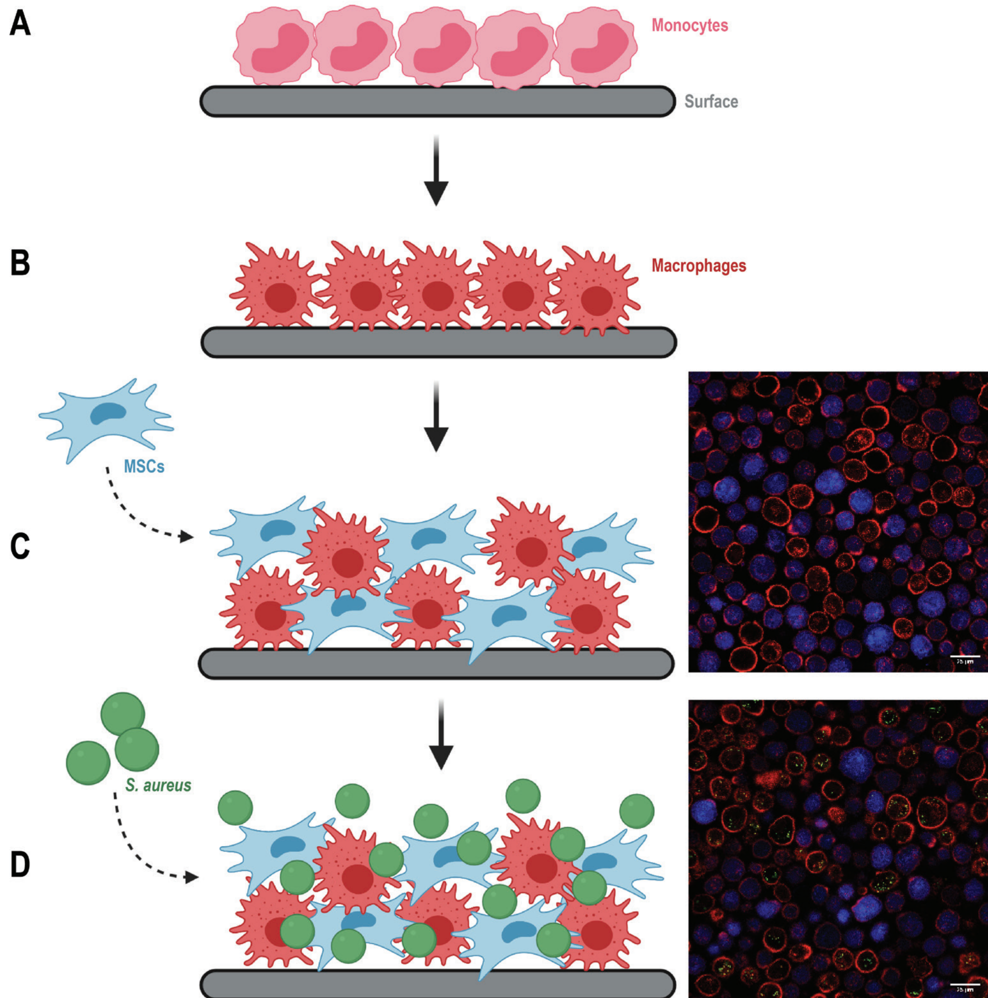


Figure 1. Schematic representation of building the multicellular *in vitro* model mimicking an implant-associated infection environment (images created with Biorender.com). (**A**) Monocytes were seeded on the selected surface and differentiated to macrophages (**B**) for 7 days. (**C**) Differentiated macrophages were re-seeded together with MSCs (left). Representative microscopy image of the co-culture (right), where MSCs were previously stained with CellTrace Violet (in blue) and all cell membranes were stained with Alexa Fluor 647-conjugated WGA (in red). (**D**) *S. aureus* was added to the co-culture (left). Representative image of the co-culture with intracellular *S. aureus* (green dots) (right).

First, monocytes were isolated from human blood and seeded on a surface to differentiate into macrophages (**Figure 1A** and **1B**). Cells were seeded either on a cell culture plate or on any biomaterial mimicking an implant. When comparing plastic with titanium, the surface itself seemed to have minimal impact on the phagocytic capacity of the macrophages (**Supplementary Figure 2A**). After differentiation, macrophages were re-seeded together with MSCs, which can differentiate into osteoblasts, to create an IAI-like environment (**Figure 1C**). In order to distinguish each element in the co-culture, we labeled at least one cell type before mixing with the second one. The co-culture was viable for up to several days, enabling the verification of cytotoxicity, modulation of cell functions, and antibacterial effects over a relatively long-time span.

The co-culture of macrophages and MSCs was then exposed to GFP-expressing bacteria (**Figure 1D**). We verified that this model could be used to study infections caused by different bacterial species relevant in the orthopedic field, such as *S. aureus* and *S. epidermidis*. Although macrophages were equally capable of associating with both bacterial species, we observed that MSCs were more susceptible to *S. aureus* than *S. epidermidis* infection (**Supplementary Figure 2B**). To assess the efficiency of antibacterial treatments in both macrophages and MSCs, all further experiments were conducted with *S. aureus*.

At the desired time points, the co-culture was processed for (confocal) microscopy (**Figure 1**), flow cytometry (**Supplementary Figure 2**), or bacterial enumeration via CFU plating (**Figure 4**). Flow cytometry offered the possibility to track intracellular bacterial survival per host-cell type over time by including gentamicin and lysostaphin treatment. These two compounds are not membrane-permeable and therefore selectively kill and lyse extracellular bacteria (49,50). We confirmed by flow cytometry that the treatment efficiently lysed bacteria, as no GFP signal could be detected from *S. aureus* already after 1 h incubation with gentamicin and lysostaphin (**Supplementary Figure 2C**). Moreover, microscopy images confirmed that all GFP signal detected by the flow cytometer originated from intracellular bacteria and not from membrane-bound or extracellular *S. aureus* (**Figure 1D**). These results confirm the validity of the model we developed to study intracellular bacteria.

As expected, after 30 min of direct contact between bacteria and host cells, almost all macrophages had engulfed at least one *S. aureus* bacterium. In comparison, less than half of the MSCs population had engulfed bacteria. After 24 h, the proportion of infected macrophages was significantly reduced both when cultured alone, and in the co-culture. Interestingly, the proportion of infected MSCs was only reduced when cultured alone, but not in co-culture (**Supplementary Figure 2D**). This highlights distinct behaviors between cells cultured alone or together.

Finding a therapeutic window for silver treatment

We tested the validity of our co-culture model by using it to determine a possible therapeutic window for silver as an antibacterial treatment. For this purpose, silver must be at a concentration that is antibacterial, but not toxic to human cells. Therefore,

we incubated *S. aureus*, macrophages, and MSCs with several concentrations of $AgNO_3$, 20 nm AgNP, and 100 nm AgNP.

We observed that at the lowest concentration tested, $AgNO_3$ reduced bacterial growth and significantly decreased the number of viable *S. aureus*,while complete inhibition of growth and killing was achieved after exposure to $AgNO_3$ concentrations higher than 0.0017 µg/mL (**Figure 2A** and **2B**). Although bacterial growth speed

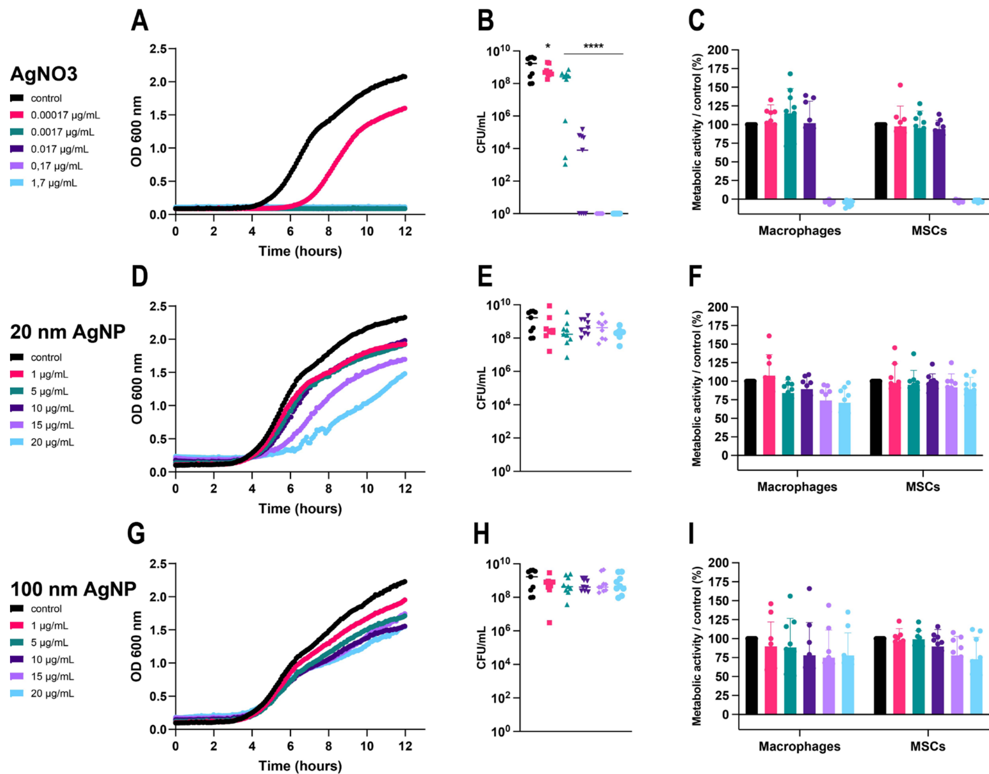


Figure 2. Evaluation of the effects of silver on *S. aureus*, macrophages, and MSCs. Bacteria and host cells were incubated with several concentrations of AgNO3 (**A-C**), 20 nm AgNP (**D-F**), and 100 nm AgNP (**G-I**). Bacterial growth (graphs on the left) was monitored by measuring OD (600 nm) continuously during 12 h and the number of surviving bacteria was quantified by CFU count (graphs on the center). (n=9, from 3 independent experiments). Data for bacterial growth were plotted with mean only, and data for CFU count were transformed into log10 and represented as mean +/- SD. Statistical significance compared to control was determined via one-way ANOVA. *p<0,05; ****p<0,0001 on the log-transformed data. The metabolic activity of macrophages and MSCs (graphs on the right) was determined by alamar blue assay after 24 h incubation with silver formulations. Assay values were normalized to untreated control cells. (n=9, from 3 independent experiments). (n=9, from 3 independent experiments). Data were represented as mean +/- SD.

was affected by increasing the concentrations of 20 nm AgNP (**Figure 2D**), no bacteriostatic or bactericidal effects were observed after incubation with 20 nm AgNP and 100 nm AgNP (**Figure 2-D, -E, -G, -H**).

On the other hand, $AgNO_3$ completely abolished the metabolic activity of host cells starting at 0.17 µg/mL (**Figure 2C**), while exposure to AgNP, regardless of size or concentration, partially affected the metabolism of both cell types. For instance, concentrations of 20 nm AgNP higher than 1 µg/mL reduced the total metabolic activity of the macrophage population by up to ~25% (**Figure 2F**). Incubation with any concentration of 100 nm AgNP reduced the viability of macrophages, while the metabolic activity of MSCs was affected only at concentrations higher than 10 µg/mL (**Figure 2I**). Nonetheless, we exclude a toxic effect derived from exposure to nanoparticles as more than 75% of the total metabolic activity remained for both the macrophages and MSCs populations.

In addition, we assessed whether silver triggered an inflammatory response by measuring the secretion of TNF-α and IL-6. Overall, non-toxic silver concentrations

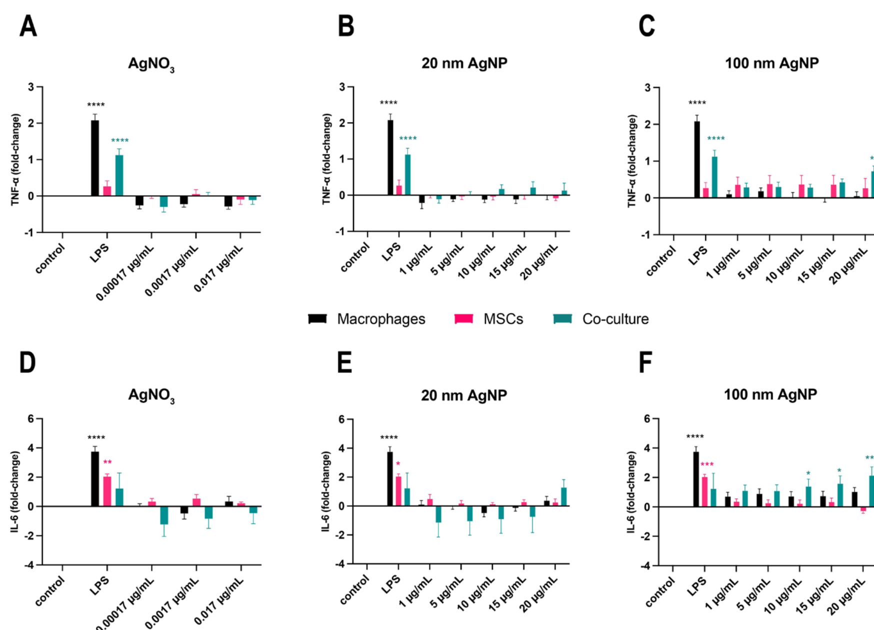


Figure 3. Evaluation of the inflammatory response caused by stimulation of host cells with non-toxic concentrations of silver. Macrophages, MSCs, and macrophages with MSCs (Co-culture) were incubated for 24 h with several concentrations of AgNO3, 20 nm AgNP, 100 nm AgNP, and 10 ng/mL LPS as positive control. Then, TNF-α (**A**, **B**, **C**) and IL-6 (**D**, **E**, **F**) levels in the supernatant were quantified by cytokine-specific ELISA and expressed as fold-change compared to control samples. (n=9, from 3 independent experiments). Data were transformed into 2-log and represented as mean +/- SEM. Statistical significance compared to control was determined via two-way ANOVA. ****$p<0.0001$; ***$p<0.001$; **$p\leq 0.005$; *$p<0,05$.

did not elicit any inflammatory response in monocultured cells compared to control samples (**Figure 3**). However, from this assay we could observe the added value of co-culturing different cell types. Addition of MSCs reduced LPS-mediated activation of macrophages, as observed by decreased production of TNF-α. In addition, including immune cells reduced IL-6 production by MSCs. Moreover, exposure to 100 nm AgNP at 20 μg/mL and at concentrations higher than 5 μg/mL significantly increased TNF-α (**Figure 3C**) and IL-6 (**Figure 3F**) levels respectively in the co-culture.

Evaluation of the effects of silver on the multicellular *in vitro* model

Once we established the effects of silver in simple *in vitro* models, we could further explore the consequences of its use in an IAI-like environment thanks to our multicellular model to study infection. Besides defining the antibacterial, non-toxic, and non-inflammatory concentrations of $AgNO_3$ and AgNPs, we explored the impact of silver on the antibacterial functions of host cells (**Figure 4A**).

Due to their toxicity to host cells, we excluded AgNO3 at concentrations of 0.17 μg/mL and 1.7 μg/mL from the assays. Only three representative concentrations of 20 nm AgNP were selected, as similar cytotoxicity and antibacterial activity were observed. Analysis of samples by flow cytometry showed a remarkable shift in the side-scatter (SSC) values for macrophages in the co-culture when exposed to AgNP for 24 h (**Supplementary Figure 3A**). This increase in cellular granularity was concentration dependent and particularly evident in the presence of 100 nm AgNP. The effect was probably caused by the internalization of nanoparticles by macrophages, as shown by bright field microscopy (**Supplementary Figure 3B**). For this reason, 100 nm AgNPs were excluded from the analysis with the multicellular model.

Despite the previously observed antibacterial activity of AgNO3 (**Figure 2A** and **2B**), this was not strong enough to counteract bacterial growth within the time frame tested in the multicellular model. In spite of the presence of AgNO3 or 20 nm AgNP, after 4 h incubation, the bacteria had already overtaken and killed most macrophages and MSCs in culture as observed by the increased amount of cells positive for sytox staining (**Supplementary Figure 4**).

Following *S. aureus* introduction into the co-culture, silver treatment did not influence bacterial uptake by host cells. We confirmed the presence of intracellular *S. aureus* in both cell types. As observed before, macrophages phagocytosed more bacteria than MSCs (**Figure 4B**). Depending on the donor, silver treatment had either no impact on phagocytosis or eventually led to an increased uptake of bacteria by both cell types. Moreover, exposure to silver did not reduce the amount of *S. aureus* able to survive intracellularly compared to the control samples (**Figure 4C**). Confocal microscopy imaging confirmed the flow cytometry results. Higher numbers of intracellular *S. aureus* were observed within macrophages than MSCs, while exposure to AgNO3 or AgNP did not influence bacterial uptake (**Figure 4D**).

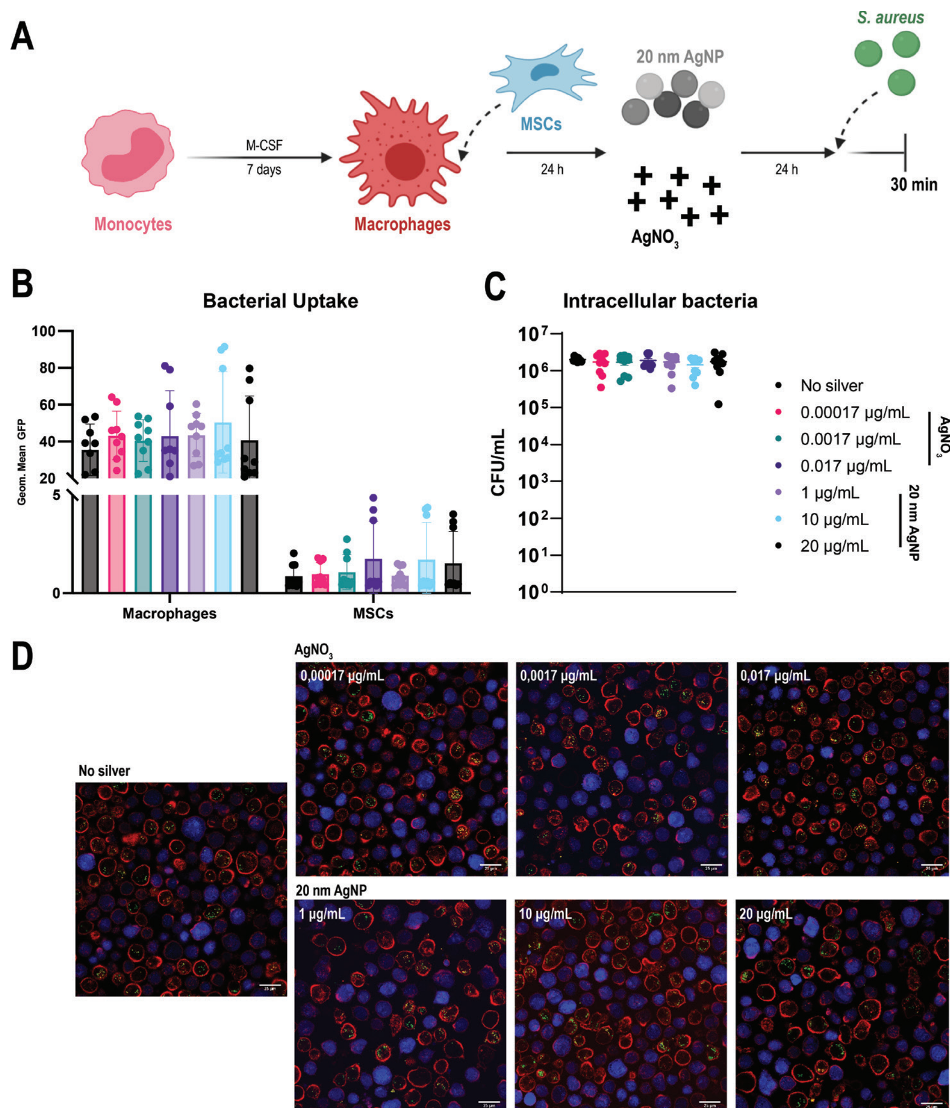


Figure 4. Evaluation of the effects of AgNO3 and 20 nm AgNP on the multicellular *in vitro* model (***A***) Schematic representation of the protocol used (images created with Biorender.com). Macrophages were combined with MSCs in culture and then incubated with AgNO3 and 20 nm AgNP for 24 h before infection with *S. aureus* for 30 min. (***B***) The amount of bacteria taken up by macrophages and MSCs is represented as the geometric mean of the GFP signal. (***C***) The number of intracellular bacteria was determined by counting CFU after cells lysis. (n = 9, from a total of 3 independent experiments). Data were represented as mean +/- SD. (***D***) Confocal imaging confirms flow cytometry observations. Macrophages (red), MSCs (blue), and *S. aureus* (green dots).

Discussion

Despite the long-known antibacterial efficacy of silver, its clinical use has lagged. One cause might be ascribed to the lack of reliable *in vitro* models to test silver's efficacy. Another limitation might be related to the need to identify a therapeutic window where pathogens are killed without affecting the viability of host cells. In this work, we developed a multicellular *in vitro* model that mimics an IAI-like scenario to investigate the efficacy of silver as a therapeutic treatment.

Traditionally, the effect of silver on bacteria or host cells was investigated separately, ignoring cell-cell interactions. This often rushed towards the identification of silver concentrations that were antibacterial and non-toxic for host cells (51–54) without addressing interactions between bacteria and host cells, and between host cell types. Although they did not address the antibacterial properties of silver, other research groups have shown that the effectiveness of novel treatments was different when assessed on cells cultured alone or in the presence of bacteria (55,56). Similarly, encouraging results have been achieved after tests run on monocultures *in vitro*, but generated opposite outcomes when these treatments were tested in animal models (44,57). In a previous study, we showed that AgNP-coated surfaces completely killed *S. aureus in vitro*, while they did not exert any bactericidal effect *in vivo*. Moreover, the presence of coated implants worsened the healing process. Afterward, we learnt that these side effects derived from previously undetected Ag toxic levels against immune cells (39). Therefore, to improve the reliability of our *in vitro* tests, we designed a comprehensive multicellular model where cell-cell interactions can be investigated.

The interaction between MSCs and macrophages have already been showed to improve osteogenic differentiation of MSCs (58,59), resolution of infection (60–62) and inflammation (63). We also assessed the additive value of culturing macrophages and MSCs together. In fact, in the presence of LPS, cells in co-culture secreted a lower amount of cytokines compared to single cells (**Figure 3**). On the other hand, although immune cells captured most of the invading pathogens, a small fraction of MSCs was still infiltrated by *S. aureus*, via a process that could have been either active or passive (9,64). In co-culture, 24 h after infection, we observed a reduction of intracellular bacteria only within macrophages, with no changes in MSCs (**Supplementary Figure 2D**). While this could be explained in immune cells by the activation of their bactericidal activity (65), further studies are needed to understand the antibacterial mechanisms activated in MSCs when seeded alone but not in co-culture. Nonetheless, we have shown that our multicellular model allows us to identify each element involved in culture as well as track the intracellular survival of bacteria over time. As cells could also be seeded on different surfaces (**Supplementary Figure 2A**), this model can be extended to study the impact of new biomaterials and/or coatings on host cells and bacteria. Here, we used the model as a preliminary screening tool for finding the optimal silver concentrations to include in antibacterial coatings for orthopedic implants.

There are several reasons to favor silver formulations as nanoparticles rather than free ions. Use of higher-sized nanoparticles with a larger surface area is thought to reduce the amount of silver ions released, and therefore reduce AgNPs toxicity against host cells (27–29). Accordingly, nanoparticles with larger sizes need to be used at higher concentrations to achieve similar efficacy to $AgNO_3$ (37,66–68). Besides a reduction in cytotoxicity, it should be considered that continuous uptake of non-degradable nanoparticles by host cells eventually leads to alterations in cell shape and morphology, as shown by our flow cytometry analysis (**Supplementary Figure 3**). Moreover, AshaRani *et al.*, showed that nanoparticle uptake *in vitro* did not correlate with substantial cell death, even after incubation with higher concentrations of AgNP with a size distribution lower than 20 nm, but forced macrophages into a state of metabolic arrest (22). Instead, nanoparticle dimensions affected *in vivo* clearance and tissue accumulation, with the risk that particles larger than 40 nm reside indefinitely within the body (69). Furthermore, continuous exposure to nanoparticles might cause the onset of a local inflammatory response in the long term with detrimental consequences for implant-tissue integration (70). According to our results, AgNPs were less toxic to host cells and bacteria than $AgNO_3$ (**Figure 2**). Although we observed only a slight reduction in metabolically active macrophages and MSCs after exposure to AgNPs, previous studies suggest that use of higher concentrations might have induced cytotoxic effects on host cells as well (40–42,71). For instance, the onset of harmful effects on host cells was evident from the inflammatory response caused by 100 nm AgNP (**Figure 3C** and **3F**). Interestingly, this side effect was detected only with the use of the co-culture model rather than with single cell assays. Furthermore, treatment with neither 20 nm AgNP or $AgNO_3$ reduced the number of intracellular bacteria found in macrophages and MSCs (**Figure 4**).

The multicellular model we used only allowed us to assess silver's efficacy for a limited period of time due to the fact that bacteria grow faster than host cells. While $AgNO_3$ displayed bacteriostatic and bactericidal effects after 12 h (**Figure 2A** and **2B**), the same concentrations failed to inhibit *S. aureus* growth after 4 h in the multicellular model (**Supplementary Figure 4**). This limited our study to bacteria that survive intracellularly, while this multicellular model might be used to assess the efficacy of treatment on both extracellular and intracellular bacteria. The observed change in the efficacy of silver might be caused by the assay conditions *in vitro*. For instance, aerobic or anaerobic culture conditions (72), aggregation of nanoparticles (73) or binding of silver ions to serum proteins (74,75) could negatively impact the bio-functionality of silver. Our study might be affected by this, since different types of media were used to assess silver toxicity against *S. aureus*, macrophages, and MSCs. Moreover, silver exposure may even affect the response of macrophages to invading pathogens. For instance, Sarkar *et al.*, showed that AgNP-treated macrophages had a reduced cytokine response and activation following *Mycobacterium tuberculosis* infection (40). On the other hand, other research groups did not observe any positive impact on phagocytosis or oxidative burst in innate immune cells after treatment with

silver (76–78).

Apart from testing the efficacy of silver as an antibacterial agent, this model could be used as an *in vitro* screening tool for several other therapeutic compounds (79) or antibacterial coatings. By developing culture models in which host-host and host-bacteria cells interact, we may gain greater insight into how implant surface features influence these cells, which has been investigated only on single elements so far (80–84). In fact, this model could be adapted to study different scenarios of IAI. In accordance with the race for the surface theory (85), bacterial colonization of the implant surface may prevent host cells adhesion and subsequently implant-tissue integration. Accordingly, Luan *et al.*, showed that variations in gold nanoparticle-coatings were able to modulate macrophages functions and bone cells adhesion according to the presence or absence of bacteria on the coated surface (55). However, these and other studies, including ours, were performed under static conditions *in vitro*. By adding a flow system that simulates *in vivo*-like conditions such as shear stress and exchange of nutrients and molecules among cells in culture, outcomes can be closer to a real IAI scenario (86–88). Finally, these multicellular models could include different cell types involved in IAI, such as osteocytes, osteoclasts, or cells from the bone marrow. Neutrophils and macrophages, for example, form the first line of defense against invading pathogens (65,89,90). Despite previous reports about neutrophil interactions with single cell types and different biomaterials (91–95), little is known about their role in complex *in vitro* models that mimic the conditions of IAI.

Conclusion

Thanks to our multicellular model combining two host-cell types, bacteria, and an implant surface, we can predict the possible benefits and pitfalls derived from the use of silver as an antibacterial agent. Although our monoculture assays suggested the existence of a therapeutic window for the use of $AgNO_3$ between 0.00017 and 0.017 µg/mL, our multicellular model revealed that neither the extracellular nor intracellular survival of *S. aureus* was affected. Moreover, we found that uptake of 20 nm AgNP had no impact on metabolic activity, phagocytic and killing capacities of macrophages, whereas increasing the size of the nanoparticles caused an inflammatory response that was only detectable when macrophages and MSCs were cultured together. By developing a model that accounts for the interactions among host cells and bacteria, *in vitro* screening tests can better simulate the complexity of *in vivo* models and better predict treatment outcomes.

Acknowledgements

The collaboration project is cofounded by the PPP Allowance made available by Health~Holland, Top Sector Life Sciences & Health, to stimulate public-private partnerships. This research also received co-funding from the EU's H2020 research and innovation program under Marie S. Curie cofund RESCUE grant agreement No 801540. This publication is also part of the project DARTBAC (with project number NWA.1292.19.354) of the research program NWA-ORC which is (partly) financed by the Dutch Research Council (NWO).

References

1. Arciola CR, Campoccia D, Montanaro L. Implant infections: Adhesion, biofilm formation and immune evasion. Nat Rev Microbiol (2018) 16:397–409. doi: 10.1038/s41579-018-0019-y
2. Moriarty TF, Kuehl R, Coenye T, Metsemakers WJ, Morgenstern M, Schwarz EM, Riool M, Zaat SAJ, Khana N, Kates SL, *et al.* Orthopaedic device-related infection: Current and future interventions for improved prevention and treatment. EFORT Open Rev (2016) 1:89–99. doi: 10.1302/2058-5241.1.000037
3. Campoccia D, Montanaro L, Arciola CR. The significance of infection related to orthopedic devices and issues of antibiotic resistance. Biomaterials (2006) 27:2331–2339. doi: 10.1016/j.biomaterials.2005.11.044
4. Masters EA, Ricciardi BF, Bentley KL de M, Moriarty TF, Schwarz EM, Muthukrishnan G. Skeletal infections: microbial pathogenesis, immunity and clinical management. Nat Rev Microbiol (2022) 20:385–400. doi: 10.1038/s41579-022-00686-0
5. de Jong NWM, van Kessel KPM, van Strijp JAG. Immune evasion by staphylococcus aureus. Gram-Positive Pathogens (2019)618–639. doi: 10.1128/9781683670131.ch39
6. de Vor L, Rooijakkers SHM, van Strijp JAG. Staphylococci evade the innate immune response by disarming neutrophils and forming biofilms. FEBS Lett (2020)1–14. doi: 10.1002/1873-3468.13767
7. Flannagan RS, Heit B, Heinrichs DE. Antimicrobial mechanisms of macrophages and the immune evasion strategies of *Staphylococcus aureus*. (2015). 826–868 p. doi: 10.3390/pathogens4040826
8. Perret M, Badiou C, Lina G, Burbaud S, Benito Y, Bes M, Cottin V, Couzon F, Juruj C, Dauwalder O, *et al.* Cross-talk between *Staphylococcus aureus* leukocidins-intoxicated macrophages and lung epithelial cells triggers chemokine secretion in an inflammasome-dependent manner. Cell Microbiol (2012) 14:1019–1036. doi: 10.1111/j.1462-5822.2012.01772.x
9. Fraunholz M, Sinha B. Intracellular *Staphylococcus aureus*: live-in and let die. Front Cell Infect Microbiol (2012) 2:43. doi: 10.3389/fcimb.2012.00043
10. Horn J, Stelzner K, Rudel T, Fraunholz M. Inside job: *Staphylococcus aureus* host-pathogen interactions. International Journal of Medical Microbiology (2018) 308:607–624. doi: 10.1016/j.ijmm.2017.11.009
11. Strobel M, Pförtner H, Tuchscherr L, Völker U, Schmidt F, Kramko N, Schnittler HJ, Fraunholz MJ, Löffler B, Peters G, *et al.* Post-invasion events after infection with *Staphylococcus aureus* are strongly dependent on both the host cell type and the infecting *S. aureus* strain. Clinical Microbiology and Infection (2016) 22:799–809. doi: 10.1016/j.cmi.2016.06.020
12. de Mesy Bentley KL, Trombetta R, Nishitani K, Bello-Irizarry SN, Ninomiya M, Zhang L, Chung HL, McGrath JL, Daiss JL, Awad HA, *et al.* Evidence of Staphylococcus Aureus Deformation, Proliferation, and Migration in Canaliculi of Live Cortical Bone in Murine Models of Osteomyelitis. Journal of Bone and Mineral Research (2017) 32:985–990. doi: 10.1002/jbmr.3055
13. Chopra I. The increasing use of silver-based products as antimicrobial agents: A useful development or a cause for concern? Journal of Antimicrobial Chemotherapy (2007) 59:587–590. doi: 10.1093/jac/dkm006
14. Amin Yavari S, Castenmiller SM, van Strijp JAG, Croes M. Combating Implant Infections: Shifting Focus from Bacteria to Host. Advanced Materials (2020) 32:1–25. doi: 10.1002/adma.202002962

15. Djošić M, Janković A, Mišković-Stanković V. Electrophoretic deposition of biocompatible and bioactive hydroxyapatite-based coatings on titanium. Materials (2021) 14: doi: 10.3390/ma14185391
16. Diez-Escudero A, Hailer NP. The role of silver coating for arthroplasty components. Bone and Joint Journal (2021) 103:423–429. doi: 10.1302/0301-620X.103B3.BJJ-2020-1370.R1
17. Fiore M, Sambri A, Zucchini R, Giannini C, Donati DM, de Paolis M. Silver-coated megaprosthesis in prevention and treatment of peri-prosthetic infections: a systematic review and meta-analysis about efficacy and toxicity in primary and revision surgery. European Journal of Orthopaedic Surgery and Traumatology (2021) 31:201–220. doi: 10.1007/s00590-020-02779-z
18. Hardes J, von Eiff C, Streitbuerger A, Balke M, Budny T, Henrichs MP, Hauschild G, Ahrens H. Reduction of periprosthetic infection with silver-coated megaprostheses in patients with bone sarcoma. J Surg Oncol (2010) 101:389–395. doi: 10.1002/jso.21498
19. Trovarelli G, Angelini A, Pala E, Cappellari A, Breda A, Ruggieri P. Infection in orthopaedic oncology: Crucial problem in modern reconstructive techniques. Eur Rev Med Pharmacol Sci (2019) 23:271–278. doi: 10.26355/EURREV_201904_17501
20. Wafa H, Grimer RJ, Reddy K, Jeys L, Abudu A, Carter SR, Tillman RM. Retrospective evaluation of the incidence of early periprosthetic infection with silver-treated endoprostheses in high-risk patients: Case-control study. Bone and Joint Journal (2015) 97-B:252–257. doi: 10.1302/0301-620X.97B2.34554
21. Eto S, Kawano S, Someya S, Miyamoto H, Sonohata M, Mawatari M. First Clinical Experience With Thermal-Sprayed Silver Oxide-Containing Hydroxyapatite Coating Implant. Journal of Arthroplasty (2016) 31:1498–1503. doi: 10.1016/j.arth.2015.12.034
22. AshaRani P, Low Kah Mun G, Hande M, Valiyaveettil S. Cytotoxicity and genotoxicity of silver nanomaterials. ACS Nano (2009)
23. Park EJ, Yi J, Kim Y, Choi K, Park K. Silver nanoparticles induce cytotoxicity by a Trojan-horse type mechanism. Toxicology in Vitro (2010) 24:872–878. doi: 10.1016/j.tiv.2009.12.001
24. Durán N, Durán M, de Jesus MB, Seabra AB, Fávaro WJ, Nakazato G. Silver nanoparticles: A new view on mechanistic aspects on antimicrobial activity. Nanomedicine (2016) 12:789–799. doi: 10.1016/j.nano.2015.11.016
25. Xiu ZM, Zhang QB, Puppala HL, Colvin VL, Alvarez PJJ. Negligible particle-specific antibacterial activity of silver nanoparticles. Nano Lett (2012) 12:4271–4275. doi: 10.1021/nl301934w
26. Liao C, Li Y, Tjong SC. Bactericidal and cytotoxic properties of silver nanoparticles. Int J Mol Sci (2019) 20: doi: 10.3390/ijms20020449
27. Ivask A, Elbadawy A, Kaweeteerawat C, Boren D, Fischer H, Ji Z, Chang CH, Liu R, Tolaymat T, Telesca D, *et al.* Toxicity mechanisms in Escherichia coli vary for silver nanoparticles and differ from ionic silver. ACS Nano (2014) 8:374–386. doi: 10.1021/nn4044047
28. McQuillan JS, Groenaga Infante H, Stokes E, Shaw AM. Silver nanoparticle enhanced silver ion stress response in Escherichia coli K12. Nanotoxicology (2012) 6:857–866. doi: 10.3109/17435390.2011.626532
29. Barros D, Pradhan A, Mendes VM, Manadas B, Santos PM, Pascoal C, Cássio F. Proteomics and antioxidant enzymes reveal different mechanisms of toxicity induced by ionic and nanoparticulate silver in bacteria. Environ Sci Nano (2019) 6:1207–1218. doi: 10.1039/c8en01067f

30. Hamad A, Khashan KS, Hadi A. Silver Nanoparticles and Silver Ions as Potential Antibacterial Agents. J Inorg Organomet Polym Mater (2020) 30:4811–4828. doi: 10.1007/s10904-020-01744-x
31. Qing Y, Cheng L, Li R, Liu G, Zhang Y, Tang X, Wang J, Liu H, Qin Y. Potential antibacterial mechanism of silver nanoparticles and the optimization of orthopedic implants by advanced modification technologies. Int J Nanomedicine (2018) 13:3311–3327. doi: 10.2147/IJN.S165125
32. Rajendran A, Kapoor U, Jothinarayanan N, Lenka N, Pattanayak DK. Effect of Silver-Containing Titania Layers for Bioactivity, Antibacterial Activity, and Osteogenic Differentiation of Human Mesenchymal Stem Cells on Ti Metal. ACS Appl Bio Mater (2019) 2:3808–3819. doi: 10.1021/acsabm.9b00420
33. Souter P, Vaughan J, Butcher K, Dowle A, Cunningham J, Dodd J, Hall M, Wilson D, Horner A, Genever P. Identification of mesenchymal stromal cell survival responses to antimicrobial silver ion concentrations released from orthopaedic implants. Sci Rep (2020) 10:1–14. doi: 10.1038/s41598-020-76087-1
34. Hackenberg S, Scherzed A, Kessler M, Hummel S, Technau A, Froelich K, Ginzkey C, Koehler C, Hagen R, Kleinsasser N. Silver nanoparticles: Evaluation of DNA damage, toxicity and functional impairment in human mesenchymal stem cells. Toxicol Lett (2011) 201:27–33. doi: 10.1016/j.toxlet.2010.12.001
35. Pauksch L, Hartmann S, Rohnke M, Szalay G, Alt V, Schnettler R, Lips KS. Biocompatibility of silver nanoparticles and silver ions in primary human mesenchymal stem cells and osteoblasts. Acta Biomater (2014) 10:439–449. doi: 10.1016/j.actbio.2013.09.037
36. Samberg ME, Loboa EG, Oldenburg SJ, Monteiro-Riviere NA. Silver nanoparticles do not influence stem cell differentiation but cause minimal toxicity. Nanomedicine (2012) 7:1197–1209. doi: 10.2217/nnm.12.18
37. Albers CE, Hofstetter W, Siebenrock KA, Landmann R, Klenke FM. In vitro cytotoxicity of silver nanoparticles on osteoblasts and osteoclasts at antibacterial concentrations. Nanotoxicology (2013) 7:30–36. doi: 10.3109/17435390.2011.626538
38. Sengstock C, Diendorf J, Epple M, Schildhauer TA, Köller M. Effect of silver nanoparticles on human mesenchymal stem cell differentiation. Beilstein Journal of Nanotechnology (2014) 5:2058–2069. doi: 10.3762/bjnano.5.214
39. Croes M, Bakhshandeh S, van Hengel IAJ, Lietaert K, van Kessel KPM, Pouran B, van der Wal BCH, Vogely HC, Van Hecke W, Fluit AC, *et al.* Antibacterial and immunogenic behavior of silver coatings on additively manufactured porous titanium. Acta Biomater (2018) 81:315–327. doi: 10.1016/j.actbio.2018.09.051
40. Sarkar S, Leo BF, Carranza C, Chen S, Rivas-Santiago C, Porter AE, Ryan MP, Gow A, Chung KF, Tetley TD, *et al.* Modulation of human macrophage responses to mycobacterium tuberculosis by silver nanoparticles of different size and surface modification. PLoS One (2015) 10:1–28. doi: 10.1371/journal.pone.0143077
41. Carrola J, Bastos V, Daniel-da-Silva AL, Gil AM, Santos C, Oliveira H, Duarte IF. Macrophage Metabolomics Reveals Differential Metabolic Responses to Subtoxic Levels of Silver Nanoparticles and Ionic Silver. Eur J Inorg Chem (2020) 2020:1867–1876. doi: 10.1002/ejic.202000095
42. Aurore V, Caldana F, Blanchard M, Kharoubi Hess S, Lannes N, Mantel PY, Filgueira L, Walch M. Silver-nanoparticles increase bactericidal activity and radical oxygen responses against bacterial pathogens in human osteoclasts. Nanomedicine (2018) 14:601–607. doi: 10.1016/j.nano.2017.11.006
43. Sarkar S, Leo BF, Carranza C, Chen S, Rivas-Santiago C, Porter AE, Ryan MP, Gow A,

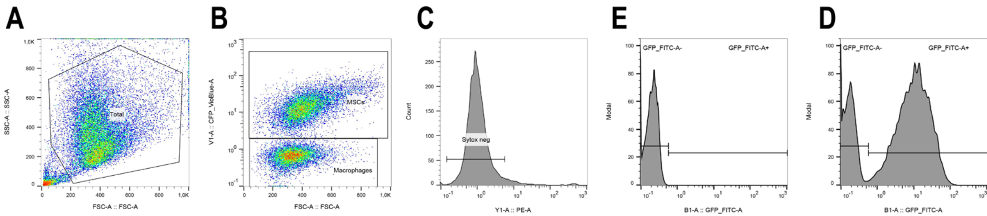
Chung KF, Tetley TD, *et al.* Modulation of human macrophage responses to mycobacterium tuberculosis by silver nanoparticles of different size and surface modification. PLoS One (2015) 10:1–28. doi: 10.1371/journal.pone.0143077
44. Chen Z, Yuen J, Crawford R, Chang J, Wu C, Xiao Y. The effect of osteoimmunomodulation on the osteogenic effects of cobalt incorporated β-tricalcium phosphate. Biomaterials (2015) 61:126–138. doi: 10.1016/j.biomaterials.2015.04.044
45. Moriarty TF, Grainger DW, Richards RG. Challenges in linking preclinical anti-microbial research strategies with clinical outcomes for device-associated infections. Eur Cell Mater (2014) 28:112–128. doi: 10.22203/eCM.v028a09
46. Grainger DW, van der Mei HC, Jutte PC, van den Dungen JJAM, Schultz MJ, van der Laan BFAM, Zaat SAJ, Busscher HJ. Critical factors in the translation of improved antimicrobial strategies for medical implants and devices. Biomaterials (2013) 34:9237–9243. doi: 10.1016/J.BIOMATERIALS.2013.08.043
47. Veening-Griffioen DH, Ferreira GS, van Meer PJK, Boon WPC, Gispen-de Wied CC, Moors EHM, Schellekens H. Are some animal models more equal than others? A case study on the translational value of animal models of efficacy for Alzheimer’s disease. Eur J Pharmacol (2019) 859:172524. doi: 10.1016/J.EJPHAR.2019.172524
48. Boero E, Brinkman I, Juliet T, van Yperen E, van Strijp JAG, Rooijakkers SHM, van Kessel KPM. Use of Flow Cytometry to Evaluate Phagocytosis of *Staphylococcus aureus* by Human Neutrophils. Front Immunol (2021) 12:1–15. doi: 10.3389/fimmu.2021.635825
49. Hamza T, Li B. Differential responses of osteoblasts and macrophages upon *Staphylococcus aureus* infection. BMC Microbiol (2014) 14:1–11. doi: 10.1186/s12866-014-0207-5
50. Hamza T, Dietz M, Pham D, Clovis N, Danley S, Li B. Intra-cellular *Staphylococcus aureus* alone causes infection *in vivo*. Eur Cell Mater (2013) 25:341–350. doi: 10.22203/ecm.v025a24
51. Huang Y, Zhang Y, Li M, Yang H, Liang J, Chen Y, Zhang Y, Huang X, Xie L, Lin H, *et al.* Physicochemical, osteogenic and antimicrobial properties of graphene oxide reinforced silver/strontium-doped hydroxyapatite on titanium for potential orthopedic applications. Surf Coat Technol (2022) 446:128788. doi: 10.1016/J.SURFCOAT.2022.128788
52. Kuo YJ, Chen CH, Dash P, Lin YC, Hsu CW, Shih SJ, Chung RJ. Angiogenesis, Osseointegration, and Antibacterial Applications of Polyelectrolyte Multilayer Coatings Incorporated With Silver/Strontium Containing Mesoporous Bioactive Glass on 316L Stainless Steel. Front Bioeng Biotechnol (2022) 10: doi: 10.3389/fbioe.2022.818137
53. Selvamani V, Kadian S, Detwiler DA, Zareei A, Woodhouse I, Qi Z, Peana S, Alcaraz AM, Wang H, Rahimi R. Laser-Assisted Nanotexturing and Silver Immobilization on Titanium Implant Surfaces to Enhance Bone Cell Mineralization and Antimicrobial Properties. Langmuir (2022) 38:4014–4027. doi: 10.1021/acs.langmuir.2c00008
54. Priebe M, Widmer J, Suhartha Löwa N, Abram SL, Mottas I, Woischnig AK, Brunetto PS, Khanna N, Bourquin C, Fromm KM. Antimicrobial silver-filled silica nanorattles with low immunotoxicity in dendritic cells. Nanomedicine (2017) 13:11–22. doi: 10.1016/J.NANO.2016.08.002
55. Luan Y, Van Der Mei HC, Dijk M, Geertsema-Doornbusch GI, Atema-Smit J, Ren Y, Chen H, Busscher HJ. Polarization of Macrophages, Cellular Adhesion, and Spreading on Bacterially Contaminated Gold Nanoparticle-Coatings in Vitro. ACS Biomater Sci Eng (2020) 6:933–945. doi: 10.1021/acsbiomaterials.9b01518
56. Zaatreh S, Wegner K, Strauß M, Pasold J, Mittelmeier W, Podbielski A, Kreikemeyer B, Bader R. Co-culture of S. epidermidis and human osteoblasts on implant surfaces: An

advanced *in vitro* model for implant-associated infections. PLoS One (2016) 11:1–16. doi: 10.1371/journal.pone.0151534
57. Tuchscherr L, Kreis CA, Hoerr V, Flint L, Hachmeister M, Geraci J, Bremer-Streck S, Kiehntopf M, Medina E, Kribus M, *et al. Staphylococcus aureus* develops increased resistance to antibiotics by forming dynamic small colony variants during chronic osteomyelitis. Journal of Antimicrobial Chemotherapy (2016) 71:438–448. doi: 10.1093/jac/dkv371
58. Nicolaidou V, Wong MM, Redpath AN, Ersek A, Baban DF, Williams LM, Cope AP, Horwood NJ. Monocytes induce STAT3 activation in human mesenchymal stem cells to promote osteoblast formation. PLoS One (2012) 7: doi: 10.1371/journal.pone.0039871
59. Loi F, Córdova LA, Ruth Z, Jukka P, Lin TH, Goodman SB, Yao Z. The effects of immunomodulation by macrophage subsets on osteogenesis *in vitro*. Stem Cell Res Ther (2016) 7:1–11. doi: 10.1186/s13287-016-0276-5
60. Krasnodembskaya A, Samarani G, Song Y, Zhuo H, Su X, Lee JW, Gupta N, Petrini M, Matthay MA. Human mesenchymal stem cells reduce mortality and bacteremia in gram-negative sepsis in mice in part by enhancing the phagocytic activity of blood monocytes. Am J Physiol Lung Cell Mol Physiol (2012) 302:1003–1013. doi: 10.1152/ajplung.00180.2011
61. Johnson V, Webb T, Norman A, Coy J, Kurihara J, Regan D, Dow S. Activated Mesenchymal Stem Cells Interact with Antibiotics and Host Innate Immune Responses to Control Chronic Bacterial Infections. Sci Rep (2017) 7:1–18. doi: 10.1038/s41598-017-08311-4
62. Chow L, Johnson V, Impastato R, Coy J, Strumpf A, Dow S. Antibacterial activity of human mesenchymal stem cells mediated directly by constitutively secreted factors and indirectly by activation of innate immune effector cells. Stem Cells Transl Med (2020) 9:235–249. doi: 10.1002/sctm.19-0092
63. Lu D, Xu Y, Liu Q, Zhang Q. Mesenchymal Stem Cell-Macrophage Crosstalk and Maintenance of Inflammatory Microenvironment Homeostasis. Front Cell Dev Biol (2021) 9: doi: 10.3389/fcell.2021.681171
64. Josse J, Velard F, Gangloff SC. *Staphylococcus aureus* vs. osteoblast: Relationship and consequences in osteomyelitis. Front Cell Infect Microbiol (2015) 5:1–17. doi: 10.3389/fcimb.2015.00085
65. Pidwill GR, Gibson JF, Cole J, Renshaw SA, Foster SJ. The Role of Macrophages in *Staphylococcus aureus* Infection. Front Immunol (2021) 11:1–30. doi: 10.3389/fimmu.2020.620339
66. Qais FA, Shafiq A, Khan HM, Husain FM, Khan RA, Alenazi B, Alsalme A, Ahmad I. Antibacterial effect of silver nanoparticles synthesized using Murraya koenigii (L.) against multidrug-resistant pathogens. Bioinorg Chem Appl (2019) 2019: doi: 10.1155/2019/4649506
67. Lu Z, Rong K, Li J, Yang H, Chen R. Size-dependent antibacterial activities of silver nanoparticles against oral anaerobic pathogenic bacteria. J Mater Sci Mater Med (2013) 24:1465–1471. doi: 10.1007/s10856-013-4894-5
68. Pazos-Ortiz E, Roque-Ruiz JH, Hinojos-Márquez EA, López-Esparza J, Donohué-Cornejo A, Cuevas-González JC, Espinosa-Cristóbal LF, Reyes-López SY. Dose-Dependent Antimicrobial Activity of Silver Nanoparticles on Polycaprolactone Fibers against Gram-Positive and Gram-Negative Bacteria. J Nanomater (2017) 2017: doi: 10.1155/2017/4752314
69. Gustafson HH, Holt-Casper D, Grainger DW, Ghandehari H. Nanoparticle uptake: The

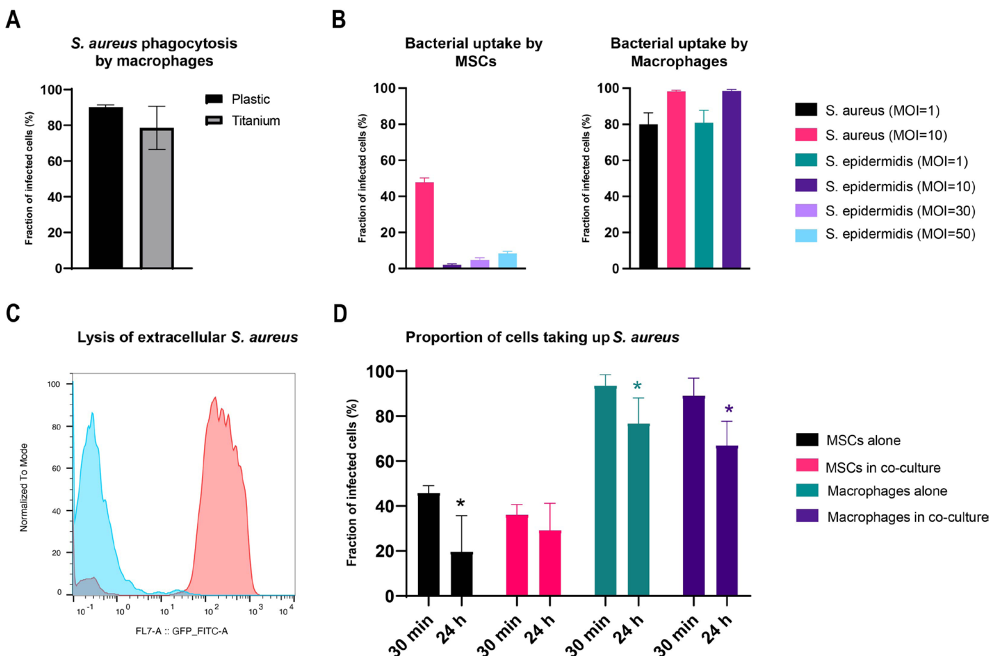
phagocyte problem. Nano Today (2015) 10:487–510. doi: 10.1016/j.nantod.2015.06.006
70. Zreiqat H, Kumar RK, Markovic B, Zicat B, Howlett CR. Macrophages at the skeletal tissue-device interface of loosened prosthetic devices express bone-related genes and their products. J Biomed Mater Res A (2003) 65:109–117. doi: 10.1002/jbm.a.10441
71. Arai Y, Miyayama T, Hirano S. Difference in the toxicity mechanism between ion and nanoparticle forms of silver in the mouse lung and in macrophages. Toxicology (2015) 328:84–92. doi: 10.1016/J.TOX.2014.12.014
72. Yang Y, Zhang C, Hu Z. Impact of metallic and metal oxide nanoparticles on wastewater treatment and anaerobic digestion. Environmental Sciences: Processes and Impacts (2013) 15:39–48. doi: 10.1039/c2em30655g
73. Brennan SA, Fhoghlú CN, DeVitt BM, O'Mahony FJ, Brabazon D, Walsh A. Instructional review: General orthopaedics silver nanoparticles and their orthopaedic applications. Bone and Joint Journal (2015) 97-B:582–589. doi: 10.1302/0301-620X.97B5.33336
74. Shahabadi N, Maghsudi M, Ahmadipour Z. Study on the interaction of silver(I) complex with bovine serum albumin by spectroscopic techniques. Spectrochim Acta A Mol Biomol Spectrosc (2012) 92:184–188. doi: 10.1016/j.saa.2012.02.071
75. Gosheger G, Hardes J, Ahrens H, Streitburger A, Buerger H, Erren M, Gunsel A, Kemper FH, Winkelmann W, Von Eiff C. Silver-coated megaendoprostheses in a rabbit model - An analysis of the infection rate and toxicological side effects. Biomaterials (2004) 25:5547–5556. doi: 10.1016/j.biomaterials.2004.01.008
76. Haase H, Fahmi A, Mahltig B. Impact of silver nanoparticles and silver ions on innate immune cells. J Biomed Nanotechnol (2014) 10:1146–1156. doi: 10.1166/jbn.2014.1784
77. Huang M, Ye K, Hu T, Liu K, You M, Wang L, Qin H. Silver nanoparticles attenuate the antimicrobial activity of the innate immune system by inhibiting neutrophil-mediated phagocytosis and reactive oxygen species production. Int J Nanomedicine (2021) 16:1345–1360. doi: 10.2147/IJN.S292482
78. Liu H, Yang D, Yang H, Zhang H, Zhang W, Fang YJ, Lin Z, Tian L, Lin B, Yan J, *et al.* Comparative study of respiratory tract immune toxicity induced by three sterilisation nanoparticles: Silver, Zinc oxide and titanium dioxide. J Hazard Mater (2013) 248–249:478–486. doi: 10.1016/j.jhazmat.2013.01.046
79. Cecotto L, van Kessel K, Wolfert MA, Vogely C, van der Wal B, Weinans H, van Strijp J, Yavari SA. Antibacterial and anti-inflammatory properties of host defense peptides against *Staphylococcus aureus*. iScience (2022) 25:105211. doi: 10.1016/j.isci.2022.105211
80. Monteiro NO, Casanova MR, Quinteira R, Fangueiro JF, Reis RL, Neves NM. Biomimetic surface topography as a potential modulator of macrophages inflammatory response to biomaterials. Biomaterials Advances (2022) 141:213128. doi: 10.1016/J.BIOADV.2022.213128
81. Zhu Y, Liang H, Liu X, Wu J, Yang C, Man Wong T, H Kwan KY, C Cheung KM, Wu S, K Yeung KW. Regulation of macrophage polarization through surface topography design to facilitate implant-to-bone osteointegration. (2021). https://www.science.org
82. Liu Y, Segura T. Biomaterials-Mediated Regulation of Macrophage Cell Fate. Front Bioeng Biotechnol (2020) 8: doi: 10.3389/fbioe.2020.609297
83. Leach JK, Whitehead J. Materials-Directed Differentiation of Mesenchymal Stem Cells for Tissue Engineering and Regeneration. ACS Biomater Sci Eng (2018) 4:1115–1127. doi: 10.1021/acsbiomaterials.6b00741
84. Tan L, Liu X, Dou H, Hou Y. Characteristics and regulation of mesenchymal stem cell plasticity by the microenvironment — specific factors involved in the regulation of MSC plasticity. Genes Dis (2022) 9:296–309. doi: 10.1016/J.GENDIS.2020.10.006

85. Gristina AG, Naylor P, Myrvik Q. Infections from biomaterials and implants: a race for the surface. Med Prog Technol (1989) 14:205–224.
86. Zhao B, van der Mei HC, Subbiahdoss G, de Vries J, Rustema-Abbing M, Kuijer R, Busscher HJ, Ren Y. Soft tissue integration versus early biofilm formation on different dental implant materials. Dental Materials (2014) 30:716–727. doi: 10.1016/J.DENTAL.2014.04.001
87. Subbiahdoss G, Fernández ICS, da Silva Domingues JF, Kuijer R, van der Mei HC, Busscher HJ. In vitro interactions between bacteria, Osteoblast-Like cells and macrophages in the pathogenesis of Biomaterial-Associated infections. PLoS One (2011) 6: doi: 10.1371/journal.pone.0024827
88. Da Silva Domingues JF, Roest S, Wang Y, Van Der Mei HC, Libera M, Van Kooten TG, Busscher HJ. Macrophage phagocytic activity toward adhering staphylococci on cationic and patterned hydrogel coatings versus common biomaterials. Acta Biomater (2015) 18:1–8. doi: 10.1016/j.actbio.2015.02.028
89. Rosowski EE. Determining macrophage versus neutrophil contributions to innate immunity using larval zebrafish. DMM Disease Models and Mechanisms (2020) 13: doi: 10.1242/dmm.041889
90. Rosales C. Neutrophil: A cell with many roles in inflammation or several cell types? Front Physiol (2018) 9:1–17. doi: 10.3389/fphys.2018.00113
91. Jhunjhunwala S. Neutrophils at the Biological-Material Interface. ACS Biomater Sci Eng (2018) 4:1128–1136. doi: 10.1021/acsbiomaterials.6b00743
92. Wesdorp MA, Schwab A, Bektas EI, Narcisi R, Eglin D, Stoddart MJ, van Osch GJVM, D'Este M. A culture model to analyze the acute biomaterial-dependent reaction of human primary neutrophils *in vitro*. Bioact Mater (2023) 20:627–637. doi: 10.1016/J.BIOACTMAT.2022.05.036
93. Abaricia JO, Shah AH, Musselman RM, Olivares-Navarrete R. Hydrophilic titanium surfaces reduce neutrophil inflammatory response and NETosis. Biomater Sci (2020) 8:2289–2299. doi: 10.1039/c9bm01474h
94. Brandau S, Jakob M, Bruderek K, Bootz F, Giebel B, Radtke S, Mauel K, Jäger M, Flohé SB, Lang S. Mesenchymal stem cells augment the anti-bacterial activity of neutrophil granulocytes. PLoS One (2014) 9: doi: 10.1371/journal.pone.0106903
95. Silva MT. When two is better than one: macrophages and neutrophils work in concert in innate immunity as complementary and cooperative partners of a myeloid phagocyte system. J Leukoc Biol (2010) 87:93–106. doi: 10.1189/jlb.0809549

Supplementary Figures

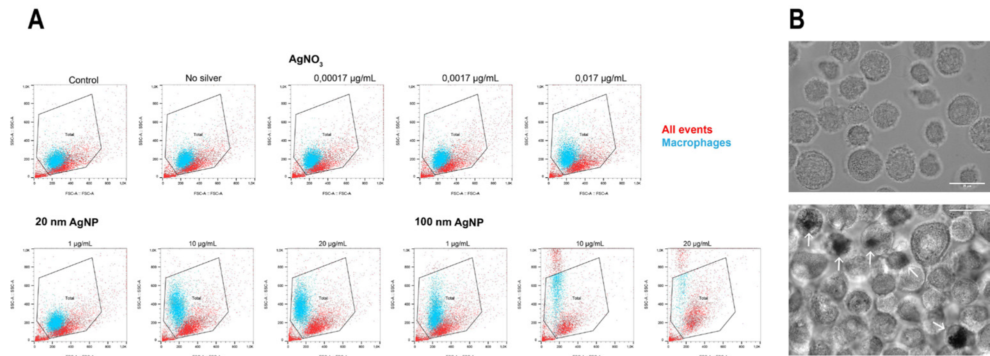


Supplementary Figure 1. Flow cytometry phagocytosis gating strategy. (**A**) Selection of the total cells population (total gate) in the linear FSC and SSC. (**B**) Selection of the two cell types in the linear FSC and fluorescence at 405 nm wavelength. (**C**) Selection of sytox negative cells within each cell type population. (**D**) Histogram setting GFP fluorescence baseline for non-infected cells. (**E**) Proportion of non-infected (left peak) and infected (right peak) cells.

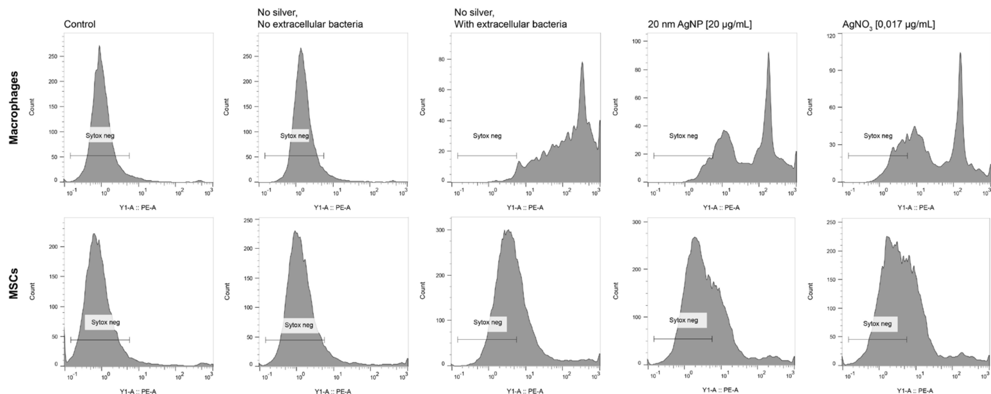


Supplementary Figure 2. Different applications of the multicellular model according to the research question, analyzed by flow cytometry. (**A**) Monocytes were seeded either on a culture plate (plastic) or on a titanium disk and differentiated to macrophages. Next, cells were exposed to *S. aureus* for 30 min and the proportion of cells that had ingested at least 1 bacterium was determined. (**B**) MSCs and macrophages were seeded alone and then exposed for 30 min at increasing MOIs of *S. aureus* and *S. epidermidis* to determine the strain dependent-uptake for each cell type. Proportion of cells that ingested at least 1 bacterium is shown. (**C**) Serum-opsonized *S. aureus* was incubated at 37ºC in presence of 100 µg/mL gentamicin

and 20 µg/mL lysostaphin. After 1 h incubation, bacterial GFP signal was measured by flow cytometry and the histograms of control (red) and treated (blue) bacteria were compared. (**D**) Macrophages and MSCs were seeded alone or in combination with each other and infected with *S. aureus*. The proportion of cells with intracellular bacteria was calculated at 30 min and 24 h after infection. Data were represented as mean +/- SD. Statistical significance compared to 30 min was determined via t-test, *p<0,01.



Supplementary Figure 3. Influence of nanoparticles uptake in macrophages. (**A**) Dot plots from co-culture samples showing the distribution in the FSC and SSC axis of the macrophage population (blue) within all the events recorded by the flow cytometer (red). (**B**) Bright field images showing the change in morphology of macrophages before (upper image) and after (lower image) 24 h incubation with AgNP 20 nm at 20 µg/mL. White arrows pointing at intracellular nanoparticles.



Supplementary Figure 4. *Selection of sytox negative macrophages* (upper row) *and MSCs* (lower row) *after incubation for 4 h with S. aureus.*

CHAPTER
3

Antibacterial and anti-inflammatory properties of host defense peptides against *Staphylococcus aureus*

Leonardo Cecotto[1,2], Kok P.M. van Kessel[2], Margreet A. Wolfert[3], H. Charles Vogely[1], Bart C.H. van der Wal[1], Harrie Weinans[1,4], Jos A.G. van Strijp[2], Saber Amin Yavari[1,5]

[1] Department of Orthopedics, University Medical Center Utrecht, Utrecht, The Netherlands
[2] Department of Medical Microbiology, University Medical Center Utrecht, Utrecht, The Netherlands
[3] Department of Chemical Biology and Drug Discovery, Utrecht University, Utrecht, The Netherlands
[4] Department of Biomechanical Engineering, Delft University of Technology, Delft, The Netherlands
[5] Regenerative Medicine Centre Utrecht, Utrecht University, Utrecht, The Netherlands

Published in: *iScience. 2022. 25(10):105211*

Abstract

Cationic host defense peptides (HDPs) are a promising alternative to antibiotics in the fight against *Staphylococcus aureus* infections. In this study, we investigated the antibacterial and immunomodulatory properties of three HDPs namely IDR-1018, CATH-2, and LL-37. While all three HDPs significantly inhibited LPS-induced activation of human macrophages, only CATH-2 prevented *S. aureus* growth. When applied to different infection models focused on intracellularly surviving bacteria, only IDR-1018 showed consistent reduction in macrophage bacterial uptake. However, this observation did not correlate to an increase in killing efficiency of intracellular *S. aureus*. Here, we conclude that despite promising antibacterial and anti-inflammatory properties of the selected HDPs, macrophages intrinsic antibacterial functions were not improved. Future studies should either focus on combining different HDPs or using them synergistically with other antibacterial agents to improve immune cells efficacy against *S. aureus* pathogenesis.

Introduction

Bacterial infections are one of the most frequent and severe complications associated with the use of biomaterials (1). Despite the significant improvement in medical and surgical management, infection incidence still arise up to 5% after orthopedic surgeries (2). The majority of biomaterials infections are caused by Staphylococci, particularly by *Staphylococcus aureus* (3). Over the years, traditional antibacterial strategies became less and less effective against *S. aureus* infections due to its ability to build resistance to antibiotics and evade immune system recognition and killing mechanisms (4–6). Moreover, *S. aureus* can invade, survive, and proliferate inside numerous cell types besides immune cells (6–8), even reaching the narrowest and deepest spaces of the osteocytes canaliculi network (9,10). Finally, *S. aureus* pathogenesis exacerbated in the presence of biomaterials because they offer an ideal substrate for bacterial adhesion and biofilm formation (11).

Despite the progress in treatment efficiency by releasing drugs locally (11–13), yet therapeutic compounds able to completely overcome pathogens defense mechanisms and survival are still missing (14,15). As we have previously shown, to enhance the therapeutic outcome, implant bio-functionalization strategies should shift focus from antibiotics that eradicate bacteria to enhance host cell response, aiming to improve intrinsic immune cells functions against pathogens invasion (16).

Cationic host defense peptides (HDPs) are naturally occurring molecules participating in the innate immune response in almost all vertebrates. The cathelicidins family of HDPs is the one characterized by a conserved "cathelin" domain with high interspecies homology (17). These molecules are generally 10 to 50 amino acids long and positively charged with amphipathic properties. These features enhance peptides interactions with negatively charged membranes of both bacterial and host cells. Thereby, HDPs could control bacterial infections via two routes: direct antimicrobial activity and regulation of immune response (18). Moreover, use of HDPs as potential alternative to antibiotics gained interest thanks to their very low microbial resistance development (19).

Among the numerous natural and synthetic HDPs described in the literature, we narrowed down the selection to three well-known cathelicidins: human LL-37, chicken CATH-2, and bovine-derived IDR-1018. These peptides retain broad-spectrum antibacterial activity by direct killing mechanisms, like CATH-2 (20), or by anti-biofilm and indirect bactericidal properties, as IDR-1018 and LL-37 (21–24). Several studies reported these peptides immunomodulatory functions as well. In fact, all three peptides modulate immune cells cytokines production by stimulating chemokine release and inhibiting LPS-mediated activation (20,25–28). LL-37 promotes internalization and intracellular killing of pathogens via an increase in ROS production, both in neutrophils (29) and macrophages (30–32). IDR-1018 contributes

to neutrophils activation and production of HDPs, including LL-37 (33). Stimulation with IDR-1018 drives macrophages phenotype to an intermediate state, enhancing both pro-inflammatory stimuli against pathogens and pro-healing properties (34). Moreover, *in vivo* wound healing improvement have been reported for both IDR-1018 and LL-37 (35,36).

Nevertheless, HDPs efficacy against bacterial infection turned out to be controversial among different research groups. Particularly, changes in testing conditions might yield different effects from the same peptide. For instance, peptides immunomodulatory properties have been described mainly on non-human cell lines, which can hide possible species- or cell-specific effects (20). At the same time, HDPs antibacterial properties have been mainly monitored in non-physiological conditions which are different compared to the *in vivo* scenarios (19,20,37). For this reason, we aimed to characterize and compare IDR-1018, CATH-2, and LL-37 immunomodulatory and antibacterial properties under the same conditions *in vitro*. Furthermore, we tested the ability of single peptides to control *S. aureus* infection either by direct killing or modulating primary human macrophage functions, with particular focus on pathogens intracellular survival.

Materials and Methods

Human monocyte-derived macrophages culture

Blood from healthy human donors was supplied by the Dutch blood bank (Sanquin, Amsterdam, The Netherlands). Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats using Ficoll-Paque density centrifugation. Monocytes were positively selected by magnetic-activated cell sorting (MACS) with anti-CD14 labelled microbeads according to manufacturer instructions.

Isolated monocytes were seeded in a 24-well plate at a density of 300,000 cells/well, except where otherwise stated. Monocytes were differentiated to macrophages by culture for 7 days in α-Minimum Essential Medium (α-MEM) supplemented with 10% (v/v) hyclone fetal bovine serum (hyFBS), 100 U/mL penicillin- streptomycin (1% p/s), and 40 ng/mL human recombinant M-CSF. Culture media was refreshed after 3-4 days.

Viability of the isolated cells was above 75% as determined by Sytox Orange dead cell stain for flow cytometry, before and after differentiation. Purity of the isolated monocytes was above 90% as checked by staining for CD14 and contamination by T-cells (CD3), B-cells (CD19), or granulocytes (CD15) (Supplementary Figure 1). Cells staining was performed as described in “Peptides influence on macrophage phenotype markers” section, having all fluorochrome-conjugated antibodies diluted 1:30 in PBS with 0,1% BSA and 1 % (v/v) heat-inactivated human serum.

Human mesenchymal stem cells culture

Mesenchymal stem cells (MSCs) were isolated from human bone marrow

aspirates that were obtained from consenting patients. Aspiration procedure was approved by the local medical research ethics committee, University Medical Center Utrecht, under the protocols METC 08-001/K and METC 07-125/C.

Aspirates were diluted in PBS, filtered through a 100 µm cell strainer and the mononuclear cell layer was collected after Ficoll-Paque density centrifugation. Approximately 250,000 mononuclear cells were plated per cm² in MSCs expansion medium consisting of α-MEM supplemented with 10% (v/v) heat-inactivated FBS (FBS), 1% p/s, 0,2 mM L-ascorbic acid-2-phosphate (ASAP). Cells starting from passage 3 were used in the experimental setups.

Before culturing together with macrophages, MSCs were fluorescently labelled with CellTrace Violet diluted in Hanks Balanced Salt Solution (HBSS) according to the manufacturer's instructions for labelling adherent cells. After staining, MSCs were detached with trypsin/EDTA 0,25% and re-seeded at a density of 100,000 cells/well according to experimental setup.

Bacterial culture

All experiments used GFP-labelled *Staphylococcus aureus* strain SH1000, transformed with a GFP-expressing plasmid pCM29 to constitutively express GFP, as previously described (38). Bacteria were grown overnight in Todd-Hewitt broth (THB) with 10 µg/mL chloramphenicol to reach stationary phase.

Peptides

IDR-1018 (sequence VRLIVAVRIWRR-NH2), CATH-2 (sequence RFGRFLRKIRRFRPKVTITIQGSARF-NH2), and LL-37 (sequence LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) purity (>95%) was verified by the manufacturers via MS and HPLC.

Peptides were diluted at different concentrations in α-MEM supplemented with 10% FBS or in THB when testing their effects on macrophages or bacteria, respectively.

Peptides direct antibacterial properties

Peptides direct antibacterial properties were determined by broth micro-dilution method. Overnight bacterial suspension was diluted in THB to reach a final inoculum concentration of 5×10^5 colony-forming units per mL (CFU/mL). Bacterial suspension and peptides dilutions were mixed in equal parts in a flat-bottom 96-well plate in triplicates in a total volume of 200 µL and incubated at 37°C. Bacterial growth was monitored by measuring OD (600nm) continuously every 5 minutes for 12 h in Clariostar plate reader (BMG labtech) with gentle shaking before each measurement.

Peptides anti-inflammatory properties and cytotoxicity

Monocytes were seeded in a 96-well plate at a density of 150,000 cells/well. After 7 days differentiation, macrophages were incubated with fresh media containing a range of peptides concentrations alone or in combination with 10 ng/

mL LPS O111:B4 from *Escherichia coli*. After 24 h stimulation, the supernatant was collected to measure LDH levels or TNF-α and IL-10 by ELISA, according to the manufacturer's instructions. Both LDH and ELISA assays were performed in three independent experiments with measurements in triplicate.

Qualitative expression of various cytokines and chemokines was measured in duplicate for selected conditions by human cytokine array G5, according to the manufacturer's instructions.

Peptides influence on macrophage phenotype markers

After 7 days differentiation, macrophages were incubated with fresh media with LPS, IL-4, and optimal concentrations of peptides alone or in combination with LPS. After 24 h stimulation, macrophages were detached from the culture plate and processed for staining into a 96-well plate. All washing steps were performed with cold 0,1% (w/v) bovine serum albumin (BSA)/PBS and centrifugation at 5 min, 500 x g. A panel of surface molecules was selected, based on previous reports for human macrophage polarization (39). The staining solutions were prepared by diluting the following fluorochrome-conjugated antibodies in PBS with 0,1% BSA and 1 % (v/v) heat-inactivated human serum: CD16 (1:50); CD80 (1:50); CD163 (1:50). As a negative control, staining solution without antibodies was used. Cells were incubated with staining solutions for 30 min on ice, in the dark. Markers expression was measured via flow cytometer (FACSVerse, BD) and data analyzed using FlowJo.

Peptides indirect antibacterial properties

To assess the peptides influence on macrophages antibacterial properties, three different infection models studying intracellular bacteria survival, as outlined in **Figures 3A**, **5A**, and **5D**, were adopted. In all models, selected concentrations of peptides were used: 65 μM IDR-1018; 10 μM CATH-2; 10 μM LL-37.

The first step concerned the "peptides during infection" model, where cells were washed twice before incubation with the peptides for 24 h and subsequently bacteria were directly added to the culture without any washing step. In a subsequent step we assessed the "peptides during differentiation", where peptides were added to the differentiation media for the first 3 days and removed after the regular media change; cells were washed twice before infection. In the third step we tested "peptides with MSCs", where macrophages were cultured in presence of MSCs and peptides for 24 h; before infection cells were washed twice. All washing steps, before or after infection, were performed using warm α-MEM.

In all three models described, the same *S. aureus* infection protocol was applied. An overnight bacterial culture was diluted in α-MEM to reach a final inoculum concentration of 1 x 107 CFU/mL and opsonized in 5% human pooled serum (HPS) for 15 min at 37°C. Opsonized *S. aureus* was added to the culture at a multiplicity of infection (MOI) = 1, meaning 1 bacterium per eukaryotic cell. To synchronize bacterial uptake, plates were centrifuged for 5 min, 110 x g at RT, and then moved to the incubator at 37°C, 5% CO2 for additional 25 min or 24 h. To study

intracellular bacterial survival, after 30 min the cells were washed twice and cultured in media supplemented with 100 µg/mL gentamicin and 20 µg/mL lysostaphin for 1 h. Afterwards, cells were washed twice and incubated in media with only 5 µg/mL gentamicin. This treatment allows only intracellular bacteria to survive, as both gentamicin and lysostaphin are unable to penetrate mammalian cell membranes within short time periods (40,41).

Samples were analyzed by flow cytometry and CFU counting at 30 min and 24 h after infection. Cells for flow cytometry were detached from the culture plate using 1mM DPBS/EDTA in combination with gentle scraping. When macrophages were cultured together with MSCs, samples were first trypsinzed and then scraped in DPBS/EDTA if cells were still attached to the bottom of the culture plate. Cells were moved to a 96-well plate and fixed in paraformaldehyde 4% before analysis. Samples were measured with MACSquant VYB (Miltenyi Biotech) flow cytometer and data analyzed with FlowJo. Gating strategy is summarized in Supplementary Figure 2. Briefly, a total of 10,000 events were collected for each sample gated on the macrophage population based on forward scatter (FSC) and side scatter (SSC) parameters. When cultured with fluorescently labelled MSCs, the macrophage population was further selected based on the signal of CellTrace Violet. Non-infected samples were used to set GFP fluorescence baseline and define the proportion of infected, GFP-positive cells.

To quantify the numbers of intracellular bacteria, cells were lysed with Triton X 0,1% and then plated on Todd-Hewitt agar plates in serial dilutions. Plates were incubated overnight at 37 °C after which colonies were counted.

Confocal images

According to the “peptides during infection” model, after 30 min infection cells were harvested and fixed as described for flow cytometry analysis. Cells membranes were stained with 3 µg/mL Alexa Fluor 647-labelled Wheat Germ Agglutinin (WGA) for 10 min at RT, on a shaking plate. Then, samples were transferred to CELLview slide previously coated with poly-L-lysine, and imaged on a Leica TCS SP5 microscope with a HCX PL AP CS 63x/1.40-0.60 OIL objective (Leica Microsystems). For each condition, the number of intracellular bacteria was counted in 50 randomly chosen cells. Images were adjusted for publication using Image J Fiji.

Quantification and statistical analysis

GraphPad Prism 9 was used to create the graphs and determine statistical significance via one-way ANOVA.

Results

Only CATH-2 had direct antibacterial properties

To evaluate the antibacterial properties of each peptide, we continuously monitored growth of *S. aureus* in the presence of different peptide concentrations over a period of 12 h. **Figure 1A** shows that only CATH-2 had direct antibacterial properties. Interestingly, CATH-2 arrested *S. aureus* growth at concentrations 10-times lower than those needed to inhibit macrophage LPS-mediated activation. Both IDR-1018 and LL-37 did not show antibacterial effects at any concentration tested (**Figures 1B** and **1C**). However, IDR-1018 was able to delay the start of *S. aureus* exponential growth only at concentrations starting at 65 μM.

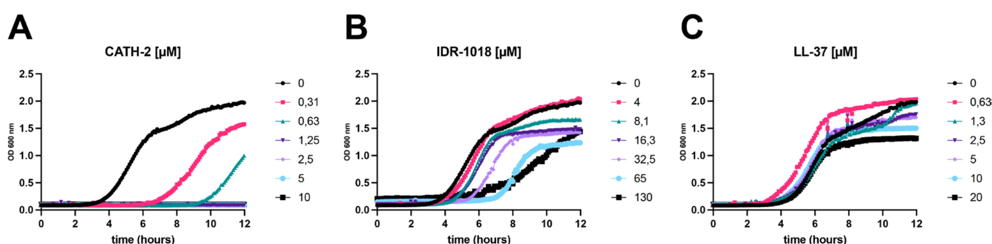


Figure 1. Only CATH-2 had direct antibacterial properties. *S. aureus* growth was monitored by measuring OD (600 nm) continuously during 12 h. Bacteria were incubated with a concentration range (given in μM) of peptides of CATH-2 (**A**), IDR-1018 (**B**), and LL-37 (**C**). (n=3). Data were plotted with mean only.

IDR-1018, CATH-2, and LL-37 inhibited macrophage LPS-mediated activation

To evaluate the potential anti-inflammatory action of each peptide, we measured the release of TNF-α and IL-10 after LPS stimulation of primary human macrophages. First, we tested a non-toxic concentration range of each peptide with LPS stimulated cells. All three peptides efficiently decreased TNF-α and IL-10 release in a dose-dependent manner (**Supplementary Figure 3A-F**). Subsequently, the optimal concentration of each peptide was tested for both LPS stimulated and non-stimulated cells. As shown in **Figure 2**, all three peptides inhibited the LPS-induced release of IL-10 (**Figure 2A**) and TNF-α (**Figure 2B**) significantly.

At the same time, peptides alone did not trigger a pro-inflammatory response (**Figures 2C and 2D**). Also, none of these conditions affected the viability of the macrophages as determined by LDH release (**Supplementary Figure 3G**). This anti-inflammatory effect of the peptides did not correlate with a clear polarization of macrophages towards a M1 or M2 phenotype, defined by CD80 and CD163 expression, respectively (**Supplementary Figure S4**).

To further characterize the potential anti-inflammatory profile of each peptide, expression levels of several cytokines, chemokines, and growth factors were measured after 24 h stimulation with LPS. Overall, it was found that all three peptides

altered LPS-induced expression of several other factors, besides TNF-α and IL-10 (**Figure 2E**). Moreover, all the peptides limited the over-activation and recruitment of immune cells to the inflammation site by reducing the expression of macrophage inflammatory protein 1β (MIP-1β), RANTES, and monocyte chemoattractant proteins (MCP-1,2,3).

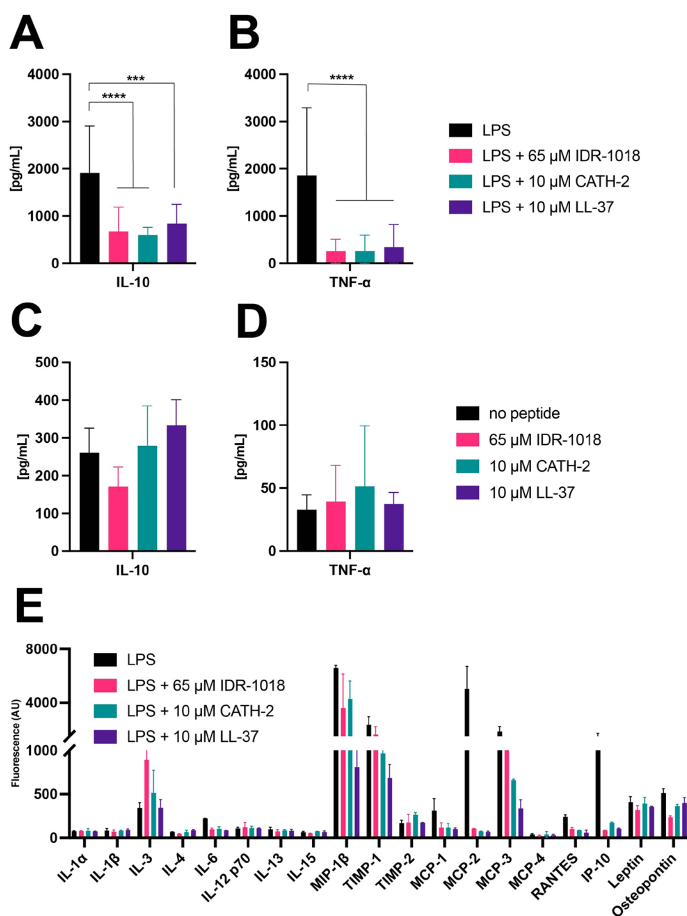


Figure 2. All peptides inhibited macrophage LPS-mediated activation. Macrophages were incubated for 24 h with LPS and peptides (**A**, **B**), or peptides alone (**C**, **D**) and TNF-α and IL-10 levels were quantified by ELISA. (n=9, from a total of 3 independent experiments). (**E**) Qualitative expression of several other cytokines was determined by cytokine array kit after 24 h stimulation with LPS and peptides. Fluorescence values are expressed as arbitrary units (AU). (n=2). Data were represented as +/- SD. One-way ANOVA was used to determine statistical significance. ****p<0,0001, ***p=0,0002.

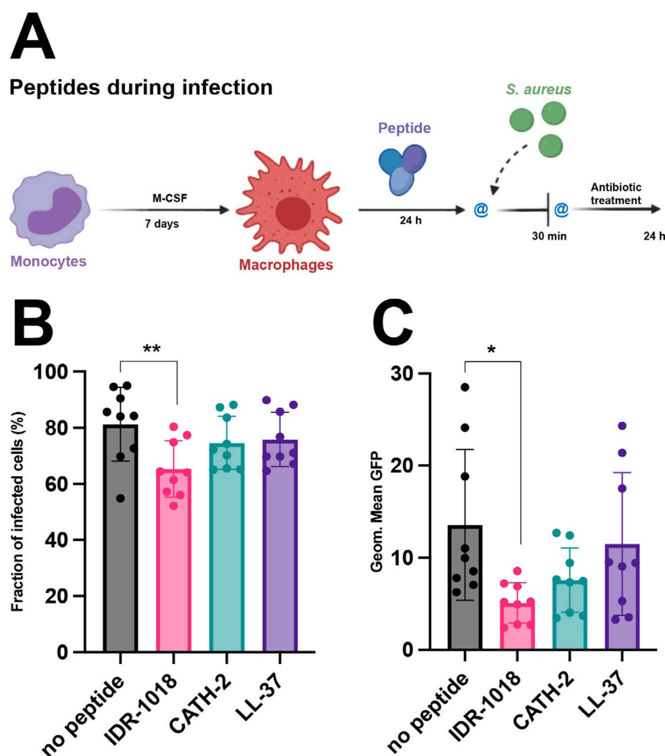


Figure 3. When used during infection, IDR-1018 reduced the number of bacteria phagocytosed by macrophages. Macrophages were first stimulated with the peptides and then infected with *S. aureus* as outlined in the "peptides during infection" model (**A**) (Created with BioRender.com) where @ represents 2 washing steps. Results from the 30 min time point are shown. The percentage of macrophages that had taken up at least 1 bacterium is depicted as the fraction of infected cells (**B**). The bacterial load is represented as the geometric mean of the GFP signal (**C**). (n=9, from a total of 3 independent experiments). Data were represented as +/- SD. One-way ANOVA was used to determine statistical significance. **p<0,01, *p<0,02.

Use of different infection models to study HDPs contribution to bacteria phagocytosis

Once the anti-inflammatory and direct antibacterial profile for each peptide was defined, we aimed to explore HDPs ability to influence macrophage antibacterial functions via different infection models focusing on bacteria surviving intracellularly. Based on the anti-inflammatory effect of each peptide (**Figure 2**), macrophages were stimulated with 65 μM IDR-1018, 10 μM CATH-2, or 10 μM LL-37 before infecting them. In all infection models, the same time points after infection were selected: 30 min to determine *S. aureus* uptake by immune cells and 24 h to evaluate macrophage bactericidal activity against intracellular bacteria.

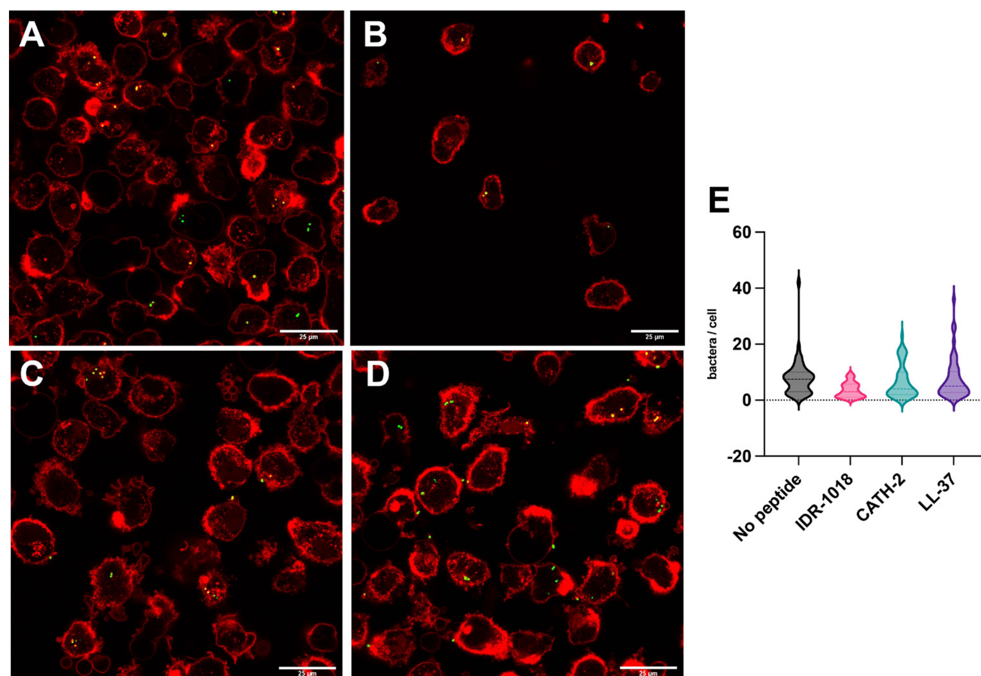


Figure 4. Confocal images confirmed the flow cytometry observations. Macrophages were incubated without peptide (**A**), with IDR-1018 (**B**), CATH-2 (**C**), or LL-37 (**D**). After 30 min infection with *S. aureus* (green dots), cells were collected, and their membranes stained with Alexa Fluor 647-labelled WGA (in red) for confocal imaging. The number of intracellular bacteria in each macrophage was manually counted from 50 randomly chosen cells (E). Data were represented with violin plots, lines at mean.

As a first approach, macrophages were treated for 24 h with peptides and subsequently infected by *S. aureus* while peptides were kept in the culture media, hence named "peptides during infection" model (**Figure 3A**). Given the proportion of macrophages that phagocytosed at least 1 bacterial cell, IDR-1018 was the only peptide that significantly reduced the number of infected cells after 30 min (**Figure 3B**). In addition, according to the geometric mean of GFP signal intensity that verifies the number of intracellular bacteria, only IDR-1018 was able to markedly decrease phagocytosis, while CATH-2 slightly reduced the bacterial load in macrophages (**Figure 3C**). After 24 h, a similar trend was observed with IDR-1018 as the only peptide that reduced the amount of phagocytosed *S. aureus* (**Supplementary Figure 5**).

Confocal microscopy imaging also confirmed the flow cytometry results. Particularly, manual counting of intracellular bacteria after 30 min infection showed

that IDR-1018 and CATH-2 reduced the bacterial uptake by macrophages, while LL-37 did not provide such effect (**Figure 4**).

Pena and colleagues observed that monocyte differentiation to macrophage in the presence of host defense peptides influenced the mature cell functions (34). Here, this aspect was evaluated in the "peptides during differentiation" model (**Figure 5A**), and the differentiated macrophages were subsequently infected with *S. aureus*. According to **Figures 5B** and **5C**, IDR-1018 significantly reduced both the proportion of infected macrophages and the number of internalized bacteria after 30 min infection. In contrast with the previous "peptides during infection" model, CATH-

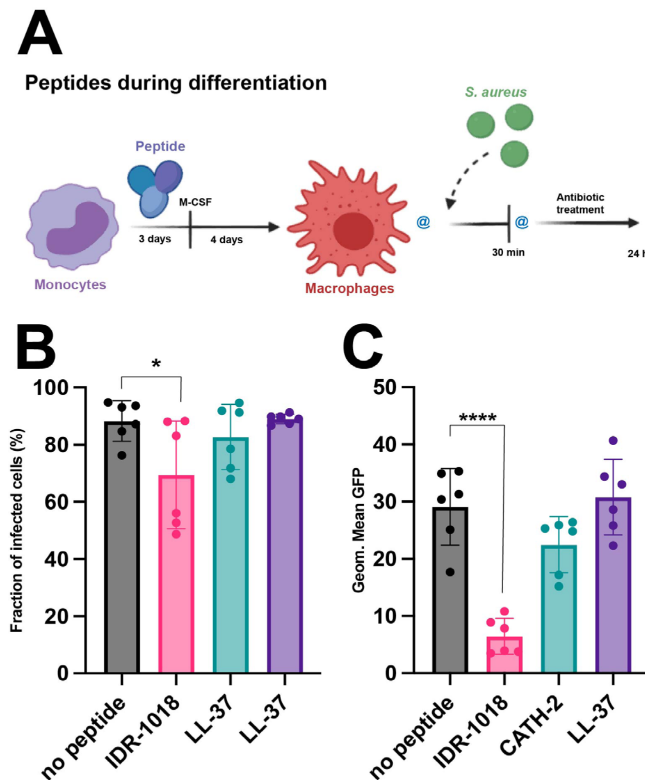


Figure 5. When the peptides were introduced during monocyte differentiation to macrophages, IDR-1018 reduced the number of bacteria phagocytosed by macrophages. Before infection, macrophages were differentiated in the presence of each peptide as outlined in the "peptides during differentiation" infection model (***A***) (Created with BioRender.com) where @ represents 2 washing steps. Results from the 30 min time point showed the fraction of infected cells (***B***) and geometric mean of GFP signal (***C***). (n=6, from a total of 2 independent experiments). Data were represented as +/- SD. One-way ANOVA was used to determine statistical significance. *p<0,03, ****p<0,0001.

2 lost its contribution to phagocytosis when it was introduced during the monocyte differentiation.

In addition to MSCs endogenous production of HDPs (*i.e.,* LL-37), there are multiple examples of MSCs immunomodulatory and antibacterial properties (42–44). Therefore, a combination of different immunomodulatory stimuli was simulated in the "peptides with MSCs" model (**Figure 6A**), where macrophages were incubated together with MSCs and peptides before infection with *S. aureus*. However, in this scenario, the contribution of the peptides to macrophage phagocytosis was almost nullified, as reported in **Figures 6B** and **6C**.

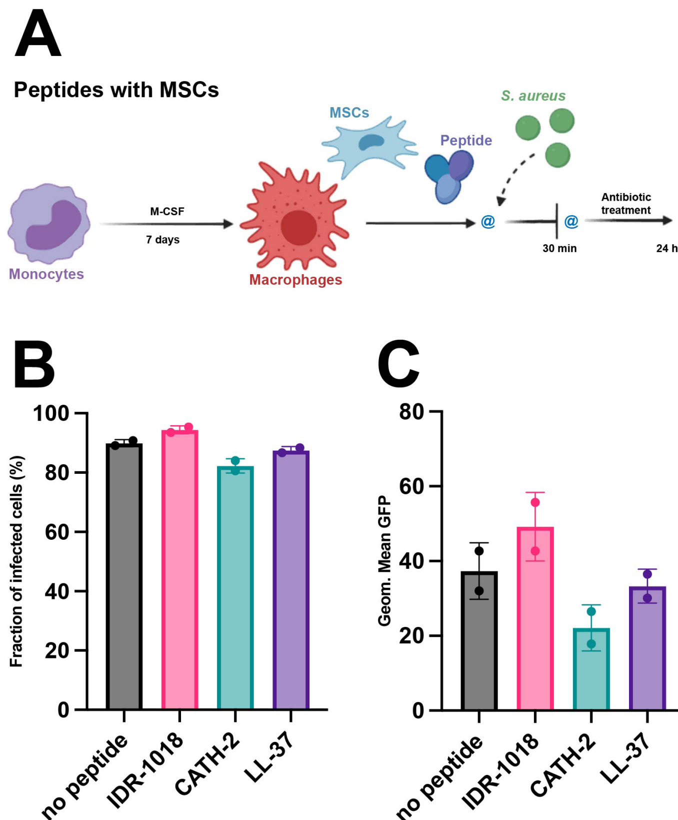


Figure 6. When used in combination with MSCs, none of the peptides influence macrophages phagocytosis. Macrophages were incubated with MSCs and peptides before infection, as schematized in the "peptides with MSCs" infection model (***A***) (Created with BioRender.com) where @ represents 2 washing steps. Results from the 30 min time point showed the fraction of infected cells (***B***) and geometric mean of GFP signal (***C***). (n=2). Data were represented as +/- SD.

Use of different infection models to study HDPs contribution to intracellular bacteria killing

Furthermore, macrophage bactericidal activity after stimulation with the peptides was investigated. In fact, cells were lysed to quantify the number of viable intracellular bacteria after 30 min and 24 h. To be able to study the intracellular killing capacity of macrophages, all extracellular bacteria and peptides were removed by wash steps and treatment with gentamicin and lysostaphin after 30 min.

Regardless of the infection model used, non-stimulated macrophages showed intrinsic ability to kill bacteria surviving intracellularly (**Figure 7**). In the "peptides during infection" model, CATH-2 significantly reduced the numbers of *S. aureus* surviving intracellularly only after 30 min, while the reduction in CFU after 24 h was not statistically significant. However, no effects were observed for IDR-1018 and LL-37 (**Figure 7A**).

No change in macrophage killing properties was observed for all the peptides in both "peptides during differentiation" (**Figure 7B**) and "peptides with MSCs" (**Figure 7C**) infection models. On the contrary, in both models macrophage stimulation with peptides showed an increased amount of *S. aureus* surviving intracellularly compared to controls after 24 h.

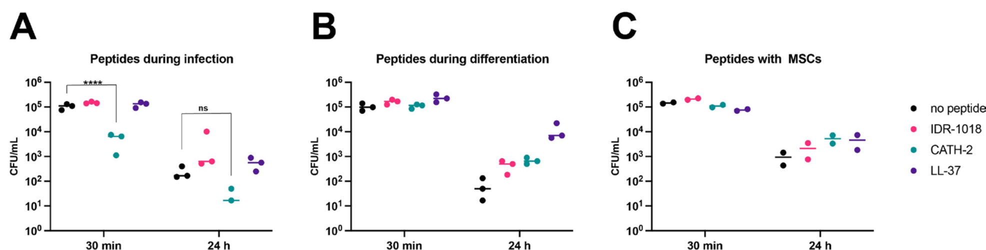


Figure 7. Regardless of the infection models used, none of the peptides reduced the number of bacteria surviving intracellularly. Macrophages were stimulated with peptides and infected according to the previously described models. After 30 min and 24 h, cells were lysed and bacteria enumerated by CFU counting for each infection model. Representative data from peptides during infection (**A**) (n=9, from a total of 3 independent experiments), peptides during differentiation (**B**) (n=6, from a total of 2 independent experiments), and peptides with MSCs (**C**) (n=2). Data were plotted with mean only. One-way ANOVA was used to determine statistical significance. ****p<0,0001.

Discussion

HDPs received attention as a promising alternative to antibiotics against bacterial infection thanks to their dual functionalities in controlling infection while modulating immune cells functions. It is foreseen that these peptides could be potentially implemented into orthopedic implants coatings to prevent implant-associated infections (IAI). So far, several HDPs with immunomodulatory and antibacterial properties have been described in the literature. However, lack of standardized methods to study their functionalities impedes the use of peptides for the next translational steps. Here, we aimed to directly compare the immunomodulatory and antibacterial functions of IDR-1018, CATH-2, and LL-37 through the same *in vitro* conditions.

Direct antibacterial effect

Although the mechanisms of antibacterial properties for several HDPs have been described (19,45–48), little is known on peptides interactions with Gram-positive bacteria, particularly with *S. aureus*. Schneider *et al.*, showed that CATH-2 bound *S. aureus* membrane through ionic interactions, causing membrane ruffling and intracellular morphological changes (49). Similar cell shrinking and membrane permeabilization effects, but against a different Gram-positive bacterium, have been described for LL-37 (50). On the other hand, the molecular mechanisms leading to the immunomodulatory effects of HDPs are more complex and not properly verified yet (19,51,52).

In this study, only CATH-2 showed direct killing effect against *S. aureus* (**Figure 1**). According to different studies that provided examples of both IDR-1018 (27,47) and LL-37 (53,54) bactericidal action, yet the culture conditions highly influenced HDPs direct antibacterial activity (19,20,37). It should be noted that in all these studies, the peptides minimum inhibitory concentration (MIC) was reported differently. This clearly stemmed from the variation in the experimental setup and bacterial strain tested. In addition, Durr *et al.* showed that bacterial killing and immune cells cytotoxicity of LL-37 were rendered at the same concentrations which undermined its broad-spectrum antimicrobial properties (53).

Anti-inflammatory effect

All the peptides showed a similar inhibitory action against LPS-mediated activation of macrophages, although higher concentrations of IDR-1018 were required as compared to CATH-2 and LL-37 (**Figures 2A** and **2B**). In addition to TNF-α and IL-10, the peptides affected the expression of several other cytokines involved into immune cells activation and recruitment at the inflammation site (**Figure 2E**). To illustrate, expression of several pro-inflammatory cytokines, such as MIP-1β, RANTES, and MCP-1,2,3 was reduced, confirming a stronger inhibition of LPS activation of macrophages by LL-37 (46). Furthermore, IL-3 expression, a basophil growth factor also involved in infection-induced response of immune cells

(55), was up-regulated by all peptides stimulation. In conclusion, this suggests that the selected peptides from one side dampen excessive inflammatory stimuli, while in parallel provide immune cells tools to resolve inflammation. A similar concept was pointed out by Pena *et al.* where IDR-1018 stimulation did not correlate to polarization of macrophages towards a clear M1 or M2 phenotype (34). Similarly, we observed that also CATH-2 and LL-37 kept macrophages into an intermediate state, between a pro- and anti-inflammatory phenotype (**Supplementary Figure 4**).

Indirect antibacterial effect

S. aureus finds protection against most antibiotics and host defenses by hiding inside the host cells. At the same time, infected cells circulating in the bloodstream are used as a “Trojan horse” by the pathogens to spread throughout the body (56). Direct targeting of intracellular bacteria still remains a challenge (57,58), therefore we used HDPs to improve macrophage intrinsic ability to kill intracellular bacteria.

When immune cells were stimulated with the peptides right before and during infection, only IDR-1018 reduced the proportion of cells that phagocytosed bacteria (**Figure 3B** and **3C**). Nevertheless, this did not correlate to a lower number of pathogens surviving within macrophages after 24 h (**Figure 7A**). Furthermore, CATH-2 only decreased the amount of intracellular *S. aureus* after 30 min, but not significantly after 24 h. Additionally, since bacteria and peptides were incubated together, it is more likely that decrease in the intracellular bacteria was caused by the direct killing effect of CATH-2 (**Figure 1A**). This could also explain the flow cytometry results (**Figure 3C**) which showed a decrease in the number of bacteria taken up by macrophages. Besides, the bactericidal capacity of CATH-2-stimulated immune cells was comparable to the non-stimulated group after 24 h (**Figure 7A**), which reflected no changes in the macrophages killing functions.

In line with the trained immunity theory introduced by Netea and colleagues (59), we studied the effect of peptides during monocyte differentiation. In this context, IDR-1018 immunomodulatory function was preserved by mature macrophages, which led to a reduced proportion of infected cells (**Figures 5B** and **5C**). However, even in this case, IDR-1018 had no impact on immune cells killing efficiency (**Figure 7B**). Similarly, monocytes differentiation in presence of CATH-2 and LL-37 had no impact on mature cells functions, neither on phagocytosis or intracellular killing.

In our multicellular *in vitro* model, consisting of macrophages cultured together with MSCs, we could not observe any indirect antibacterial effect derived from peptides stimulation. No difference among groups was observed both in terms of proportion of infected cells (**Figures 6B** and **6C**) and *S. aureus* intracellular survival (**Figure 7C**). Despite the multiple advantages described by direct or indirect culture of macrophages and MSCs (42–44,60), one might speculate that MSCs interferes with peptides immunomodulatory effects, yet further studies are needed to validate this hypothesis.

As we aim to find valuable therapeutic agents in human treatment, we selected primary immune cells as benchmark for our experiments. Therefore, we could

not verify previous studies that showed LL-37 promoting clearance of intracellular *S. aureus* in a macrophage human cell line (32) and bacterial phagocytosis in a macrophage murine cell line (30). This discrepancy might be ascribed either to the cell-type specificity of peptides, or differences in behavior between primary cells and cell lines from different species (61–64). On the other hand, bactericidal properties of CATH-2 on the human macrophages, to our knowledge, has not been explored yet.

Together with macrophages, neutrophils play a central role into the first response against invading pathogens. However, neutrophils were not used in this study as their shorter life span make them a less favorable candidate for *S. aureus* intracellular survival compared to macrophages (56,65). Nonetheless, it has been shown that both IDR-1018 and LL-37 improved neutrophils antibacterial functions. For instance, while IDR-1018 enhanced killing of intracellular E. coli (33), LL-37 improved neutrophils ROS production and *S. aureus* uptake (66). However, they have not studied a correlation between the higher ROS produced and intracellular killing.

Future outlook

Use of HDPs as one of the most promising alternatives to the antibiotics has been receiving many attentions recently thanks to their influence on both host and bacterial cells, as well as lower risk in developing bacterial resistance (67–69). Here, the anti-inflammatory and antibacterial properties of three HDPs were studied and reported, however their efficacy could be potentially improved. Particularly, they could be used in combinatorial or synergistic strategies with each other or other conventional antibacterial agents. For instance, combination of HDPs with antibiotics (70,71), other HDPs (72), or other immune cell components (73) already showed improved antibacterial efficiency compared to when the components were used alone. Alternatively, modification to HDPs sequence also showed improved immunomodulatory and antibacterial effects (74–77). On the other hand, loading HDPs into antibacterial coatings should be considered as new local drug delivery strategy to prevent IAI (16,78).

Conclusion

In this work the immunomodulatory and antibacterial properties of IDR-1018, CATH-2, and LL-37 peptides were studied on the same *in vitro* conditions. Although the strong anti-inflammatory properties of all peptides were verified, they did not improve macrophages antibacterial functions. In fact, only CATH-2 showed promising direct antibacterial properties against *S. aureus*. Furthermore, it was shown that IDR-1018 influenced macrophages phagocytosis ability by reducing the number of engulfed bacteria. However, none of the tested peptides enhanced macrophage’s ability to kill intracellular *S. aureus*.

Limitations of the study

One may argue that the inhibition of LPS-mediated activation of macrophages was not caused by peptides direct interaction with the immune cells. It was shown via a mechanism named “silent killing” that CATH-2 and LL-37 bind LPS to inhibit macrophages receptors activation (79). On the contrary, IDR-1018 did not showed significant binding affinity to LPS (47).

As peptides decrease LPS-induced cytokines production in a dose-dependent manner, one should investigate the antibacterial properties of macrophages similarly. Any changes in the infection models, such as MOI, time points, presence of peptides during infection, etc., may alter the outcomes as well. At the same time, use of different monocytes isolation techniques and differentiating factors may influence phenotype and functions of mature macrophages (39,80).

Acknowledgements

We thank Lisanne de Vor for assistance with acquiring images by confocal microscopy, and Dr. Michiel Croes for the meaningful discussions and data interpretations on the first stages of the research.

The collaboration project is cofounded by the PPP Allowance made available by Health~Holland, Top Sector Life Sciences & Health, to stimulate public-private partnerships. This research also received co-funding from the EU’s H2020 research and innovation programme under Marie S. Curie cofund RESCUE grant agreement No 801540. This publication is part of the project DARTBAC (with project number NWA.1292.19.354) of the research programme NWA-ORC which is (partly) financed by the Dutch Research Council (NWO).

References

1. Arciola CR, Campoccia D, Montanaro L. Implant infections: Adhesion, biofilm formation and immune evasion. Nat Rev Microbiol (2018) 16:397–409. doi: 10.1038/s41579-018-0019-y
2. Moriarty TF, Kuehl R, Coenye T, Metsemakers WJ, Morgenstern M, Schwarz EM, Riool M, Zaat SAJ, Khana N, Kates SL, *et al.* Orthopaedic device-related infection: Current and future interventions for improved prevention and treatment. EFORT Open Rev (2016) 1:89–99. doi: 10.1302/2058-5241.1.000037
3. Campoccia D, Montanaro L, Arciola CR. The significance of infection related to orthopedic devices and issues of antibiotic resistance. Biomaterials (2006) 27:2331–2339. doi: 10.1016/j.biomaterials.2005.11.044
4. de Jong NWM, van Kessel KPM, van Strijp JAG. Immune Evasion by *Staphylococcus aureus*. Microbiol Spectr (2019) 7:1–27. doi: 10.1128/microbiolspec.gpp3-0061-2019
5. Flannagan RS, Heit B, Heinrichs DE. Intracellular replication of *Staphylococcus aureus* in mature phagolysosomes in macrophages precedes host cell death, and bacterial escape and dissemination. Cell Microbiol (2016) 18:514–535. doi: 10.1111/cmi.12527
6. Horn J, Stelzner K, Rudel T, Fraunholz M. Inside job: *Staphylococcus aureus* host-pathogen interactions. International Journal of Medical Microbiology (2018) 308:607–624. doi: 10.1016/j.ijmm.2017.11.009
7. Fraunholz M, Sinha B. Intracellular *Staphylococcus aureus*: live-in and let die. Front Cell Infect Microbiol (2012) 2:43. doi: 10.3389/fcimb.2012.00043
8. Strobel M, Pförtner H, Tuchscherr L, Völker U, Schmidt F, Kramko N, Schnittler HJ, Fraunholz MJ, Löffler B, Peters G, *et al.* Post-invasion events after infection with *Staphylococcus aureus* are strongly dependent on both the host cell type and the infecting *S. aureus* strain. Clinical Microbiology and Infection (2016) 22:799–809. doi: 10.1016/j.cmi.2016.06.020
9. de Mesy Bentley KL, Trombetta R, Nishitani K, Bello-Irizarry SN, Ninomiya M, Zhang L, Chung HL, McGrath JL, Daiss JL, Awad HA, *et al.* Evidence of Staphylococcus Aureus Deformation, Proliferation, and Migration in Canaliculi of Live Cortical Bone in Murine Models of Osteomyelitis. Journal of Bone and Mineral Research (2017) 32:985–990. doi: 10.1002/jbmr.3055
10. Muthukrishnan G, Masters EA, Daiss JL, Schwarz EM. Mechanisms of Immune Evasion and Bone Tissue Colonization That Make *Staphylococcus aureus* the Primary Pathogen in Osteomyelitis. Curr Osteoporos Rep (2019) 17:395–404. doi: 10.1007/s11914-019-00548-4
11. Souza JGS, Bertolini MM, Costa RC, Nagay BE, Dongari-Bagtzoglou A, Barão VAR. Targeting implant-associated infections: titanium surface loaded with antimicrobial. iScience (2021) 24: doi: 10.1016/j.isci.2020.102008
12. Wassif RK, Elkayal M, Shamma RN, Elkheshen SA. Recent advances in the local antibiotics delivery systems for management of osteomyelitis. Drug Deliv (2021) 28:2392–2414. doi: 10.1080/10717544.2021.1998246
13. Amin Yavari S, Castenmiller SM, van Strijp JAG, Croes M. Combating Implant Infections: Shifting Focus from Bacteria to Host. Advanced Materials (2020) 32:1–25. doi: 10.1002/adma.202002962
14. Kamaruzzaman NF, Kendall S, Good L. Targeting the hard to reach: challenges and novel strategies in the treatment of intracellular bacterial infections. Br J Pharmacol (2017) 174:2225–2236. doi: 10.1111/bph.13664

15. Abed N, Couvreur P. Nanocarriers for antibiotics: A promising solution to treat intracellular bacterial infections. Int J Antimicrob Agents (2014) 43:485–496. doi: 10.1016/j.ijantimicag.2014.02.009
16. Amin Yavari S, Castenmiller SM, van Strijp JAG, Croes M. Combating Implant Infections: Shifting Focus from Bacteria to Host. Advanced Materials (2020) 32:1–25. doi: 10.1002/adma.202002962
17. Kościuczuk EM, Lisowski P, Jarczak J, Strzałkowska N, Jóźwik A, Horbańczuk J, Krzyżewski J, Zwierzchowski L, Bagnicka E. Cathelicidins: family of antimicrobial peptides. A review. Mol Biol Rep (2012) 39:10957–10970. doi: 10.1007/s11033-012-1997-x
18. Hancock REW, Sahl HG. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nat Biotechnol (2006) 24:1551–1557. doi: 10.1038/nbt1267
19. Mookherjee N, Anderson MA, Haagsman HP, Davidson DJ. Antimicrobial host defence peptides: functions and clinical potential. Nat Rev Drug Discov (2020) 19:311–332. doi: 10.1038/s41573-019-0058-8
20. Coorens M, Scheenstra MR, Veldhuizen EJA, Haagsman HP. Interspecies cathelicidin comparison reveals divergence in antimicrobial activity, TLR modulation, chemokine induction and regulation of phagocytosis. Sci Rep (2017) 7:1–11. doi: 10.1038/srep40874
21. Reffuveille F, De La Fuente-Nűnez C, Mansour S, Hancock REW. A broad-spectrum antibiofilm peptide enhances antibiotic action against bacterial biofilms. Antimicrob Agents Chemother (2014) 58:5363–5371. doi: 10.1128/AAC.03163-14
22. Kang J, Dietz MJ, Li B. Antimicrobial peptide LL-37 is bactericidal against *Staphylococcus aureus* biofilms. PLoS One (2019) 14:1–13. doi: 10.1371/journal.pone.0216676
23. Biswas D, Ambalavanan P, Ravins M, Anand A, Sharma A, Lim KXZ, Tan RYM, Lim HY, Sol A, Bachrach G, *et al.* LL-37-mediated activation of host receptors is critical for defense against group A streptococcal infection. Cell Rep (2021) 34:108766. doi: 10.1016/j.celrep.2021.108766
24. Alford MA, Choi KYG, Trimble MJ, Masoudi H, Kalsi P, Pletzer D, Hancock REW. Murine Model of Sinusitis Infection for Screening Antimicrobial and Immunomodulatory Therapies. Front Cell Infect Microbiol (2021) 11:1–15. doi: 10.3389/fcimb.2021.621081
25. van Dijk A, Molhoek EM, Veldhuizen EJA, Bokhoven JLMT van, Wagendorp E, Bikker F, Haagsman HP. Identification of chicken cathelicidin-2 core elements involved in antibacterial and immunomodulatory activities. Mol Immunol (2009) 46:2465–2473. doi: 10.1016/j.molimm.2009.05.019
26. Hancock REW, Haney EF, Gill EE. The immunology of host defence peptides: Beyond antimicrobial activity. Nat Rev Immunol (2016) 16:321–334. doi: 10.1038/nri.2016.29
27. Choe H, Narayanan AS, Gandhi DA, Weinberg A, Marcus RE, Lee Z, Bonomo RA, Greenfield EM. Immunomodulatory Peptide IDR-1018 Decreases Implant Infection and Preserves Osseointegration. Clin Orthop Relat Res (2015) 473:2898–2907. doi: 10.1007/s11999-015-4301-2
28. Mookherjee N, Hancock REW. Cationic host defence peptides: Innate immune regulatory peptides as a novel approach for treating infections. Cellular and Molecular Life Sciences (2007) 64:922–933. doi: 10.1007/s00018-007-6475-6
29. Alalwani SM, Sierigk J, Herr C, Pinkenburg O, Gallo R, Vogelmeier C, Bals R. The antimicrobial peptide LL-37 modulates the inflammatory and host defense response of human neutrophils. Eur J Immunol (2010) 40:1118–1126. doi: 10.1002/eji.200939275
30. Ugarova T, Moreno B, Podolnikova N, Lishko V. Identification of human cathelicidin

peptide LL-37 as a ligand for macrophage integrin αMβ2 (Mac-1, CD11b/CD18) that promotes phagocytosis by opsonizing bacteria. Res Rep Biochem (2016) Volume 6:39–55. doi: 10.2147/rrbc.s107070

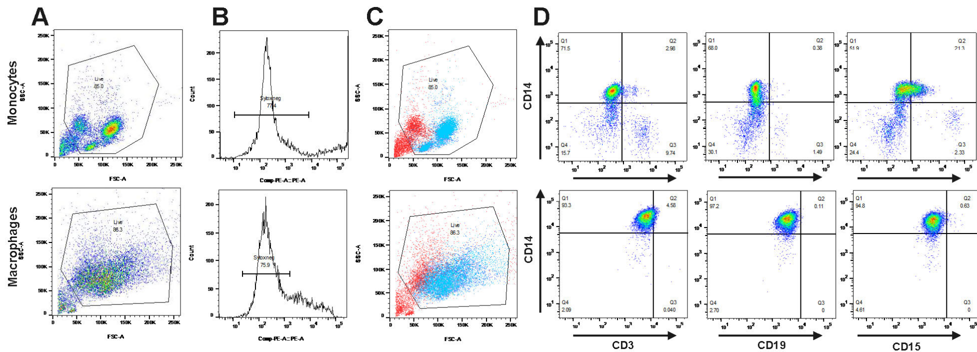
31. Wan M, van der Does AM, Tang X, Lindbom L, Agerberth B, Haeggström JZ. Antimicrobial peptide LL-37 promotes bacterial phagocytosis by human macrophages. J Leukoc Biol (2014) 95:971–981. doi: 10.1189/jlb.0513304
32. Tang X, Basavarajappa D, Haeggström JZ, Wan M. P2X 7 Receptor Regulates Internalization of Antimicrobial Peptide LL-37 by Human Macrophages That Promotes Intracellular Pathogen Clearance . The Journal of Immunology (2015) 195:1191–1201. doi: 10.4049/jimmunol.1402845
33. Niyonsaba F, Madera L, Afacan N, Okumura K, Ogawa H, Hancock REW. The innate defense regulator peptides IDR-HH2, IDR-1002, and IDR-1018 modulate human neutrophil functions. J Leukoc Biol (2013) 94:159–170. doi: 10.1189/jlb.1012497
34. Pena OM, Afacan N, Pistolic J, Chen C, Madera L, Falsafi R, Fjell CD, Hancock REW. Synthetic Cationic Peptide IDR-1018 Modulates Human Macrophage Differentiation. PLoS One (2013) 8:1–10. doi: 10.1371/journal.pone.0052449
35. Steinstraesser L, Hirsch T, Schulte M, Kueckelhaus M, Jacobsen F, Mersch EA, Stricker I, Afacan N, Jenssen H, Hancock REW, *et al.* Innate defense regulator peptide 1018 in wound healing and wound infection. PLoS One (2012) 7:1–7. doi: 10.1371/journal.pone.0039373
36. Ramos R, Silva JP, Rodrigues AC, Costa R, Guardão L, Schmitt F, Soares R, Vilanova M, Domingues L, Gama M. Wound healing activity of the human antimicrobial peptide LL37. Peptides (NY) (2011) 32:1469–1476. doi: 10.1016/j.peptides.2011.06.005
37. Bowdish DME, Davidson DJ, Scott MG, Hancock REW. Immunomodulatory activities of small host defense peptides. Antimicrob Agents Chemother (2005) 49:1727–1732. doi: 10.1128/AAC.49.5.1727-1732.2005
38. Boero E, Brinkman I, Juliet T, van Yperen E, van Strijp JAG, Rooijakkers SHM, van Kessel KPM. Use of Flow Cytometry to Evaluate Phagocytosis of *Staphylococcus aureus* by Human Neutrophils. Front Immunol (2021) 12:1–15. doi: 10.3389/fimmu.2021.635825
39. Ambarus CA, Krausz S, van Eijk M, Hamann J, Radstake TRDJ, Reedquist KA, Tak PP, Baeten DLP. Systematic validation of specific phenotypic markers for *in vitro* polarized human macrophages. J Immunol Methods (2012) 375:196–206. doi: 10.1016/j.jim.2011.10.013
40. Hamza T, Li B. Differential responses of osteoblasts and macrophages upon *Staphylococcus aureus* infection. BMC Microbiol (2014) 14:1–11. doi: 10.1186/s12866-014-0207-5
41. Hamza T, Dietz M, Pham D, Clovis N, Danley S, Li B. Intra-cellular *Staphylococcus aureus* alone causes infection *in vivo*. Eur Cell Mater (2013) 25:341–350. doi: 10.22203/ecm.v025a24
42. Alcayaga-Miranda F, Cuenca J, Khoury M. Antimicrobial activity of mesenchymal stem cells: Current status and new perspectives of antimicrobial peptide-based therapies. Front Immunol (2017) 8:1–15. doi: 10.3389/fimmu.2017.00339
43. Weiss ARR, Dahlke MH. Immunomodulation by Mesenchymal Stem Cells (MSCs): Mechanisms of action of living, apoptotic, and dead MSCs. Front Immunol (2019) 10:1–10. doi: 10.3389/fimmu.2019.01191
44. Chow L, Johnson V, Impastato R, Coy J, Strumpf A, Dow S. Antibacterial activity of human mesenchymal stem cells mediated directly by constitutively secreted factors and indirectly by activation of innate immune effector cells. Stem Cells Transl Med (2020) 9:235–249. doi: 10.1002/sctm.19-0092

45. AlMatar M, Makky EA, Yakıcı G, Var I, Kayar B, Köksal F. Antimicrobial peptides as an alternative to anti-tuberculosis drugs. Pharmacol Res (2018) 128:288–305. doi: 10.1016/j.phrs.2017.10.011
46. Scheenstra MR, van den Belt M, Tjeerdsma-van Bokhoven JLM, Schneider VAF, Ordonez SR, van Dijk A, Veldhuizen EJA, Haagsman HP. Cathelicidins PMAP-36, LL-37 and CATH-2 are similar peptides with different modes of action. Sci Rep (2019) 9:1–12. doi: 10.1038/s41598-019-41246-6
47. Wieczorek M, Jenssen H, Kindrachuk J, Scott WRP, Elliott M, Hilpert K, Cheng JTJ, Hancock REW, Straus SK. Structural studies of a peptide with immune modulating and direct antimicrobial activity. Chem Biol (2010) 17:970–980. doi: 10.1016/j.chembiol.2010.07.007
48. Sancho-Vaello E, Gil-Carton D, François P, Bonetti EJ, Kreir M, Pothula KR, Kleinekathöfer U, Zeth K. The structure of the antimicrobial human cathelicidin LL-37 shows oligomerization and channel formation in the presence of membrane mimics. Sci Rep (2020) 10:1–16. doi: 10.1038/s41598-020-74401-5
49. Schneider VAF, Coorens M, Tjeerdsma-van Bokhoven JLM, Posthuma G, van Dijk A, Veldhuizen EJA, Haagsman HP. Imaging the Antistaphylococcal Activity of CATH-2: Mechanism of Attack and Regulation of Inflammatory Response. mSphere (2017) 2: doi: 10.1128/msphere.00370-17
50. Barns KJ, Weisshaar JC. Real-time attack of LL-37 on single Bacillus subtilis cells. Biochim Biophys Acta Biomembr (2013) 1828:1511–1520. doi: 10.1016/j.bbamem.2013.02.011
51. Choi KYG, Mookherjee N. Multiple immune-modulatory functions of cathelicidin host defense peptides. Front Immunol (2012) 3:1–4. doi: 10.3389/fimmu.2012.00149
52. Steinstraesser L, Kraneburg U, Jacobsen F, Al-Benna S. Host defense peptides and their antimicrobial-immunomodulatory duality. Immunobiology (2011) 216:322–333. doi: 10.1016/j.imbio.2010.07.003
53. Dürr UHN, Sudheendra US, Ramamoorthy A. LL-37, the only human member of the cathelicidin family of antimicrobial peptides. Biochim Biophys Acta Biomembr (2006) 1758:1408–1425. doi: 10.1016/j.bbamem.2006.03.030
54. Noore J, Noore A, Li B. Cationic antimicrobial peptide ll-37 is effective against both extraand intracellular staphylococcus aureus. Antimicrob Agents Chemother (2013) 57:1283–1290. doi: 10.1128/AAC.01650-12
55. Siracusa MC. Basophils – Role in Immunity. Encyclopedia of Immunobiology (2016) 1:326–333. doi: 10.1016/B978-0-12-374279-7.03006-X
56. Thwaites GE, Gant V. Are bloodstream leukocytes Trojan Horses for the metastasis of *Staphylococcus aureus*? Nat Rev Microbiol (2011) 9:215–222. doi: 10.1038/nrmicro2508
57. Bongers S, Hellebrekers P, Leenen LPH, Koenderman L, Hietbrink F. Intracellular penetration and effects of antibiotics on staphylococcus aureus inside human neutrophils: A comprehensive review. Antibiotics (2019) 8:1–22. doi: 10.3390/antibiotics8020054
58. Liu Y, Jia Y, Yang K, Wang Z. Heterogeneous Strategies to Eliminate Intracellular Bacterial Pathogens. Front Microbiol (2020) 11:1–13. doi: 10.3389/fmicb.2020.00563
59. Netea MG, Domínguez-Andrés J, Barreiro LB, Chavakis T, Divangahi M, Fuchs E, Joosten LAB, van der Meer JWM, Mhlanga MM, Mulder WJM, *et al.* Defining trained immunity and its role in health and disease. Nat Rev Immunol (2020) 20:375–388. doi: 10.1038/s41577-020-0285-6
60. Lu D, Xu Y, Liu Q, Zhang Q. Mesenchymal Stem Cell-Macrophage Crosstalk and Maintenance of Inflammatory Microenvironment Homeostasis. Front Cell Dev Biol (2021) 9: doi: 10.3389/fcell.2021.681171

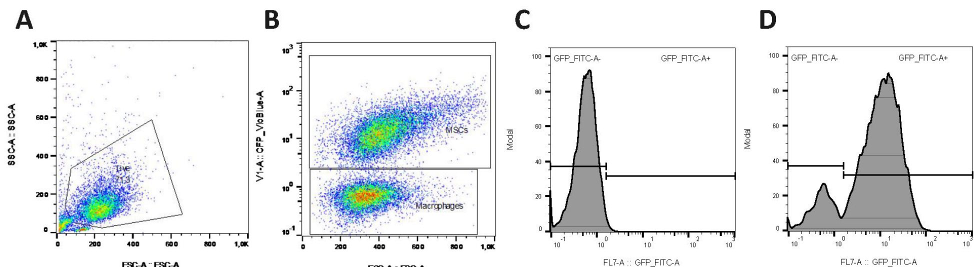
61. Andreu N, Phelan J, De Sessions PF, Cliff JM, Clark TG, Hibberd ML. Primary macrophages and J774 cells respond differently to infection with Mycobacterium tuberculosis. Sci Rep (2017) 7:1–12. doi: 10.1038/srep42225
62. Fransen L, Ruysschaert MR, Van der Heyden J, Fiers W. Recombinant tumor necrosis factor: Species specificity for a variety of human and murine transformed cell lines. Cell Immunol (1986) 100:260–267. doi: 10.1016/0008-8749(86)90025-0
63. McCarron EP, Williams DP, Antoine DJ, Kipar A, Lemm J, Stehr S, Welters ID. Exploring the translational disconnect between the murine and human inflammatory response: Analysis of LPS dose–response relationship in murine versus human cell lines and implications for translation into murine models of sepsis. J Inflamm Res (2015) 8:201–209. doi: 10.2147/JIR.S89097
64. Mestas J, Hughes CCW. Of Mice and Not Men: Differences between Mouse and Human Immunology. The Journal of Immunology (2004) 172:2731–2738. doi: 10.4049/jimmunol.172.5.2731
65. Summers C, Rankin SM, Condliffe AM, Singh N, Peters AM, Chilvers ER. Neutrophil kinetics in health and disease. Trends Immunol (2010) 31:318–324. doi: 10.1016/j.it.2010.05.006
66. Alalwani SM, Sierigk J, Herr C, Pinkenburg O, Gallo R, Vogelmeier C, Bals R. The antimicrobial peptide LL-37 modulates the inflammatory and host defense response of human neutrophils. Eur J Immunol (2010) 40:1118–1126. doi: 10.1002/eji.200939275
67. Bagheri M, Beyermann M, Dathe M. Immobilization reduces the activity of surface-bound cationic antimicrobial peptides with no influence upon the activity spectrum. Antimicrob Agents Chemother (2009) 53:1132–1141. doi: 10.1128/AAC.01254-08
68. Willcox MDP, Hume EBH, Aliwarga Y, Kumar N, Cole N. A novel cationic-peptide coating for the prevention of microbial colonization on contact lenses. J Appl Microbiol (2008) 105:1817–1825. doi: 10.1111/j.1365-2672.2008.03942.x
69. Ferreira L, Zumbuehl A. Non-leaching surfaces capable of killing microorganisms on contact. J Mater Chem (2009) 19:7796–7806. doi: 10.1039/b905668h
70. Zharkova MS, Orlov DS, Golubeva OY, Chakchir OB, Eliseev IE, Grinchuk TM, Shamova O V. Application of antimicrobial peptides of the innate immune system in combination with conventional antibiotics-a novel way to combat antibiotic resistance? Front Cell Infect Microbiol (2019) 9: doi: 10.3389/fcimb.2019.00128
71. Pletzer D, Mansour SC, Hancock REW. Synergy between conventional antibiotics and anti-biofilm peptides in a murine, sub-cutaneous abscess model caused by recalcitrant ESKAPE pathogens. PLoS Pathog (2018) 14:1–14. doi: 10.1371/journal.ppat.1007084
72. Yu G, Baeder DY, Regoes RR, Rolff J. Combination effects of antimicrobial peptides. Antimicrob Agents Chemother (2016) 60:1717–1724. doi: 10.1128/AAC.02434-15
73. Doolin T, Amir HM, Duong L, Rosenzweig R, Urban LA, Bosch M, Pol A, Gross SP, Siryaporn A. Mammalian histones facilitate antimicrobial synergy by disrupting the bacterial proton gradient and chromosome organization. Nat Commun (2020) 11:1–16. doi: 10.1038/s41467-020-17699-z
74. Narayana JL, Mishra B, Lushnikova T, Golla RM, Wang G. Modulation of antimicrobial potency of human cathelicidin peptides against the ESKAPE pathogens and *in vivo* efficacy in a murine catheter-associated biofilm model. Biochim Biophys Acta Biomembr (2019) 1861:1592–1602. doi: 10.1016/j.bbamem.2019.07.012
75. Wang X, Junior JCB, Mishra B, Lushnikova T, Epand RM, Wang G. Arginine-lysine positional swap of the LL-37 peptides reveals evolutional advantages of the native sequence and leads to bacterial probes. Biochim Biophys Acta Biomembr (2017)

1859:1350–1361. doi: 10.1016/j.bbamem.2017.04.018
76. Koro C, Hellvard A, Delaleu N, Binder V, Scavenius C, Bergum B, Gówczyk I, Roberts HM, Chapple ILC, Grant MM, *et al.* Carbamylated LL-37 as a modulator of the immune response. Innate Immun (2016) 22:218–229. doi: 10.1177/1753425916631404
77. van Harten RM, Tjeerdsma-van Bokhoven JLM, de Greeff A, Balhuizen MD, van Dijk A, Veldhuizen EJA, Haagsman HP, Scheenstra MR. D-enantiomers of CATH-2 enhance the response of macrophages against Streptococcus suis serotype 2. J Adv Res (2022) 36:101–112. doi: 10.1016/j.jare.2021.05.009
78. Onaizi SA, Leong SSJ. Tethering antimicrobial peptides: Current status and potential challenges. Biotechnol Adv (2011) 29:67–74. doi: 10.1016/j.biotechadv.2010.08.012
79. Coorens M, Schneider VAF, de Groot AM, van Dijk A, Meijerink M, Wells JM, Scheenstra MR, Veldhuizen EJA, Haagsman HP. Cathelicidins Inhibit Escherichia coli –Induced TLR2 and TLR4 Activation in a Viability-Dependent Manner . The Journal of Immunology (2017) 199:1418–1428. doi: 10.4049/jimmunol.1602164
80. Nielsen MC, Andersen MN, Møller HJ. Monocyte isolation techniques significantly impact the phenotype of both isolated monocytes and derived macrophages *in vitro*. Immunology (2020) 159:63–74. doi: 10.1111/imm.13125

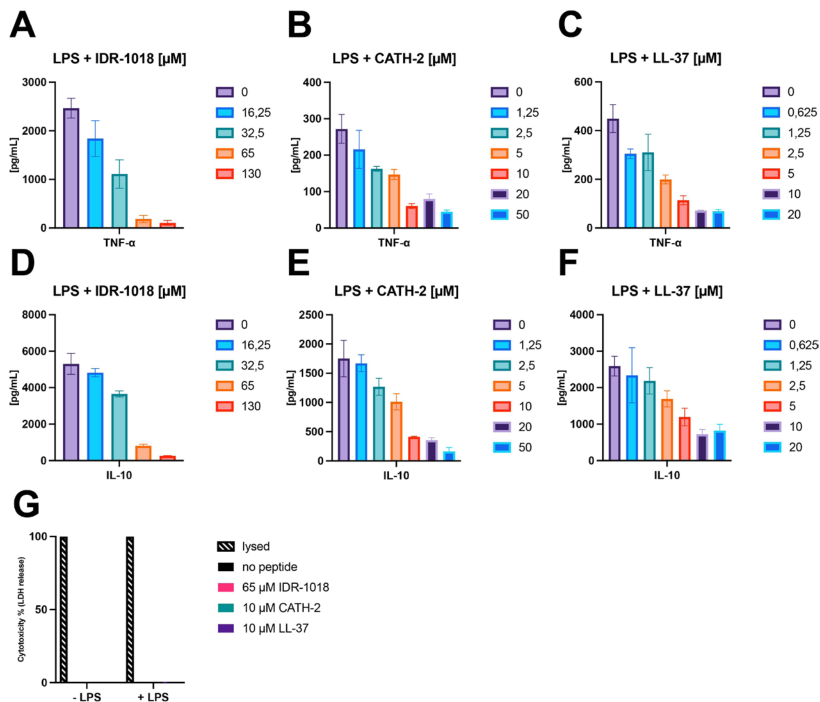
Supplementary Figures



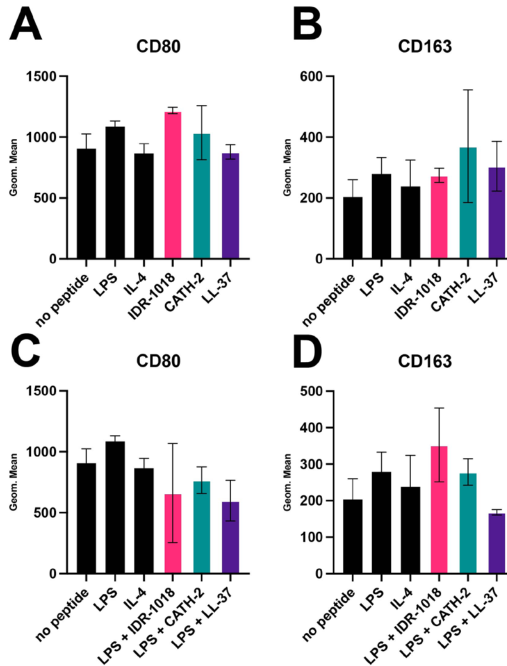
Supplementary Figure 1. Flow cytometry gating strategy to assess monocytes isolation quality. Same gating strategy was applied for monocytes (upper row) and macrophages (lower row). (**A**) Selection of total cells population (total) in the linear FSC and SSC. (**B**) Selection of sytox negative cells within the total population. (**C**) Distribution of sytox negative population (blue) within total cells population (red) recorded by the flow cytometer. (**D**) Cells within the sytox negative gate were further divided based on their combined expression of CD14 and CD3/CD19/CD15.



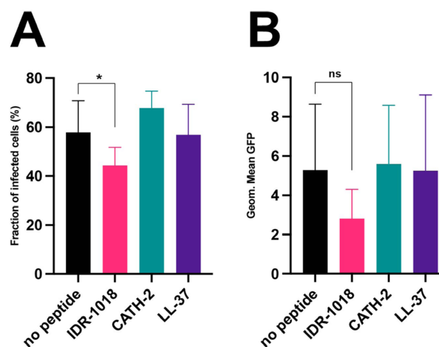
Supplementary Figure 2. Flow cytometry phagocytosis gating strategy. (**A**) Selection of macrophage population (live gate) in the linear FSC and SSC. (**B**) When cultured with MSCs, the macrophage population was further selected based on the signal of CellTrace Violet. (**C**) Histogram setting GFP fluorescence baseline for non-infected cells. (**D**) Proportion of non-infected (left peak) and infected (right peak) cells.



Supplementary Figure 3. Identification of optimal peptide concentration needed to inhibit macrophages LPS-mediated activation. All peptides tested decreased LPS-induced production of TNF-α (**A-C**) and IL-10 (**D-F**) in a dose-dependent manner. (n=3). Any of the selected concentrations of each peptide, in presence or absence of LPS, affected macrophages viability as determined by the levels of LDH released in the culture media (**G**). (n=9, from a total of 3 independent experiments). Error bars represent SD.



Supplementary Figure 4. Anti-inflammatory effect of peptides did not correlate to macrophage phenotype polarization. Macrophages were stimulated 24 h with LPS, IL-4, and selected concentrations of each peptide alone (**A**, **B**) or in combination with LPS (**C**, **D**). Then, macrophage marker expression was measured by flow cytometry to determine macrophage polarization towards a M1 (CD80) or M2 (CD163) phenotype. (n=3). Error bars represent SD.



Supplementary Figure 5. IDR-1018 reduced the number of bacteria phagocytosed by macrophages after 24h. Proportion of infected cells (**A**) and geometric mean (**B**) after 24 h infection following the “peptides during infection” model protocol. *p<0,03. (n=9). Error bars represent SD.

CHAPTER
4

The relative role of human neutrophils and macrophages in phagocytosis and killing of *Staphylococcus aureus*

Leonardo Cecotto[1,2], Kok P.M. van Kessel[2], Lisanne de Vor[2], Claudio Passerini[2], H.Charles Vogely[1], Bart C.H. van der Wal[1], Harrie Weinans[1,3], Suzan H.M. Rooijakkers[2], Saber Amin Yavari[1,4], Jos A.G. van Strijp[2]

[1] Department of Orthopedics, University Medical Center Utrecht, Utrecht, The Netherlands
[2] Department of Medical Microbiology, University Medical Center Utrecht, Utrecht, The Netherlands
[3] Department of Biomechanical Engineering, Delft University of Technology, Delft, The Netherlands
[4] Regenerative Medicine Centre Utrecht, Utrecht University, Utrecht, The Netherlands

Submitted

Abstract

Staphylococcus aureus is known for its resistance against antibiotic treatment and its host immune evasion strategies. A valid therapeutic alternative aims to enhance the effectiveness of our phagocytes against *S. aureus* via pathogen-specific monoclonal antibodies. Before screening different antibodies for their efficacy, we set out to characterize the relative role of different professional phagocytes in the elimination of *S. aureus*. Neutrophils are short-lived cells with high microbicidal activity, while macrophages have a longer life span but slower antibacterial mechanisms. Classically, neutrophils functions are studied in suspension assays, while macrophages are adherent cells. These differences hinder a fair comparison on the functions of these two phagocytes *in vitro*. We showed that neutrophils can be studied as adherent cells and vice versa macrophages in suspension conditions. This did not affect their expression of Fcγ and complement receptors, and consequently their capacity to recognize and engulf bacteria. In suspension, phagocytosis by neutrophils and macrophages was exclusively dependent on bacterial opsonization with serum factors. Moreover, neutrophils engulfed and killed more bacteria than macrophages. On the other hand, opsonin-independent uptake of *S. aureus* was observed by both phagocytes when studied in adhering conditions. The differences in bacterial uptake between neutrophils and macrophages were less evident, and no activation of their microbicidal activity was detected within the time frame examined. In conclusion, we showed that the antibacterial functions of neutrophils and macrophages changed according to the *in vitro* setup adopted. This should be considered when screening for the efficacy of antibodies against *S. aureus*.

Introduction

The first description of macrophages' and neutrophils' antibacterial properties dates back to 1883 when Elie Metchnikoff observed the phagocytosis ability of these two cell types (1,2). Over the years, the contribution of these two types of immune cells in the fight against bacterial infections has been more appreciated. After pathogens invade into the body, neutrophils and macrophages are rapidly recruited to control the infection by removing the bulk of invading bacteria. Besides, these phagocytes secrete cytokines and chemokines to mobilize more immune cells (3). A cooperative interaction between neutrophils and macrophages is essential in clearing both intracellular and extracellular pathogens (3,4). It has been often stated that absence of one or the other cell type would negatively impact infection resolution (5–7).

Neutrophils are the most abundant leukocytes present in the bloodstream, with around 10^{11} new cells produced daily from the bone marrow (8), while monocytes/macrophages represent around the 10% of circulating immune cells (9). This imbalance in numbers is compensated by their different life span. While neutrophils survive in the bloodstream from few hours up to 5 days, although the exact range is still under debate (10), macrophages live from a few days to even years, i.e. tissue resident macrophages, depending on their location (11). Neutrophils have a higher microbicidal activity via rapid generation of reactive oxygen species (ROS) and release of granule-derived mediators, like proteases and antimicrobial peptides, eventually leading to the death of the cell itself with the formation of neutrophils extracellular traps (NETs) (12). On the other hand, macrophages have a slower bactericidal activity, and their killing mechanisms mostly rely on the acidification of the phagolysosome, following activation of several proteases, enzymes, and production of reactive nitrogen species (RNS) (9).

Despite the numerous antibacterial strategies employed by the human immune system, *Staphylococcus aureus* is known for its ability to survive antibiotic treatment. In addition, this pathogen can escape from immune cells recognition and killing, often by hiding within the host cells (13–19). Therefore, *S. aureus* causes severe infections to the skin, soft tissues, and biomaterials, particularly after orthopedic surgery using implants (15,20). This necessitates finding alternative treatments to fight against *S. aureus* infections. Using and/or enhancing the effectiveness of our innate immune system, especially our phagocytes is an attractive option that was coined over a century ago. George Bernard Shaw in The Doctor's Dilemma stated that "There is at bottom only one genuinely scientific treatment for all diseases, and that is to stimulate the phagocytes, drugs are a delusion" (21). The proposed treatment option is to enhance opsonization. Now, after numerous successes in cancer treatment in the last decades, we can manipulate opsonization by using therapeutic monoclonal antibodies to directly and indirectly (via complement activation) enhance phagocytosis

(22–24). Furthermore, pathogen-specific vaccines represent a promising solution to reduce the risk of resistance development while boosting our own immune system resources.

Despite a significant progress in the development of monoclonal antibodies (mAbs) and a wide variety of antigens to stimulate specific cellular immunity against *S. aureus*, yet none of these treatments have been successfully translated into clinical practice (25). The efficacy of both mAbs administration and antibody induction through vaccination relies upon the functional properties of the introduced antibodies. One critical obstacle in the advancement of an effective therapeutic compound could be the lack of adequate or accurate preclinical models recapitulating the human immune system. For instance, majority of *in vivo* experiments use mice in their infection models which fall short due to differences between the human and murine immune system and its response against *S. aureus* infection. As a consequence, vaccine's efficacy or side effects might be overlooked (25–27). Not surprisingly, *in vitro* assay conditions could change immune cells phagocytosis dynamics (28), or use of cell lines instead of primary cells might change antibody effects when the research shifts towards *in vivo* studies (29,30). But, before we can screen different antibodies for their efficacy, we should characterize the relative role of innate immune cells in the elimination of *S. aureus*. To do so, we compared under the same conditions *in vitro* the response of primary human neutrophils and macrophages towards *S. aureus*. The results might shed light towards the role of these phagocytes in the fight against *S. aureus*, and therefore advise future strategies towards the development of a vaccine therapy.

Materials and Methods

Isolation of monocyte-derived macrophages

Blood from healthy human donors was supplied by the Dutch blood bank (Sanquin, Amsterdam, The Netherlands). Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats using Ficoll-Paque (Cytiva, 17544203) density centrifugation. Monocytes were positively selected by magnetic-activated cell sorting (MACS) with anti-CD14 labelled microbeads (Miltenyi Biotec, 130050201) according to manufacturer instructions.

Isolated monocytes were seeded in a 24-well plate at a density of 3 x 10^5 cells/well when performing the assays in adhesion, or in a 6 cm cell culture-treated petri dish at a density of 5 x 10^5 cells/well when performing the assays in suspension. Monocytes were differentiated to macrophages by culture for 7 days at 37°C, 5% CO2 in RPMI (Gibco, 52400) supplemented with 10% hyclone fetal bovine serum (FBS, Biowest, HYCLSV30160), 100 U/mL penicillin and 100 µg/mL streptomycin (1% p/s, Gibco), and 40 ng/mL human recombinant M-CSF (Peprotech, 300-25). Culture media was refreshed after 3-4 days.

Viability of the isolated cells was determined by sytox orange dead cell stain for flow cytometry (ThermoFisher, S34861), before and after differentiation. Purity of

the isolated monocytes was checked by staining for CD14-APC (BD, 555399) and contamination by T-cells (CD3-FITC, BioLegend, 300440), B-cells (CD19-FITC, BD, 555412), or granulocytes (CD15-FITC, BD, 332778). Cells staining was performed as described in section “Immune cells receptor expression”, having all fluorochrome-conjugated antibodies diluted 1:30 in phosphate-buffered saline buffer (PBS) with 0,1% bovine serum albumin (BSA) and 1% heat-inactivated human serum.

Isolation of human neutrophils

Neutrophils isolation was performed as previously described by Surewaard *et al.* (31). Briefly, blood from healthy human donors was collected by venipuncture and collected in sodium heparin tubes after informed consent. Approval from the Medical Ethics Committee of the University Medical Center Utrecht was obtained (METC protocol 07-125/C approved on March 1, 2010). Neutrophils were isolated from whole blood using a dual Ficoll/Histopaque (Sigma, 11191) density centrifugation and residual erythrocytes were lysed by hyper osmotic shock with cold deionized water. Isolated neutrophils were kept in suspension in RPMI supplemented with 0,05% human serum albumin (©, Sanquin) when performing the assays in suspension. On the other hand, when performing the assays in adhesion, neutrophils were seeded in a 24-well plate at a density of 3 x 10^5 cells/well in RPMI with 10% FBS and let adhere to the plastic bottom for >1 h before performing the experiment.

Viability and purity of the isolated neutrophils was verified as previously described for monocytes.

Bacterial culture

All experiments used GFP-labelled *Staphylococcus aureus* strain Newman, transformed with a GFP-expressing plasmid pCM29 to constitutively express GFP, as previously described (32). Bacteria were grown overnight in Todd-Hewitt broth (THB) with 10 µg/mL chloramphenicol to reach stationary phase.

Phagocytosis assays in adhesion and suspension

Neutrophils and macrophages phagocytosis ability was assessed with cells in suspension or adhering to the culture plate, as schematized in **Figures 3A** and **3C**. Cells were incubated with RPMI + 0,05% HSA for assays in suspension or RPMI + 10% FBS for assays in adhesion. Every experiment was performed with immune cells isolated from different donors.

In both conditions, 500 µL of overnight bacterial culture was centrifuged for 2 min, 17000 x g at RT, and resuspended in RPMI + 0,05% HSA (assay in suspension) or RPMI (assay in adhesion) to reach a final inoculum concentration of 1 x 10^7 colony forming units (CFU)/mL. Then bacteria were opsonized for 15 min at 37°C with a range of concentrations of normal human serum (NHS), immunoglobulins of class G- and M- (IgG-/IgM-) depleted NHS (ΔNHS), and heat-inactivated ΔNHS (HI ΔNHS). The IgG/IgM components were removed from NHS as previously described by our group (33), while HI ΔNHS was prepared by heating serum for 30 min at 56°C.

Subsequently, bacteria were combined with cells at a cell-to-bacterium ratio of 1-10, meaning 10 bacteria per phagocyte. When measuring phagocytosis in suspension, plates were incubated on a shaking plateau at 37°C until the end of the experiment. Differently, to synchronize bacterial uptake with adhering cells, the plates were centrifuged for 5 min, 110 x g at RT, and then moved to the incubator at 37°C, 5% CO2 for the remaining time of the assay.

To analyze samples by flow cytometry, phagocytosis in suspension was stopped by adding ice-cold 1,5% paraformaldehyde to the wells without washing steps. Then, plates were kept at 4°C for about 15 min before analysis. On the other hand, adhering cells were detached from the culture plate using ice-cold 10 mM DPBS/EDTA in combination with gentle scraping. Then, samples were moved to a 96-well plate and fixed in 1,5% paraformaldehyde before analysis.

Samples were measured with MACSquant VYB (Miltenyi Biotech) flow cytometer and data analyzed with FlowJo (v.10.1., FlowJo LLC). Gating strategy is summarized in **Supplementary figure 1**. Briefly, a total of 10000 events were collected for each sample gated on the total cells population based on forward scatter (FSC) and side scatter (SSC) parameters. Non-infected samples were used to set GFP fluorescence baseline and define the proportion of GFP-positive (GFP+) cells, meaning cells that took up at least 1 bacterium. The same FSC, SSC, and GFP settings were used to measure phagocytosis by both neutrophils and macrophages. Finally, to allow a fair comparison between neutrophils and macrophages' phagocytic ability, the values derived from the geometric mean of the GFP signal were normalized according to the formula adapted from (34,35):

$$Fluorescence = \frac{X - X0}{X0}$$

where X represents the values recorded for each condition in presence of *S. aureus* and X0 represents the background value of cells without bacteria.

Phagocytosis by microscopy

To study neutrophils and macrophages phagocytosis by microscopy, the assays in suspension and adhesion were performed as previously described with few variations. Instead of using a range of different sera concentrations, *S. aureus* was opsonized only with 5% NHS. At the end of the assay, samples were not fixed in paraformaldehyde but re-suspended in PBS with 1% HSA and immediately centrifuged into glass slides using a cytospin-3 (Shandon), air dried and subsequently stained with the rapid Diff-Quick (Dade Behring) procedure. Pictures were taken with a Sony Nex-5 camera mounted without lens on an Olympus BX50 microscope.

Quantification of non-phagocytosed bacteria

The assay was performed as described for the phagocytosis in suspension, but the reaction was stopped by addition of 0,3 µg/mL LDS-751 nuclear stain (Thermofisher, L7595) diluted in 1,5% paraformaldehyde. Then, plates were kept at 4°C for about 15 min before analysis, as previously described.

Samples were measured with MACSquant VYB flow cytometer and data analyzed with FlowJo. Gating strategy is summarized in **Supplementary figure 2**. Briefly, 30 μL from each sample were collected for analysis and visualized in a logarithmic scale of the FSC and SSC parameters to display both bacteria and phagocytes populations. As previously described (32), phagocytes were distinguished from bacteria based on the LDS-751 fluorescence (**Supplementary figure 2B**). A separate gate was based on the GFP signal of *S. aureus* without phagocytes (**Supplementary figure 2C**). Only events within the LDS+ or GFP+ gates were displayed based on SSC and GFP parameters. Then, the number of events were counted in each quadrant: phagocytes without intracellular bacteria (Q1, LDS+/GFP-); phagocytes with intracellular bacteria (Q2, LDS+/GFP+); non-phagocytosed bacteria (Q3, LDS-/GFP+). The fraction of cells without intracellular bacteria was calculated dividing the events counted in Q1 by the total number of cells (Q1+Q2). The fraction of non-phagocytosed bacteria was calculated dividing the events counted in Q3 by those counted in Q3 at 0% NHS (total number of bacteria introduced in the assay).

Phagocytes receptor expression

Cells kept in suspension or in adhesion could differ in their basal Fcγ and complement (CR) receptors expression and thereby influence their phagocytic capacity. Therefore, cells were analyzed for their surface expression of FcγRI with CD64-FITC (BD, 555527), FcγRII with CD32-APC (BD, 559769), FcγRIIIb with CD16-FITC (BioLegend, 360716), CR1 with CD35-PE (BD, 559872), CR2 with CD21-FITC (Abcam, ab65809), CR3 with CD11b-APC (BD, 550019), CR4 with CD11c-PE/Cy7 (BioLegend, 337215), and CD14-APC as control antigen.

All washing steps were performed with cold 0,1% BSA/PBS and centrifugation at 5 min, 500 x g. The staining solutions were prepared by diluting 1:30 the above-mentioned fluorochrome-conjugated antibodies in PBS with 0,1% BSA and 1% heat-inactivated human serum. As a negative control, staining solution without antibodies was used. Cells were incubated with staining solutions for 30 min on ice, in the dark. Markers expression was measured via flow cytometer (FACSVerse, BD) and data analyzed using FlowJo. To fairly compare receptors expression in neutrophils and macrophages, the values derived from the geometric mean of the fluorescent signal for each antibody were normalized according to the formula described in the phagocytosis assays.

Killing assay in suspension and adhesion

To study the ability of neutrophils and macrophages to kill intracellular *S. aureus*, the assays were performed similarly to the phagocytosis experiments in suspension and adhesion, with few variations. In suspension, samples were added into 2 mL non-siliconized tubes (Sigma) instead of a 96-well plate, while in adhesion the experiments were performed in a 24-well plate. *S. aureus* was opsonized with 5% NHS and combined with cells at a cell-to-bacterium ratio of 1-1. Samples were incubated on a shaking plateau at 37°C, 5% CO2 for 15 min and 1 h for the suspension

assay, or 1 and 2 h for the adhesion assay. At each time point, the cell membranes of the phagocytes were lysed by addition of ice-cold 0,3% saponin (Sigma, 47036) in deionized water. Samples were lysed for 5-10 min in ice, serially diluted in PBS and plated in duplicates. The next day, the number of colonies was counted.

When measuring bacterial killing in suspension, saponin was directly added to the samples without any washing step. Therefore, both extracellular and engulfed bacteria were counted. Although with this low cell to bacteria ratio, very few bacteria remain on the outside. On the contrary, when killing was measured with phagocytes in adhesion, the supernatant with non-phagocytosed bacteria was removed before incubation with saponin, thus only intracellular bacteria were collected and counted. To equally compare the killing efficacy of the two phagocytes in both assays, the number of viable bacteria was normalized by the CFU/mL counted after 15 min in suspension and 1 h in adhesion. Data are expressed as percentage of bacteria that survived killing by neutrophils and macrophages.

Graphs, illustrations, and statistical analysis

GraphPad Prism 9 was used to create the graphs and determine statistical significance using two-way ANOVA or t-test. $p<0.05$ was considered statistically significant. Illustrations were created in Biorender.com.

Results

Innate immune cells selection and culture conditions to study phagocytosis

Human monocyte-derived macrophages were isolated from whole blood following Ficoll density centrifugation and positive selection by anti-CD14 microbeads. The isolation protocol yielded more than 75% viable cells. Although CD14-positive cells represented the majority of the population, B- and T-cells, and neutrophils were still detected in the cell suspension. However, after 7 days culture in presence of M-CSF only monocyte-derived macrophages survived, as observed by a population with more than 90% viable cells expressing only CD14. Human neutrophils were isolated from whole blood using a dual Ficoll/Histopaque density centrifugation. The isolation protocol yielded a population of viable cells that expressed CD15 but not CD14, selected as commonly accepted markers to identify human neutrophils (36,37). Therefore, in our assays we could study the phagocytosis of primary human monocyte-derived macrophages (CD14+) and neutrophils (CD15+) (**Figure 1A-D**).

Next, we defined the optimal assay conditions to study the phagocytic ability of both cell types in suspension and in adhesion. While neutrophils assays are typically performed in suspension (38), monocytes-derived macrophages are differentiated and studied in adherent conditions (39). We observed that macrophages could be detached and incubated in suspension with the same buffer (RPMI + 0.05% HSA) normally used to study neutrophils. On the contrary, when RPMI +10% FBS was

used to incubate cells in suspension the flow cytometer detected non-homogeneous populations of neutrophils and almost no events for macrophages. Neutrophils could be studied in adhering conditions by letting the cells adhere to the culture plate for at least 1 h in presence of the same buffer (RPMI + 10% FBS) normally used for macrophages. Instead, non-homogeneous or scattered events were detected when phagocytes were incubated in adhesion in presence of RPMI + 0.05% HSA (**Figure 1E**).

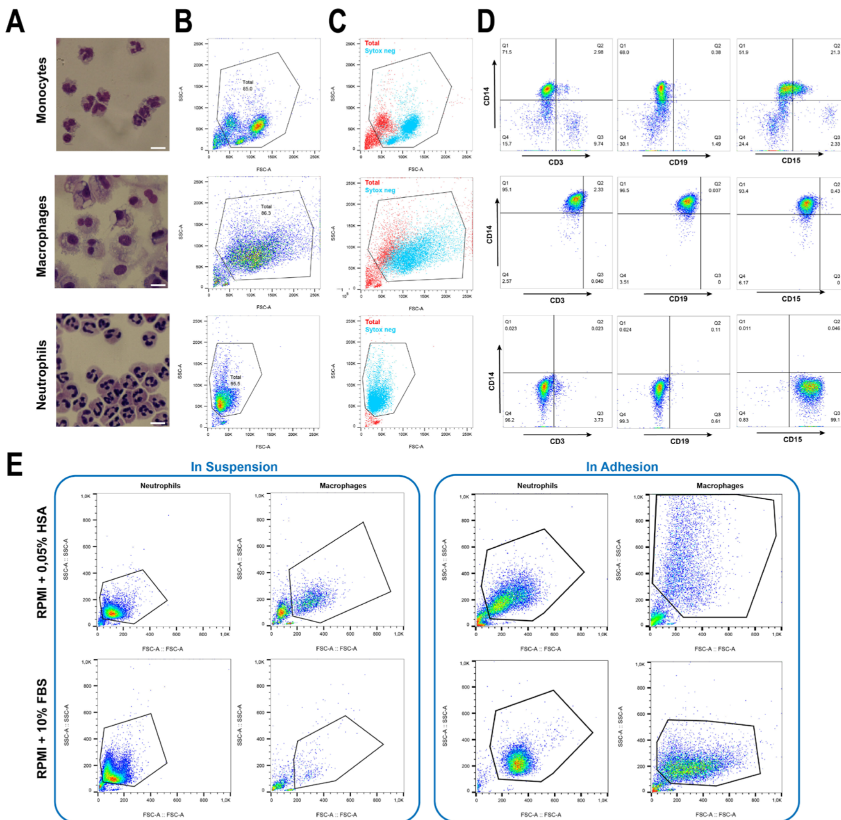


Figure 1. Isolation and culture conditions of neutrophils and macrophages. (**A**) Representative image at light microscopy of cell type. Scale bar is 20 µm. (**B**) Selection of cells of interest (total) in the linear FSC and SSC. (**C**) Distribution of sytox negative population (blue) within all events (red) recorded by flow cytometer. (**D**) Cells within the sytox negative gate were further divided based on their combined expression of CD14 and CD3/CD19/CD15. Same gating strategy was applied for monocytes (upper row), macrophages (middle row), and neutrophils (lower row). (**E**) Selection of events of interest in the linear FSC and SSC when assays were performed in suspension (first box) or in adhesion (second box). Cells were incubated with RPMI + 10% FBS (first row) or with RPMI + 0,05% HSA (second row).

Change in assay conditions did not affect the expression of Fcγ- and complement-receptors (FcγR and CR) in neutrophils and macrophages (**Figure 2** and **Table 1**). FcγRI expression was only observed in macrophages, as in neutrophils this receptor is expressed at very low levels in physiological conditions (40), increasing only during inflammation (41). In line with literature, we showed that both cell types did not express CR2 (42).

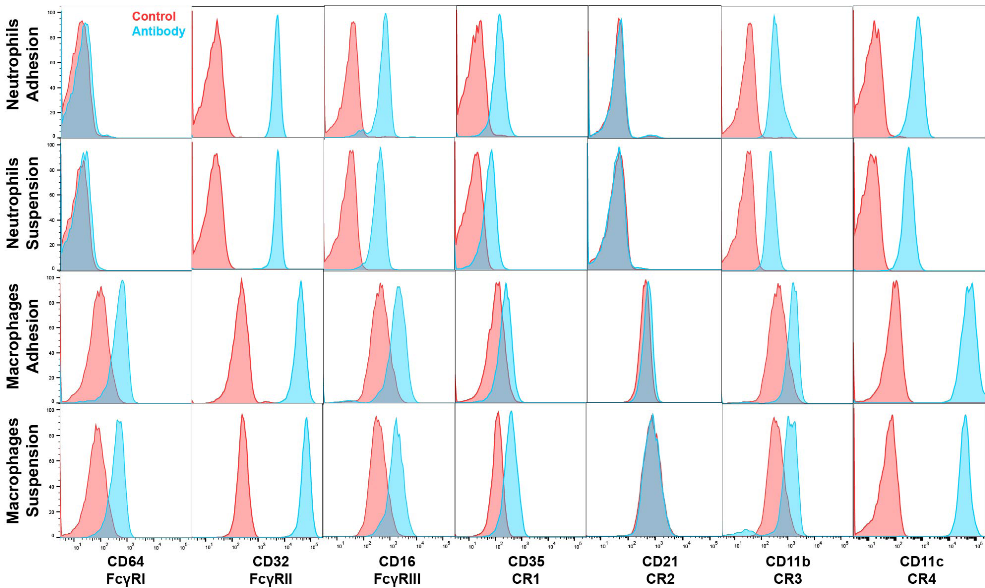


Figure 2. Expression of FcγR and CR in neutrophils and macrophages according to assay conditions. Overlay of the histograms of control with no antibody (red) and antibody-stained (blue) cells. Data are representative of three independent experiments.

	CD64 FcγRI	CD32 FcγRII	CD16 FcγRIII	CD35 CR1	CD21 CR2	CD11b CR3	CD11c CR4
Neutrophils in adhesion	0,54	336,61	19,00	14,63	0,08	14,69	8,45
Neutrophils in suspension	0,45	395,23	20,42	6,61	-0,01	12,91	3,16
Macrophages in adhesion	4,95	176,82	2,75	1,33	0,25	2,16	9,60
Macrophages in suspension	5,77	237,60	3,50	2,28	0,02	0,91	6,18

Table 1. Fluorescence values normalized relative to the control for the expression of FcγR and CR in neutrophils and macrophages in different assay conditions. Values are derived from histograms shown in Figure 2.

To compare the phagocytic ability of neutrophils and macrophages we determined the time necessary to observe serum-mediated uptake of bacteria. To do so, we looked at the number of *S. aureus* phagocytosed by immune cells described by the increase in intensity of the GFP signal. However, the different autofluorescence properties of neutrophils and macrophages complicated the gating strategy and analysis (43–45). Regardless of the fluorescence channel considered, macrophages always displayed higher fluorescence values compared to neutrophils (**Figure 3A**). Therefore, to fairly compare the number of bacteria phagocytosed by neutrophils and macrophages, the fluorescence values recorded in presence of *S. aureus* were compensated by the autofluorescence of each cell type.

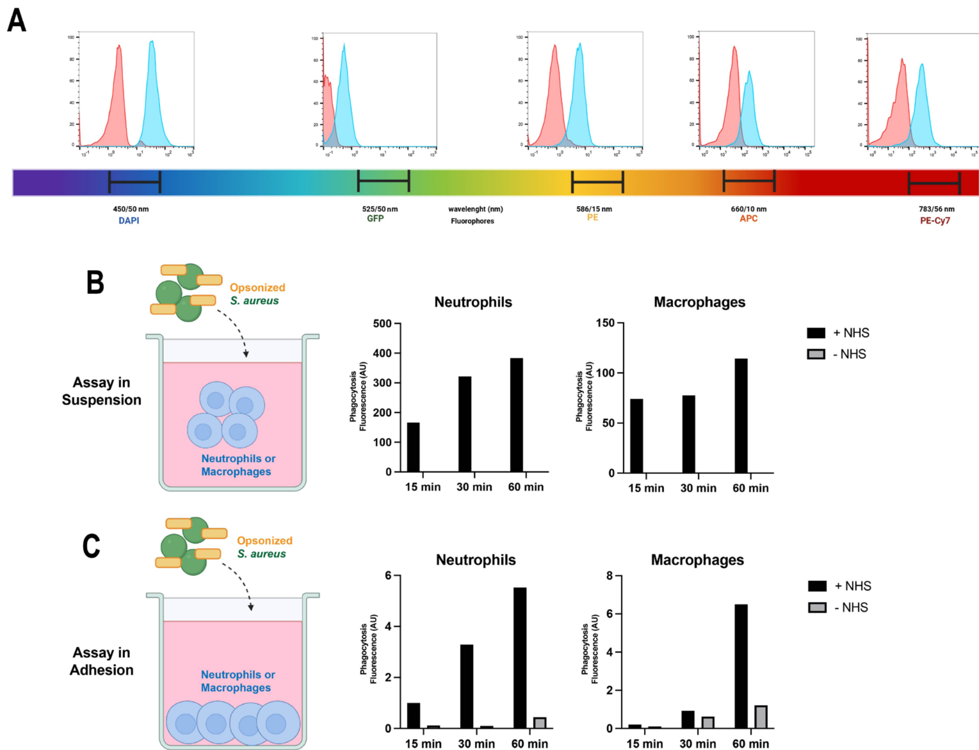


Figure 3. Parameters to study the phagocytosis of *S. aureus* by neutrophils and macrophages. (**A**) Overlay of histograms showing the background fluorescence signal of neutrophils (red) and macrophages (blue) measured at different wavelengths. Phagocytosis of serum-opsonized, or non-opsonized *S. aureus* was studied in neutrophils and macrophages in suspension (**B**) or adhering (**C**) conditions. Schematic representation of the assays on the left. Bacterial uptake over time was represented as the geometric mean of the GFP signal. Data are representative of two independent experiments.

When the assay was performed in suspension, after 15 min it was already possible to observe a clear serum-dependent phagocytosis of *S. aureus* by both neutrophils and macrophages (**Figure 3B**). In contrast, when phagocytosis was studied with cells incubated in adhesion, a higher difference between serum-dependent and -independent uptake of bacteria was observed after 60 min in both cell types (**Figure 3C**). Moreover, both phagocytes engulfed more bacteria when the assay were performed in suspension than in adhesion.

Comparison of the phagocytic ability of neutrophils and macrophages

Based on the previous results, the phagocytic ability of neutrophils and macrophages was studied for 15 min in suspension and 60 min in adhesion. Bacterial recognition and uptake by phagocytes can be induced both directly by binding of complement-derived opsonins and IgG class of antibodies to the bacterial membrane, and indirectly by IgG/IgM-mediated activation of the complement cascade (46). Therefore, *S. aureus* was opsonized with increasing concentrations of NHS (serum with both antibodies, and complement components), ΔNHS (serum depleted of IgG and IgM antibodies, but active complement), or HI ΔNHS (serum depleted of IgG and IgM antibodies, and inactivated complement components).

In suspension, a similar proportion of neutrophils and macrophages took up at least 1 bacterium in a NHS-concentration dependent way, although at concentrations lower than 2,5% NHS we could observe more GFP+ macrophages than neutrophils. On the contrary, none or limited phagocytosis was mediated by ΔNHS and HI ΔNHS opsonization even if we could detect higher percentages of macrophages with intracellular bacteria than neutrophils (**Figure 4A**). Nonetheless, in presence of 2,5% and 5% NHS we could observe a significantly higher number of intracellular bacteria in neutrophils compared to macrophages (**Figure 4B**).

In adhesion, we observed phagocytosis by both cell types regardless of the serum components used to opsonize bacteria. Almost all macrophages and ˜30% of neutrophils ingested at least 1 bacterium already in absence of serum. The number of neutrophils taking up *S. aureus* increased in a serum-concentration dependent way, which was pronounced in presence of NHS (**Figure 4C**). In absence of serum, macrophages phagocytosed more bacteria than neutrophils. However, combination of antibodies and complement proteins to opsonize *S. aureus*, increased neutrophils' phagocytic ability leading to a comparable number of intracellular bacteria between neutrophils and macrophages at 5% NHS (**Figure 4D**).

To verify a correct interpretation of the flow cytometry data, the phagocytic ability of neutrophils and macrophages was studied by light microscopy. The assays were performed both in suspension and adhesion in presence of *S. aureus* opsonized with 5% NHS. In suspension, we could confirm that neutrophils phagocytosed more bacteria than macrophages (**Figure 5A**). On the contrary, in adhesion we counted slightly more intracellular bacteria in macrophages compared to neutrophils (**Figure 5B**).

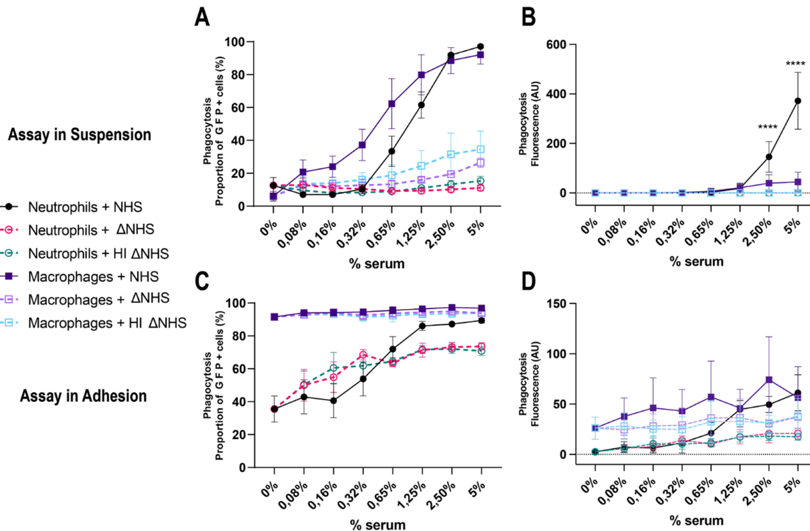


Figure 4. Comparison of the phagocytosis of *S. aureus* by neutrophils and macrophages, measured by flow cytometry. Bacteria were opsonized with several concentrations of NHS, ΔNHS, and HI ΔNHS, and added to phagocytes at a cell-to-bacterium ratio of 1-10. Bacterial uptake was evaluated after 15 min in suspension and 60 min in adhesion assays. (**A**, **C**) The percentage of cells that had taken up at least 1 bacterium is depicted as the fraction of infected cells. (**B**, **D**) The amount of bacteria engulfed by phagocytes is measured by the intensity of fluorescence of the GFP signal, expressed in arbitrary units (AU) as values were compensated for cell type's autofluorescence. (n=3). Error bars represent SEM. Statistical significance between phagocytes at each serum concentration was determined via two-way ANOVA. ****p<0.0001.

To bypass data normalization to compensate for differences in cell type's autofluorescence, we studied phagocytosis via quantification by flow cytometry of the number of bacteria that were not taken up by neutrophils and macrophages. With this method, we could confirm that by increasing NHS concentrations, the number of neutrophils and macrophages without intracellular bacteria decreased in similar proportions. Consequently, by increasing the pathogens recognition and uptake capacity of the two phagocytes, the number of extracellular bacteria decreased. With this method we observed that the proportions of non-phagocytosed bacteria by neutrophils were constantly lower than those of macrophages (**Figure 6A**). Therefore, we could confirm that neutrophils phagocytosed more bacteria than macrophages when the assay was performed in suspension. As previously observed (**Figure 4A** and **4B**), in suspension both neutrophils and macrophages did not phagocytose bacteria opsonized with ΔNHS and HI ΔNHS (**Figure 6B** and **6C**).

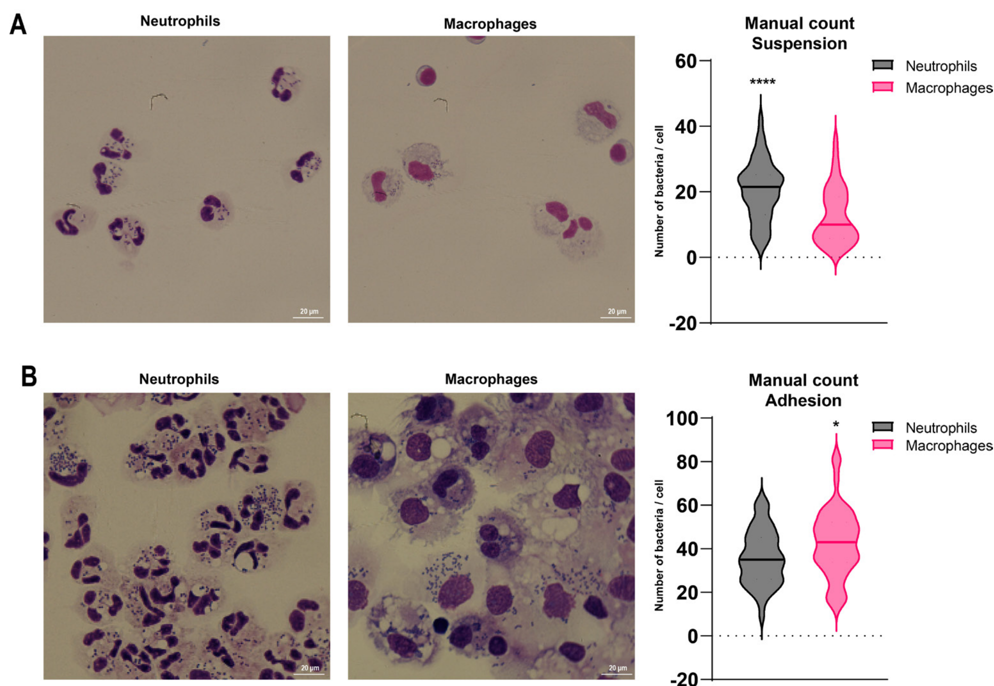


Figure 5. Comparison of the phagocytosis of *S. aureus* by neutrophils and macrophages, measured Comparison of the phagocytosis of *S. aureus* by neutrophils and macrophages, measured by light microscopy. Bacteria were opsonized with 5% NHS and added to phagocytes at a cell-to-bacterium ratio of 1-10. Bacterial uptake was evaluated after 15 min in suspension (**A**) and 60 min in adhesion (**B**) assays. The number of phagocytosed bacteria by neutrophils (left) and macrophages (right) was manually counted from 50 randomly chosen cells. Scale bar is 20 µm. Violin plot, lines at mean. Statistical significance between phagocytes was determined via t-test. *p<0.05; ****p<0.0001.

Comparison of neutrophils and macrophages microbicidal activity

Once we defined the phagocytic capacity of neutrophils and macrophages, we investigated the ability of the two cell types to kill the internalized *Staphylococci*. When phagocytosis was studied in suspension, we could observe a faster activation of the microbicidal activity in neutrophils compared to macrophages. Already after 1 h, we could observe a 40% reduction of the proportion of live *S. aureus* in presence of neutrophils, while macrophages had no impact on bacterial survival (**Figure 7A**). In adhesion, no reduction in the proportion of intracellular bacteria was recorded in both phagocytes within the time point considered. On the contrary, the number of bacteria counted after 2 h increased in both neutrophils and macrophages (**Figure 7B**).

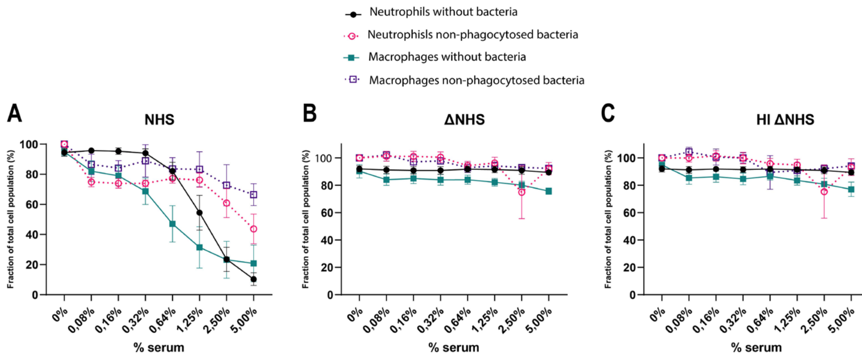


Figure 6. Comparison of neutrophils and macrophages phagocytosis, measured by quantification of non-phagocytosed bacteria. *S. aureus* was opsonized with several concentrations of NHS (**A**), ΔNHS (**B**), and HI ΔNHS (**C**) and added to the phagocytes at a cell-to-bacterium ratio of 1-10. Phagocytosis was studied after 15 min incubation of bacteria and phagocytes in suspension. The percentage of cells without intracellular bacteria and the percentage of free bacteria that had not been taken up by cells are depicted as the fraction of total cell population. (n=3). Error bars represent SEM. No statistical significance between phagocytosed or non-phagocytosed bacteria at each serum concentration was found via two-way ANOVA.

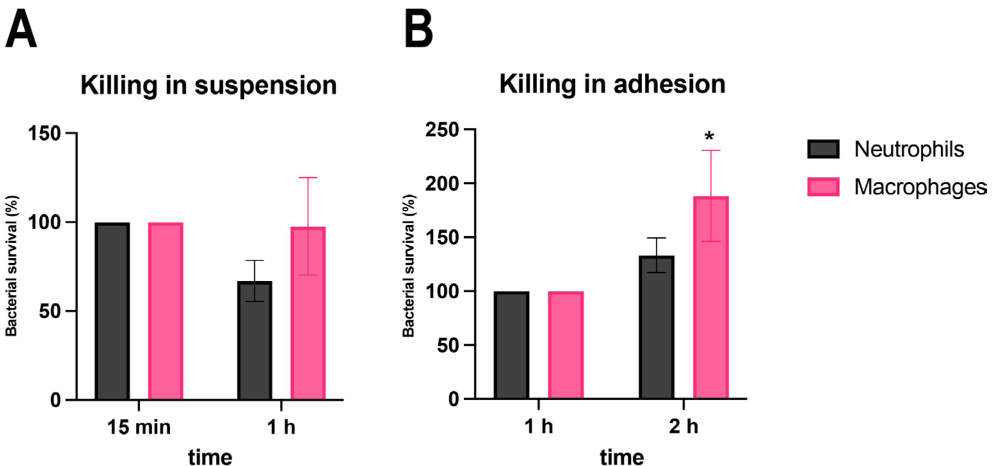


Figure 7. Comparison of the killing activity of *S. aureus* by neutrophils and macrophages. Cells were incubated with S. aureus opsonized with 5% NHS for 15 min and 1 h in suspension (**A**) and 1 h and 2 h in adhesion (**B**). After lyisis of the cell membranes of the phagocytes, the number of viable bacteria was normalized by the CFU/mL counted after 15 min in suspension and 1 h in adhesion. Dara are depicted as percentage of bacterial survival. (n=3). Error bars represent SEM. Statistical significance between phagocytes was determined via two-way ANOVA. *p<0.05.

Discussion

In this study we set out to compare the interaction between macrophages or neutrophils with *Staphylococcus aureus* bacteria. Phagocytosis assay conditions for neutrophils and macrophages in literature are very different for these two cell types (28,38,39). We decided to use both a suspension assay as well as adherence assay in order to be able to compare both cell types. By flow cytometry analysis, both phagocytes engulfed a higher number of bacteria when the assay was performed in suspension rather than in adhesion. Moreover, bacterial recognition and uptake by cells in suspension was exclusively dependent on the opsonization with serum factors. In contrast, other non-opsonic mechanisms were also involved when phagocytosis was studied in adhesion. Neutrophils engulfed and killed more bacteria than macrophages in suspension assays, while the difference between the two cell types was less evident in adhesion assays.

In general, the phagocytic and microbicidal activity of neutrophils and macrophages *in vitro* is determined with cells under suspension or adherent conditions, respectively. However, *in vivo* both pathogens and immune cells adhere, interact, and move through the extracellular matrix (ECM) network which constant modifications in mechanical properties correlate to changes in cells behavior (47). This means that the *in vivo* reality is not exactly mirrored in either of these *in vitro* assays. We showed that we could study the role of neutrophils and macrophages against *S. aureus* both in suspension and in adhesion (**Figure 1**). In both conditions, the expression of FcγRs or CRs and therefore bacterial antibody- and complement-mediated uptake capacity of the two phagocytes did not change (**Figure 2** and **Table 1**). However, this affected the phagocytosis kinetics. During assays in suspension, human cells and bacteria were incubated on a shaking plateau which promoted the frequency of interaction between the two, resulting in faster phagocytic uptake. In the adherence assays, a longer incubation time was needed for the pathogen to make contact with the adherent cells on the surface of the culture plate despite the initial centrifugation step (**Figure 3**).

Both neutrophils and macrophages showed a serum-concentration dependent phagocytosis of *S. aureus*. The highest bacterial uptake was achieved in presence of both an active complement system and IgG/IgM antibodies (**Figure 4**). Absence of these two components resulted in limited or no bacterial uptake when cells were in suspension. In contrast, in adhesion already at low serum concentrations intracellular bacteria were present in both cell types regardless of the serum components used to opsonize *S. aureus*. Besides opsonic receptors, phagocytosis by adhering cells also involved a mechanism that was independent of opsonization and might be associated to non-opsonic receptors, like C-type lectins, selectins, or scavenger receptors (28,48–51). For instance, expression of adhesion molecules in neutrophils increases to favor cells movements on surfaces. At the same time, these receptors also play a role in the first steps of the phagocytosis process (52). Although macrophages showed a higher expression of CR4/CD11c, the main

receptor involved in cells adhesion (53–55), change in assay conditions did not affect its expression in both phagocytes (**Figure 2**). It should also be noted that cells were detached before assessing receptor expression, therefore our results might not be fully representative of an adherent cell. On the other hand, macrophages are known for their opsonin-independent phagocytosis of particles (9,48,56), which might explain their higher bacterial uptake observed in absence of serum.

As previously described by our group, the values derived from the geometric mean of the GFP signal rather than the proportion of GFP+ cells better describe the phagocytic capacity of neutrophils and macrophages (32). For instance, cells with 1 or 10 intracellular bacteria would equally count as GFP+ events but they would generate different fluorescence intensities. However, because of the great difference in autofluorescence we had to normalize the data, according to the formula adapted from (34,35), to allow a fair comparison between the two phagocytes (**Figure 3A**). Nonetheless, overlaying the histograms derived by the GFP signal at each condition we could observe that increase in the NHS concentrations used to opsonize bacteria correlates to a shift of events from the left (GFP-) to the right (GFP+) side of the axis, and therefore to an increase of the intensity of the GFP signal (**Supplementary figure 3**). This serum concentration-dependent uptake of *S. aureus* was more evident in neutrophils than macrophages, as these last ones showed an higher non-opsonic uptake of bacteria in assays in adhesion. Therefore, interpretation of the results via histograms representation confirmed the validity of the formula used to compensate for cell type's autofluorescence.

To further validate our results, we measured the phagocytic capacity of the two cell types with different methods that did not rely on fluorescence parameters. Firstly, we determined phagocytosis by light microscopy and counted the number of intracellular bacteria within each cell. Secondly, we shifted the flow cytometry analysis from the proportion of cells taking up bacteria to the analysis of free remaining extracellular bacteria and counted the number of non-phagocytosed bacteria in flow cytometry. Due to assay limitation, this last method could not be used with cells in adhesion as extracellular bacteria were removed to detach cells before analysis.

When the assays were performed in suspension, all three methods consistently showed that neutrophils engulfed more bacteria than macrophages. According to the method used, the difference in bacterial uptake was 2 to 5 times higher in neutrophils than macrophages (**Figure 4B**, **5A**, and **6A**). Instead, when the assays were performed in adhesion, the difference in phagocytosis between the two cell types was less evident. While flow cytometry analysis showed a comparable number of intracellular bacteria between neutrophils and macrophages, a slightly higher number of intracellular *S. aureus* was counted in macrophages after microscopy observations (**Figures 4D**, **5B**). Discrepancy in results might be ascribed to limitations of flow cytometry in detecting only engulfed bacteria. Both extracellular bacteria in contact with the cell membrane and intracellular bacteria are recognized as GFP+ events. However, light microscopy images showed only cells with intracellular bacteria. Similar conclusions were drawn in previous studies where phagocytosis of *S.*

aureus by neutrophils (32) and macrophages (57) was studied by flow cytometry and confirmed by confocal microscopy. Alternative methods can be used to discriminate phagocytosed from adherent or extracellular particles. For example, pHrodo labelling relies on acidification of phagosomes/endosomes to detect phagocytosis (58). With this analysis method, Lea *et al.* observed that neutrophils phagocytosed more bacteria than macrophages when cultured in adhesion (59).

Besides a higher bacterial uptake in suspension, neutrophils showed a faster and stronger activation of the microbicidal mechanisms compared to macrophages. Differently, in adhesion no killing activity could be observed in both cell types in the time frame tested (**Figure 7**). As non-opsonic receptors are also involved in bacterial recognition, we might speculate that their activation is not closely associated with the activation of antibacterial mechanisms in immune cells (60,61). This might delay the activation of the killing process in adhering cells. For instance, in previous work, we observed killing of intracellular *S. aureus* by macrophages after 24 h (57).Therefore we, and others, conclude that neutrophils are fast killers and macrophages take far more time to achieve this, but also live longer (9,13,59).

All above conclusions are based on experiments after bacteria were opsonized with complement proteins and polyclonal antibodies normally found in the serum of healthy individuals. However, use of monoclonal antibodies (mAb) as a therapeutic treatment for *S. aureus* might further improve bacterial recognition and phagocytosis by immune cells, as previously demonstrated by Zwarthoff *et al.*, (62). This could eventually lead to the development of long-term immunity (63). As we showed that assay conditions *in vitro* affect the antibacterial response of phagocytes, this might be true also when testing the efficacy of mAbs against *S. aureus*. Use of more reliable *in vitro* tests, involving primary human neutrophils and macrophages, might correlate with an higher success rate in vaccines development. So far, despite numerous mAb targeting different *S. aureus*' surface antigens, secreted toxins, and immune evasion proteins are currently under investigation in clinical trials (64–67), yet none received regulatory approval (25).

Acknowledgements

The collaboration project is cofounded by the PPP Allowance made available by Health~Holland, Top Sector Life Sciences & Health, to stimulate public-private partnerships. This research also received co-funding from the EU's H2020 research and innovation program under Marie S. Curie cofund RESCUE grant agreement No 801540. This publication is also part of the project DARTBAC (with project number NWA.1292.19.354) of the research program NWA-ORC which is (partly) financed by the Dutch Research Council (NWO).

References

1. Cavaillon J-M. The historical milestones in the understanding of leukocyte biology initiated by Elie Metchnikoff. J Leukoc Biol (2011) 90:413–424. doi: 10.1189/jlb.0211094
2. Silva MT, Correia-Neves M. Neutrophils and macrophages: The main partners of phagocyte cell systems. Front Immunol (2012) 3:2008–2013. doi: 10.3389/fimmu.2012.00174
3. Silva MT. When two is better than one: macrophages and neutrophils work in concert in innate immunity as complementary and cooperative partners of a myeloid phagocyte system. J Leukoc Biol (2010) 87:93–106. doi: 10.1189/jlb.0809549
4. Rosowski EE. Determining macrophage versus neutrophil contributions to innate immunity using larval zebrafish. DMM Disease Models and Mechanisms (2020) 13: doi: 10.1242/dmm.041889
5. Verma AK, Bansal S, Bauer C, Muralidharan A, Sun K. Influenza Infection Induces Alveolar Macrophage Dysfunction and Thereby Enables Noninvasive Streptococcus pneumoniae to Cause Deadly Pneumonia . The Journal of Immunology (2020) 205:1601–1607. doi: 10.4049/jimmunol.2000094
6. Jorch SK, Surewaard BGJ, Hossain M, Peiseler M, Deppermann C, Deng J, Bogoslowski A, van der Wal F, Omri A, Hickey MJ, et al. Peritoneal GATA6+ macrophages function as a portal for Staphylococcus aureus dissemination. Journal of Clinical Investigation (2019) 129:4643–4656. doi: 10.1172/JCI127286
7. Ridder MJ, McReynolds AKG, Dai H, Pritchard MT, Markiewicz MA, Bose JL. Kinetic Characterization of the Immune Response to Methicillin-Resistant Staphylococcus aureus Subcutaneous Skin Infection. Infect Immun (2022) doi: 10.1128/iai.00065-22
8. Rosales C. Neutrophil: A cell with many roles in inflammation or several cell types? Front Physiol (2018) 9:1–17. doi: 10.3389/fphys.2018.00113
9. Pidwill GR, Gibson JF, Cole J, Renshaw SA, Foster SJ. The Role of Macrophages in Staphylococcus aureus Infection. Front Immunol (2021) 11:1–30. doi: 10.3389/fimmu.2020.620339
10. Koenderman L, Tesselaar K, Vrisekoop N. Human neutrophil kinetics: a call to revisit old evidence. Trends Immunol (2022) 43:868–876. doi: 10.1016/J.IT.2022.09.008
11. Patel AA, Ginhoux F, Yona S. Monocytes, macrophages, dendritic cells and neutrophils: an update on lifespan kinetics in health and disease. Immunology (2021) 163:250–261. doi: 10.1111/imm.13320
12. Amulic B, Cazalet C, Hayes GL, Metzler KD, Zychlinsky A. Neutrophil function: From mechanisms to disease. Annu Rev Immunol (2012) 30:459–489. doi: 10.1146/annurev-immunol-020711-074942
13. de Jong NWM, van Kessel KPM, van Strijp JAG. Immune Evasion by Staphylococcus aureus. Microbiol Spectr (2019) 7:1–27. doi: 10.1128/microbiolspec.gpp3-0061-2019
14. Horn J, Stelzner K, Rudel T, Fraunholz M. Inside job: Staphylococcus aureus host-pathogen interactions. International Journal of Medical Microbiology (2018) 308:607–624. doi: 10.1016/j.ijmm.2017.11.009
15. Pietrocola G, Nobile G, Rindi S, Speziale P. Staphylococcus aureus manipulates innate immunity through own and host-expressed proteases. Front Cell Infect Microbiol (2017) 7:1–15. doi: 10.3389/fcimb.2017.00166
16. Fraunholz M, Sinha B. Intracellular Staphylococcus aureus: live-in and let die. Front Cell Infect Microbiol (2012) 2:43. doi: 10.3389/fcimb.2012.00043
17. Strobel M, Pförtner H, Tuchscherr L, Völker U, Schmidt F, Kramko N, Schnittler HJ, Fraunholz MJ, Löffler B, Peters G, et al. Post-invasion events after infection with

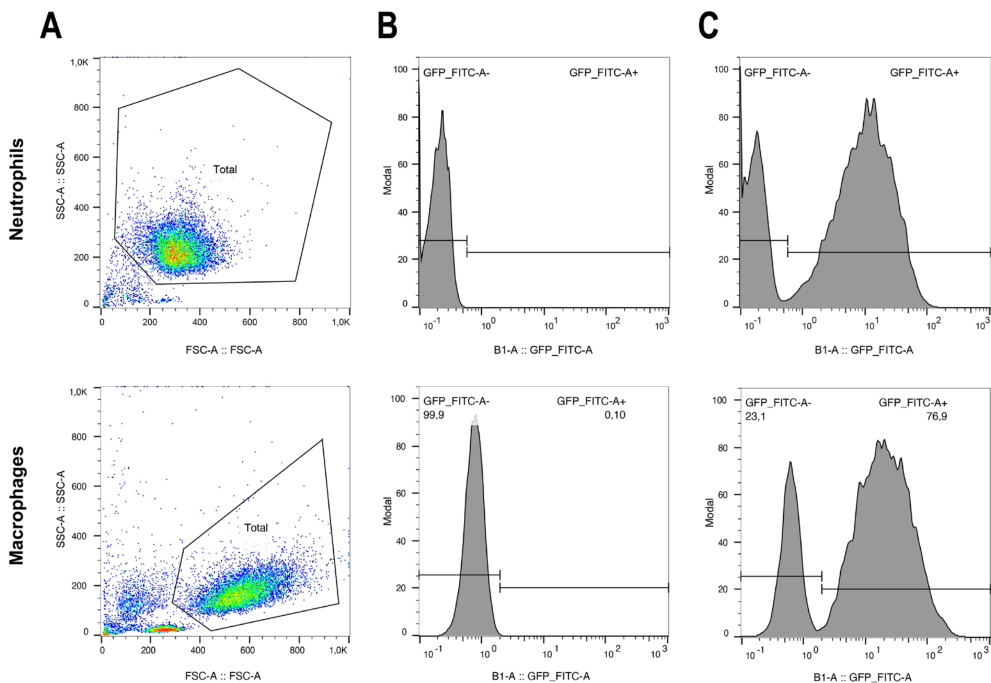
Staphylococcus aureus are strongly dependent on both the host cell type and the infecting S. aureus strain. Clinical Microbiology and Infection (2016) 22:799–809. doi: 10.1016/j.cmi.2016.06.020
18. Flannagan RS, Heit B, Heinrichs DE. Intracellular replication of Staphylococcus aureus in mature phagolysosomes in macrophages precedes host cell death, and bacterial escape and dissemination. Cell Microbiol (2016) 18:514–535. doi: 10.1111/cmi.12527
19. Flannagan RS, Heit B, Heinrichs DE. Antimicrobial mechanisms of macrophages and the immune evasion strategies of Staphylococcus aureus. (2015). 826–868 p. doi: 10.3390/pathogens4040826
20. Josse J, Valour F, Maali Y, Diot A, Batailler C, Ferry T, Laurent F. Interaction between staphylococcal biofilm and bone: How does the presence of biofilm promote prosthesis loosening? Front Microbiol (2019) 10: doi: 10.3389/fmicb.2019.01602
21. Shaw GB. The Doctor's Dilemma. 1st Edition Thus. Penguin Books (1958).
22. Tsao LC, Force J, Hartman ZC. Mechanisms of therapeutic antitumor monoclonal antibodies. Cancer Res (2021) 81:4641–4651. doi: 10.1158/0008-5472.CAN-21-1109
23. Jin S, Sun Y, Liang X, Gu X, Ning J, Xu Y, Chen S, Pan L. Emerging new therapeutic antibody derivatives for cancer treatment. Signal Transduct Target Ther (2022) 7: doi: 10.1038/s41392-021-00868-x
24. Rogers LM, Veeramani S, Weiner GJ. Complement in monoclonal antibody therapy of cancer. Immunol Res (2014) 59:203–210. doi: 10.1007/s12026-014-8542-z
25. Clegg J, Soldaini E, McLoughlin RM, Rittenhouse S, Bagnoli F, Phogat S. Staphylococcus aureus Vaccine Research and Development: The Past, Present and Future, Including Novel Therapeutic Strategies. Front Immunol (2021) 12:1–19. doi: 10.3389/fimmu.2021.705360
26. Proctor RA. Immunity to Staphylococcus aureus : Implications for Vaccine Development . Microbiol Spectr (2019) 7: doi: 10.1128/microbiolspec.gpp3-0037-2018
27. Salgado-Pabón W, Schlievert PM. Models matter: The search for an effective Staphylococcus aureus vaccine. Nat Rev Microbiol (2014) 12:585–591. doi: 10.1038/nrmicro3308
28. Lu T, Porter AR, Kennedy AD, Kobayashi SD, DeLeo FR. Phagocytosis and killing of staphylococcus aureus by human neutrophils. J Innate Immun (2014) 6:639–649. doi: 10.1159/000360478
29. Fowler VG, Allen KB, Moreira ED, Moustafa M, Isgro F, Boucher HW, Corey GR, Carmeli Y, Betts R, Hartzel JS, et al. Effect of an investigational vaccine for preventing Staphylococcus aureus infections after cardiothoracic surgery: A randomized trial. JAMA - Journal of the American Medical Association (2013) 309:1368–1378. doi: 10.1001/jama.2013.3010
30. Zeng H, Yang F, Feng Q, Zhang J, Gu J, Jing H, Cai C, Xu L, Yang X, Xia X, et al. Rapid and broad immune efficacy of a recombinant five-antigen vaccine against Staphylococcus aureus infection in animal models. Vaccines (Basel) (2020) 8: doi: 10.3390/vaccines8010134
31. Surewaard BGJ, van Strijp JAG, Nijland R. Studying interactions of Staphylococcus aureus with neutrophils by flow cytometry and time lapse microscopy. J Vis Exp (2013)1–5. doi: 10.3791/50788
32. Boero E, Brinkman I, Juliet T, van Yperen E, van Strijp JAG, Rooijakkers SHM, van Kessel KPM. Use of Flow Cytometry to Evaluate Phagocytosis of Staphylococcus aureus by Human Neutrophils. Front Immunol (2021) 12:1–15. doi: 10.3389/fimmu.2021.635825
33. Zwarthoff SA, Magnoni S, Aerts PC, van Kessel KPM, Rooijakkers SHM. "Method for

Depletion of IgG and IgM from Human Serum as Naive Complement Source.," Methods in Molecular Biology. Humana Press Inc. (2021). p. 21–32 doi: 10.1007/978-1-0716-1016-9_2
34. Reinhard NR, Mastop M, Yin T, Wu Y, Bosma EK, Gadella TWJ, Goedhart J, Hordijk PL. The balance between Gαi-Cdc42/Rac and Gα1/2/13-RhoA pathways determines endothelial barrier regulation by sphingosine-1-phosphate. Mol Biol Cell (2017) 28:3371–3382. doi: 10.1091/mbc.E17-03-0136
35. Postma M, Roelofs J, Goedhart J, Gadella TWJ, Visser AJWG, Van Haastert PJM. Uniform cAMP Stimulation of Dictyostelium Cells Induces Localized Patches of Signal Transduction and Pseudopodia. Mol Biol Cell (2003) 14:5019–5027. doi: 10.1091/mbc.E03-08
36. Grecian R, Whyte MKB, Walmsley SR. The role of neutrophils in cancer. Br Med Bull (2018) 128:5–14. doi: 10.1093/bmb/ldy029
37. McKenna E, Mhaonaigh AU, Wubben R, Dwivedi A, Hurley T, Kelly LA, Stevenson NJ, Little MA, Molloy EJ. Neutrophils: Need for Standardized Nomenclature. Front Immunol (2021) 12:1–14. doi: 10.3389/fimmu.2021.602963
38. Kuhns DB, Priel DAL, Chu J, Zarember KA. Isolation and Functional Analysis of Human Neutrophils. Curr Protoc Immunol (2015) 111:7.23.1-7.23.16. doi: 10.1002/0471142735.im0723s111
39. Safi W, Kuehnl A, Nüssler A, Eckstein HH, Pelisek J. Differentiation of human CD14+ monocytes: an experimental investigation of the optimal culture medium and evidence of a lack of differentiation along the endothelial line. Exp Mol Med (2016) 48: doi: 10.1038/EMM.2016.11
40. Kerntke C, Nimmerjahn F, Biburger M. There Is (Scientific) Strength in Numbers: A Comprehensive Quantitation of Fc Gamma Receptor Numbers on Human and Murine Peripheral Blood Leukocytes. Front Immunol (2020) 11:1–17. doi: 10.3389/fimmu.2020.00118
41. Wang Y, Jönsson F. Expression, role, and regulation of neutrophil Fcγ receptors. Front Immunol (2019) 10:1–13. doi: 10.3389/fimmu.2019.01958
42. Vandendriessche S, Cambier S, Proost P, Marques PE. Complement Receptors and Their Role in Leukocyte Recruitment and Phagocytosis. Front Cell Dev Biol (2021) 9:1–25. doi: 10.3389/fcell.2021.624025
43. Aubin JE. Autofluorescence of Viable Cultured Mammalian Cells. (1979). 36–43 p.
44. Li F, Yang M, Wang L, Williamson I, Tian F, Qin M, Shah PK, Sharifi BG. Autofluorescence contributes to false-positive intracellular Foxp3 staining in macrophages: A lesson learned from flow cytometry. J Immunol Methods (2012) 386:101–107. doi: 10.1016/j.jim.2012.08.014
45. Benson RC, Meyer RA, Zaruba ME, Mckhann GM. Cellular Autofluorescence-Is It Due To vi2. (1979). 44–48 p.
46. Vidarsson G, Dekkers G, Rispens T. IgG subclasses and allotypes: From structure to effector functions. Front Immunol (2014) 5: doi: 10.3389/fimmu.2014.00520
47. Pfisterer K, Shaw LE, Symmank D, Weninger W. The Extracellular Matrix in Skin Inflammation and Infection. Front Cell Dev Biol (2021) 9:1–19. doi: 10.3389/fcell.2021.682414
48. Uribe-Querol E, Rosales C. Phagocytosis: Our Current Understanding of a Universal Biological Process. Front Immunol (2020) 11:1–13. doi: 10.3389/fimmu.2020.01066
49. Cougoule C, Wiedemann A, Lim J, Caron E. Phagocytosis, an alternative model system for the study of cell adhesion. Semin Cell Dev Biol (2004) 15:679–689. doi: 10.1016/j.

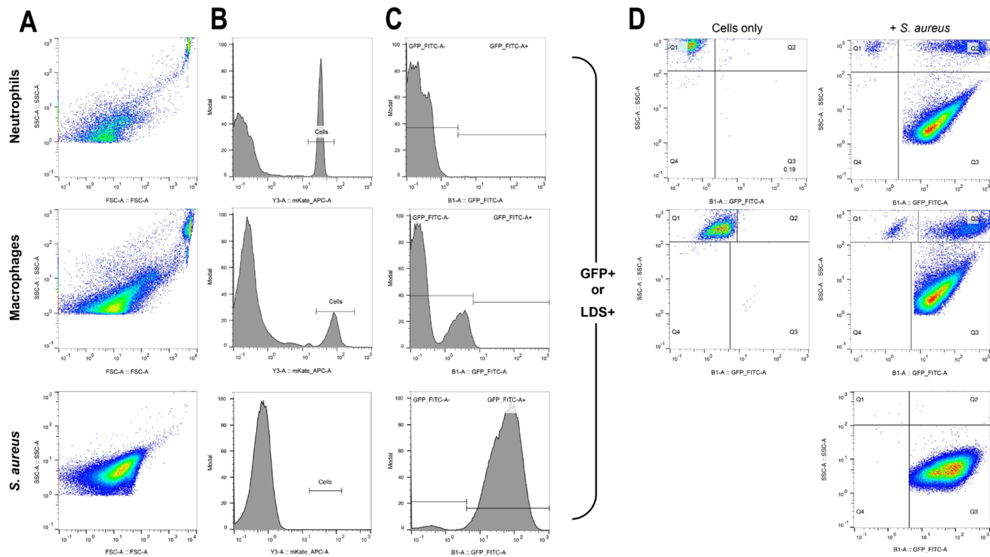
semcdb.2004.09.001
50. Hoffmann C, Ohlsen K, Hauck CR. Integrin-mediated uptake of fibronectin-binding bacteria. Eur J Cell Biol (2011) 90:891–896. doi: 10.1016/J.EJCB.2011.03.001
51. Taban Q, Mumtaz PT, Masoodi KZ, Haq E, Ahmad SM. Scavenger receptors in host defense: from functional aspects to mode of action. Cell Communication and Signaling (2022) 20: doi: 10.1186/s12964-021-00812-0
52. Kraus RF, Gruber MA. Neutrophils—From Bone Marrow to First-Line Defense of the Innate Immune System. Front Immunol (2021) 12:1–35. doi: 10.3389/fimmu.2021.767175
53. Lukácsi S, Nagy-Baló Z, Erdei A, Sándor N, Bajtay Z. The role of CR3 (CD11b/CD18) and CR4 (CD11c/CD18) in complement-mediated phagocytosis and podosome formation by human phagocytes. Immunol Lett (2017) 189:64–72. doi: 10.1016/j.imlet.2017.05.014
54. Lukácsi S, Gerecsei T, Balázs K, Francz B, Szabó B, Erdei A, Bajtay Z. The differential role of CR3 (CD11b/CD18) and CR4 (CD11c/CD18) in the adherence, migration and podosome formation of human macrophages and dendritic cells under inflammatory conditions. PLoS One (2020) 15:1–21. doi: 10.1371/journal.pone.0232432
55. Sándor N, Lukácsi S, Ungai-Salánki R, Orgován N, Szabó B, Horváth R, Erdei A, Bajtay Z. CD11c/CD18 dominates adhesion of human monocytes, macrophages and dendritic cells over CD11b/CD18. PLoS One (2016) 11:1–17. doi: 10.1371/journal.pone.0163120
56. Schulz D, Severin Y, Zanotelli VRT, Bodenmiller B. In-Depth Characterization of Monocyte-Derived Macrophages using a Mass Cytometry-Based Phagocytosis Assay. Sci Rep (2019) 9: doi: 10.1038/s41598-018-38127-9
57. Cecotto L, van Kessel K, Wolfert MA, Vogely C, van der Wal B, Weinans H, van Strijp J, Yavari SA. Antibacterial and anti-inflammatory properties of host defense peptides against Staphylococcus aureus. iScience (2022) 25:105211. doi: 10.1016/j.isci.2022.105211
58. Kapellos TS, Taylor L, Lee H, Cowley SA, James WS, Iqbal AJ, Greaves DR. A novel real time imaging platform to quantify macrophage phagocytosis. Biochem Pharmacol (2016) 116:107–119. doi: 10.1016/j.bcp.2016.07.011
59. Lea S, Gaskell R, Hall S, Maschera B, Hessel E, Singh D. Assessment of bacterial exposure on phagocytic capability and surface marker expression of sputum macrophages and neutrophils in COPD patients. Clin Exp Immunol (2021) 206:99–109. doi: 10.1111/cei.13638
60. Nguyen GT, Green ER, Mecsas J. Neutrophils to the ROScue: Mechanisms of NADPH oxidase activation and bacterial resistance. Front Cell Infect Microbiol (2017) 7: doi: 10.3389/fcimb.2017.00373
61. Zani IA, Stephen SL, Mughal NA, Russell D, Homer-Vanniasinkam S, Wheatcroft SB, Ponnambalam S. Scavenger receptor structure and function in health and disease. Cells (2015) 4:178–201. doi: 10.3390/cells4020178
62. Zwarthoff SA, Widmer K, Kuipers A, Strasser J, Ruyken M, Aerts PC, C de Haas CJ, Ugurlar D, den Boer MA, Vidarsson G, et al. C1q binding to surface-bound IgG is stabilized by C1r 2 s 2 proteases. Proc Natl Acad Sci U S A (2021) 118: doi: 10.1073/pnas.2102787118/-/DCSupplemental
63. Pelegrin M, Naranjo-Gomez M, Piechaczyk M. Antiviral Monoclonal Antibodies: Can They Be More Than Simple Neutralizing Agents? Trends Microbiol (2015) 23:653–665. doi: 10.1016/j.tim.2015.07.005
64. Peck M, Rothenberg ME, Deng R, Lewin-Koh N, She G, Kamath A V, Carrasco-Triguero M, Saad O, Castro A, Teufel L, et al. A Phase 1, Randomized, Single-Ascending-Dose Study To Investigate the Safety, Tolerability, and Pharmacokinetics of DSTA4637S, an Anti-Staphylococcus aureus Thiomab Antibody-Antibiotic Conjugate, in Healthy Volunteers.

Antimicrob Agents Chemother (2019) 63:e02588-18. doi: 10.1128/AAC.02588-18
65. François B, Mercier E, Gonzalez C, Asehnoune K, Nseir S, Fiancette M, Desachy A, Plantefève G, Meziani F, de Lame PA, et al. Safety and tolerability of a single administration of AR-301, a human monoclonal antibody, in ICU patients with severe pneumonia caused by Staphylococcus aureus: first-in-human trial. Intensive Care Med (2018) 44:1787–1796. doi: 10.1007/s00134-018-5229-2
66. François B, Jafri HS, Chastre J, Sánchez-García M, Eggimann P, Dequin PF, Huberlant V, Viña Soria L, Boulain T, Bretonnière C, et al. Efficacy and safety of suvratoxumab for prevention of Staphylococcus aureus ventilator-associated pneumonia (SAATELLITE): a multicentre, randomised, double-blind, placebo-controlled, parallel-group, phase 2 pilot trial. Lancet Infect Dis (2021) 21:1313–1323. doi: 10.1016/S1473-3099(20)30995-6
67. Yu X-Q, Robbie GJ, Wu Y, Esser MT, Jensen K, Schwartz HI, Bellamy T, Hernandez-Illas M, Jafri HS. Safety, Tolerability, and Pharmacokinetics of MEDI4893, an Investigational, Extended-Half-Life, Anti-Staphylococcus aureus Alpha-Toxin Human Monoclonal Antibody, in Healthy Adults. Antimicrob Agents Chemother (2017) 61:e01020-16. doi: 10.1128/AAC.01020-16

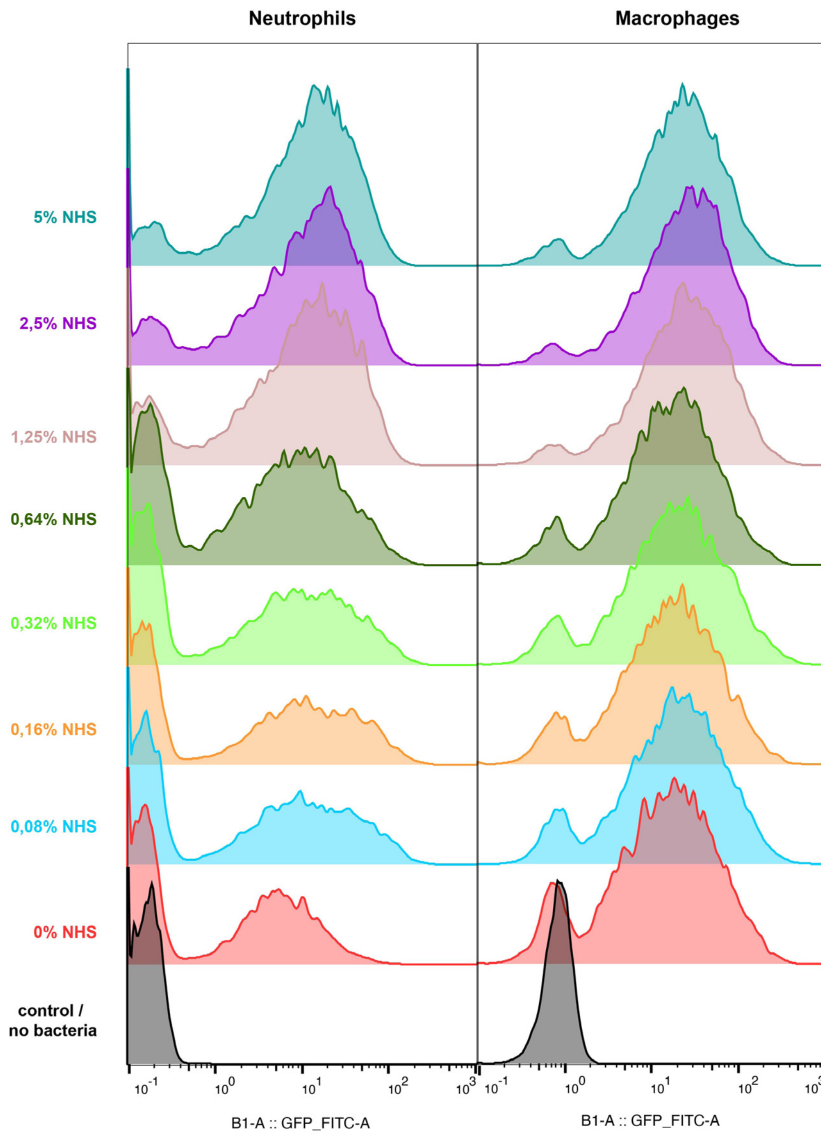
Supplementary Figures



Supplementary Figure 1. Gating strategy to compare neutrophils (upper row) and macrophages (lower row) phagocytosis by flow cytometry. (**A**) Selection of cells population (Total gate) in the linear FSC and SSC. (**B**) Histogram setting GFP fluorescence baseline for cells incubated without bacteria. (**C**) Proportion of GFP-negative (left peak) and GFP-positive (right peak) cells, with and without intracellular bacteria respectively. Same gating strategy was applied for both neutrophils and macrophages.



Supplementary Figure 2. Gating strategy to compare neutrophils and macrophages phagocytosis by quantification of non-phagocytosed bacteria. (**A**) Acquisition of events by flow cytometer in the logarithmic FSC and SSC. (**B**) Histogram defining LDS-positive cells. (**C**) Histogram setting GFP fluorescence baseline for cells incubated without bacteria or bacteria only. (**D**) Gating selection of GFP-positive or LDS-positive events visualized based on the logarithmic SSC and GFP parameters. Gating shows: cells without intracellular bacteria (Q1); Cells with intracellular bacteria (Q2); Non-phagocytosed bacteria (Q3). Same gating strategy was applied for neutrophils (upper row), macrophages (middle row), and S. aureus only (lower row).



Supplementary figure 3. Representative histograms of a phagocytosis assay in adhesion showing the variation of GFP signal after bacterial opsonization with NHS.

CHAPTER
5

Unraveling the therapeutic potential of monoclonal antibodies: comparing neutrophils and macrophages in the context of *Staphylococcus aureus* infections

Leonardo Cecotto[1,2], Elisabeth H. Jin[2], Claudio Passerini[2], Carla J.C. de Haas[2], Piet C. Aerts[2], Kok P.M. van Kessel[2], H.Charles Vogely[1], Bart C.H. van der Wal[1], Suzan H.M. Rooijakkers[2], Saber Amin Yavari[1,3], Jos A.G. van Strijp[2]

[1] Department of Orthopedics, University Medical Center Utrecht, Utrecht, The Netherlands
[2] Department of Medical Microbiology, University Medical Center Utrecht, Utrecht, The Netherlands
[3] Regenerative Medicine Centre Utrecht, Utrecht University, Utrecht, The Netherlands

Manuscript in preparation

Abstract

Staphylococcus aureus is known for its antibiotic resistance, immune evasion strategies, and ability to survive within host cells. Pathogen-specific monoclonal antibodies (mAb) have been proposed as a potential therapeutic strategy to enhance the antibacterial functions of phagocytes against *S. aureus*. In this study, IgG1- and IgG3-mAbs targeting different cell-wall structures were generated and tested on phagocytes under different *in vitro* conditions. When phagocytosis was studied in adhesion, no clear difference was observed between neutrophils and macrophages or IgG1- and IgG3-mediated uptake. On the contrary, assays in suspension clearly demonstrated the distinct roles of opsonins in promoting bacterial uptake. Expression of virulence factors SpA and Sbi by *S. aureus* inhibited IgG1- but not IgG3-mediated phagocytosis. Consequently, bacterial opsonization with IgG3 mAbs improved phagocytosis, ROS production, and partially killing of *S. aureus* by both neutrophils and macrophages. Overall, neutrophils showed stronger antibacterial properties than macrophages, which may represent an ideal environment for *S. aureus* intracellular survival and persistence. To prevent this, we designed bispecific antibodies (bAbs) aiming to specifically deliver bacteria to neutrophils or macrophages. Unfortunately, the current choices failed to drive phagocyte-specific uptake of *S. aureus*. In conclusion, we showed that *in vitro* assay conditions affect the antibacterial functions of phagocytes, and suspension assays better reflect the antibacterial role of mAb-mediated phagocytosis than adhesion assays. These findings provide insight into the potential therapeutic applications of mAbs for *S. aureus* infections and highlight the distinct roles of neutrophils and macrophages in infection resolution.

Introduction

Staphylococcus aureus is a Gram-positive bacterium that belongs to the ESKAPE family of pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* species) known for their high virulence and antibiotic resistance, posing a global threat to human health (1). *S. aureus* is responsible for various infections in the skin, soft tissues, and biomaterials, particularly following orthopedic surgeries involving implants (2,3). In the pursuit of alternatives to traditional antibacterial treatments, pathogen-specific antibodies represent a promising solution to reduce the risk of resistance development while boosting our own immune system functions. This is exemplified by the successful vaccines against *Neisseria meningitidis* or *Haemophilus influenzae B* and many other viral vaccines that are now adopted in common medical practice to prevent and control infectious diseases (4). Hence, it is possible that monoclonal antibodies (mAb) against *S. aureus* may lead to long-term immunity (5).

Immunoglobulin G (IgG) is one of the most abundant proteins in human serum (6). Upon binding to the bacterial surface, circulating IgGs can directly stimulate phagocytosis via interaction with Fcγ receptors (FcγR) on phagocytes. Furthermore, this class of antibodies can activate the classical pathway of the complement system, leading to deposition of C3b (and iC3b) on the bacterial surface to favor complement receptors (CR)-mediated uptake by phagocytes (7). Development of IgG monoclonal antibodies (mAb) that specifically target epitopes on the surface of pathogens has been shown to enhance bacterial recognition by phagocytes and therefore clearance of the infection (8–10). To successfully improve the antibacterial functions of the immune system while avoiding resistance mechanisms, mAbs should be designed to target epitopes on the bacterial surface that are less prone to modifications.

Previous studies identified mAbs targeting the glycosyl modifications on cell-wall anchored teichoic acids (WTA, mAb 4461 and 4497) (11,12) and the serine-aspartate dipeptide repeat portion of proteins (SDR, mAb rF1) (13) on the surface of *S. aureus*. Both targets play an essential role in *S. aureus* survival and virulence. Almost 60% of the dry weight of the pathogen is represented by the cell wall, made of a peptidoglycan layer and teichoic acids (14). Although the composition of teichoic acids varies according to the bacterial strain (15), WTA showed limited structural variation among different *S. aureus* strains (16). WTA consists of a polymerized ribitol phosphate (RboP) backbone that can be modified by the addition of positively charged D-alanine and N-acetylglucosamine (GlcNAc) residues as a defense mechanism against host-mediated immune detection and clearance (17). On the other hand, microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) represent a family of cell wall-anchored proteins used by *S. aureus* to adhere to host extracellular matrix (ECM) components, leading to invasion of host

cells and tissue colonization. Among the MSCRAMMs group, *S. aureus* expresses several proteins, such as clumping factor A (ClfA), ClfB, SdrC, SdrD, and SdrE characterized by a large portion of SDR repeats (18,19). These SDR domains are heavily glycosylated to protect the proteins from host proteases, like cathepsin G, preserving bacterial host tissue interactions (13).

Use of mAbs to opsonize *S. aureus* have been shown to enhance bacterial uptake and killing by neutrophils (8,20,21). Nonetheless, the innate immune response against *S. aureus* involves collaboration between neutrophils and macrophages (22,23). Neutrophils have a short life span but a strong and fast activation of microbicidal mechanisms, enabling them to drastically reduce most invading pathogens. In contrast, macrophages with a longer life span and weaker antibacterial functions fight bacteria that survived neutrophils attacks (24,25). For the same reasons, neutrophils are less susceptible than macrophages to *S. aureus* intracellular survival and associated risk of recalcitrant infections. Consequently, an ideal antibacterial therapy may start with mAb targeting *S. aureus* and evolve towards the development of bispecific antibodies (bAbs), able to induce the uptake of opsonized pathogens preferentially by the cell type with the strongest capacity to clear the infection.

In this study we aimed to characterize and compare the enhancement in the antibacterial functions of neutrophils and macrophages after opsonization of *S. aureus* with the mAbs rF1, 4461, and 4497. As we previously showed (26), human neutrophils and macrophages' antibacterial functions change according to the *in vitro* setup adopted. Therefore, in this study, we evaluated the mAb-contribution to elimination of *S. aureus* by neutrophils and macrophages, studied in suspension and adherent assays. Finally, as a proof of concept, we tested the ability of bAbs targeting *S. aureus* and neutrophils or *S. aureus* and macrophages to drive phagocyte-specific bacterial uptake.

Materials and Methods

Isolation of monocyte-derived macrophages

Blood from healthy human donors was supplied by the Dutch blood bank (Sanquin, Amsterdam, The Netherlands). Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats using Ficoll-Paque (Cytiva, 17544203) density centrifugation. Monocytes were positively selected by magnetic-activated cell sorting (MACS) with anti-CD14 labelled microbeads (Miltenyi Biotec, 130050201) according to manufacturer instructions.

Isolated monocytes were seeded in a 24-well plate at a density of 3×10^5 cells/well when performing the assays in adhesion. They were also seeded in a 6 cm cell culture-treated petri dish at a density of 5×10^5 cells/well when performing the assays in suspension. Monocytes were differentiated to macrophages by culture for 7 days at 37°C, 5% CO_2 in RPMI (Gibco, 52400) supplemented with 10% hyclone fetal bovine serum (FBS, Biowest, HYCLSV30160), 100 U/mL penicillin and 100 µg/mL streptomycin (1% p/s, Gibco), and 40 ng/mL human recombinant M-CSF

(Peprotech, 300-25). Culture media was refreshed on days 3 and 5 of differentiation.

After 7 days of differentiation, when performing assays in adhesion, macrophages were left on the same culture plate in RPMI + 10% FBS (RPMI-FBS). Differently, when performing assays in suspension, macrophages were detached using ice-cold 10 mM DPBS/EDTA combined with gentle scraping. Cells were pelleted for 5 min, 500 x g, resuspended in RPMI with 0,05% human serum albumin (RPMI-HSA, ©, Sanquin), and counted before performing the experiment.

Isolation of human neutrophils

Neutrophils isolation was performed as previously described by Surewaard *et al.* (27). Briefly, blood from healthy human donors was collected by venipuncture and collected in sodium heparin tubes after informed consent. Approval from the Medical Ethics Committee of the University Medical Center Utrecht was obtained (METC protocol 07-125/C approved on March 1, 2010). Neutrophils were isolated from whole blood using a dual Ficoll/Histopaque (Sigma, 11191) density centrifugation and residual erythrocytes were lysed by hyper osmotic shock with cold deionized water. Isolated neutrophils were kept in suspension in RPMI-HSA when performing the assays in suspension. On the other hand, when performing the assays in adhesion, neutrophils were seeded in a 24-well plate at a density of 3×10^5 cells/well in RPMI-FBS and allowed to adhere to the plastic bottom for >1 h before performing the experiment.

Bacterial culture

The majority of experiments were run using *Staphylococcus aureus* strain Newman, transformed with a GFP-expressing plasmid pCM29 to constitutively express GFP, as previously described (28). Alternatively, we used mCherry-expressing *S. aureus* Newman for reactive oxygen species (ROS) assays, gamma-irradiated *S. aureus* Newman to verify the markers expression on cells, or GFP-expressing *S. aureus* Newman with genes coding for protein A (SpA) and *S. aureus* binder of IgG (Sbi) knocked-out (29) to assess IgG1-mediated phagocytosis by neutrophils and macrophages.

All bacterial strains were grown overnight in Todd-Hewitt broth (THB) with 10 µg/mL chloramphenicol to reach stationary phase, except gamma-irradiated bacteria that were stored at OD=0,96 at -20°C and thawed on the day of the experiment. The day after, 500 µL of overnight bacterial culture was centrifuged for 2 min, 17000 x g at RT, and resuspended in RPMI-HSA (assay in suspension) or RPMI (assay in adhesion) to reach a final inoculum concentration of 1×10^7 colony-forming units (CFU)/mL.

Production of human monoclonal antibodies binding to *S. aureus*

IgG1 and IgG3 mAbs rF1, 4461, and 4497 were produced as described previously (20,21). As a negative control for antibody specificity, one antibody recognizing the hapten dinitrophenol (DNP) was produced (30).

To verify binding of mAbs to *S. aureus*, 1,5 x 10^5 bacteria were resuspended in RPMI-HSA in a round-bottom 96-well plate and combined with 7 nM of each antibody. Samples were incubated for 30 min at 4°C, shaking. After washing with buffer, mAbs binding to bacteria was verified by addition of a secondary antibody, AlexaFluor647 (AF647)-conjugated goat anti-human kappa F(ab)2, diluted 1:1000 (Southern Biotech, 2062-31). Samples were incubated with the secondary antibody for 30 min at 4°C, shaking. Then, samples were washed with buffer and fixed in 1,5% paraformaldehyde (PFA) in PBS. Samples were measured via flow cytometer (FACSVerse, BD) and data were analyzed with FlowJo (v.10.1., FlowJo LLC). Bacteria were selected based on logarithmic forward scatter (FSC) and side scatter (SSC) parameters and GFP expression. Binding affinity was determined based on the geometric mean of AF647.

Phagocytosis of *S. aureus* by neutrophils and macrophages

Neutrophils and macrophages phagocytosis ability was assessed with cells in suspension or adhering to the culture plate as previously described (26). Briefly, phagocytes were incubated with previously opsonized *S. aureus* at a cell-to-bacterium ratio of 1-10, meaning 10 bacteria per phagocyte. To study mAb-mediated phagocytosis, bacteria were opsonized for 15 min at 37°C with a range of concentrations expressed in nM of IgG1 and IgG3 mAbs. To study the role of complement- and antibody-mediated phagocytosis, mAbs were incubated with 1% of IgG- and IgM-depleted normal human serum (ΔNHS). As a positive control, bacteria were opsonized with 1% NHS, containing non-specific antibodies for *S. aureus* and active complement system. Phagocytes and bacteria were incubated at 37°C for 15 min in suspension assays, and 1 h in adhesion assays. Then, samples were fixed in 1,5% PFA and phagocytosis was measured with MACSquant VYB (Miltenyi Biotech) flow cytometer and data were analyzed with FlowJo. Gating strategy is summarized in **Supplementary figure 1**. Briefly, a total of 10000 events were collected for each sample gated on the total cell population based on forward scatter (FSC) and side scatter (SSC) parameters (**Supplementary figure 1A**). Samples without bacteria were used to set the GFP fluorescence baseline, while the geometric mean of the GFP signal was used to define the number of bacteria phagocytosed (**Supplementary figure 1C**). Due to intrinsic differences in autofluorescence between neutrophils and macrophages, the values derived from the geometric mean of the GFP signal were normalized according to the formula adapted from (31,32):

$$Fluorescence = \frac{X - X0}{\mathrm{X0}}$$

where X represents the values recorded for each condition in the presence of *S. aureus* and X0 represents the background value of cells without bacteria. This data normalization allowed a fair comparison of the phagocytic ability of neutrophils and macrophages.

Activation markers in neutrophils and macrophages

Pathogen-presentation via mAbs might play a role in priming and activation of phagocytes, and thereby influence their response to clearing the infection. Therefore, neutrophils were analyzed for their surface expression of CD11b-APC (BD, 550019) and CD62L-FITC (BD, 555543), and macrophages for their expression of CD38-FITC (BD, 560982), CD64-PE (Biolegend, 399503), and CD206-APC (Biolegend, 321110).

Following the protocol for assays in suspension, gamma-irradiated *S. aureus* was opsonized with 10 nM IgG1- and IgG3-mAbs, in the presence or absence of 5% ΔNHS, and incubated at a cell-to-bacteria ratio of 1-10 for 30 min with neutrophils and 4 h with macrophages at 37°C, shaking. As controls, cells were incubated without bacteria or with 5% NHS-opsonized bacteria. The expression of surface markers on macrophages is greatly affected by the stimulation time used, therefore 4 h represents the earliest time point useful to observe an effect (33).

All washing steps were performed with cold 0,1% BSA/PBS and centrifugation at 5 min, 500 x g. The staining solutions were prepared by diluting 1:30 the above-mentioned fluorochrome-conjugated antibodies in PBS with 0,1% BSA and 1% heat-inactivated NHS. As a negative control, a staining solution without antibodies was used. Cells were incubated with staining solutions for 30 min on ice, in the dark. Markers expression was measured via flow cytometer (FACSVerse, BD) and data analyzed using FlowJo. The values derived from the geometric mean of the fluorescent signal for each antibody were normalized by the control group without bacteria. Data were represented as a -fold change compared to control.

ROS production by neutrophils and macrophages

Besides priming phagocytes, we investigated the influence of mAbs on the activation of killing mechanisms by phagocytes by measuring the ROS production. Following the protocol for assays in suspension, mCherry-expressing *S. aureus* was opsonized with 10 nM IgG1- and IgG3-mAbs, in presence or absence of 5% ΔNHS, or with 5% NHS as a control. Then, bacteria were combined with phagocytes at a cell-to-bacteria ratio of 1-10, together with 625 ng/mL Dihydrorhodamine 123 (DHR123, Invitrogen, D23806). Samples were incubated for 30 min, at 37°C, 5% CO2. As controls, cells were incubated without bacteria, with 5% NHS-opsonized bacteria, or without bacteria and without DHR123. ROS production was determined based on the geometric mean of the GFP signal measured via MACSquant VYB (Miltenyi Biotech) flow cytometer and data analyzed with FlowJo. To fairly compare ROS production between the two phagocytes, the fluorescent signal was normalized on the values of the control without bacteria and DHR123, which defines cell's autofluorescence, according to the formula described for the phagocytosis assays.

Killing of *S. aureus* by neutrophils and macrophages

Neutrophils and macrophages' ability to kill intracellular *S. aureus* was only tested with cells in suspension. Bacteria were opsonized for 15 min at 37°C with 10 nM

IgG1 and IgG3 mAbs, in the presence or absence of 5% ΔNHS, and with 5% NHS as a positive control. Then, phagocytes and opsonized bacteria were combined in 1.5 mL siliconized tubes at a cell-to-bacterium ratio of 1-1 and incubated for 90 min at 37°C in the presence of 5% CO2. To quantify the number of bacteria killed, the cell membranes of the phagocytes were lysed by addition of ice-cold 0.3% saponin (Sigma, 47036) in deionized water. Samples were lysed for 5-10 min in ice, serially diluted in PBS and plated in duplicates. The next day, the number of colonies was counted. Data were normalized by the CFU/mL counted from the initial bacterial inoculum. They were expressed as the percentage of bacteria that survived killing by neutrophils and macrophages.

Production of bispecific antibodies targeting *S. aureus* and neutrophils or *S. aureus* and macrophages

The sequences to produce antibodies targeting neutrophils and macrophages were derived from the patents EP0585570A1 (CD66b) and US20080025913A1 (DC-SIGN). We used the duobody technology as developed by Genmab (34). Bispecific antibodies (bAbs) were generated by combining brF1 (*S. aureus*), bCD66b (neutrophils), and bDC-SIGN (macrophages). The antibodies were produced from a IgG1 backbone modified to prevent binding of SpA and Sbi and to allow the formation of the bispecific antibody. Two-point mutations, H435R and Y436F, were introduced in the VH region to prevent SpA and Sbi binding (35,36). To favor bAb formation, mutations 409R in brF1 and 405L in bCD66b and bDC-SIGN were introduced in the CH3 domain of the Fc region of the antibodies. Once the disulfide bonds in the hinge region are broken, the Fc chain of brF1 prefers to pair with the Fc chain of bCD66b or bDC-SIGN, rather than with itself (37).

bAbs were generated after incubation for 5 h at 31°C with 75 mM cysteamine hydrochloride (2-MEA, Sigma, M6500), a mild reducing agent used to break the disulfide bonds and allow antibodies recombination. The reaction resulted in the formation of two bAbs, brF1+bCD66b targeting *S. aureus* and neutrophils and brF1+bDC-SIGN targeting *S. aureus* and macrophages. As a control to verify efficient bAb formation, antibodies were incubated without 2-MEA to generate brF1 / bCD66b and brF1 / bDC-SIGN.

Finally, each bAb was incubated with 1 µg/mL Immunoglobulin G-degrading enzyme of Streptococcus pyogenes (IdeS) for 1 h at 37°C, to cleave the Fc region and generate F(ab)2 fragments of each bAb (38). Next, the solution was purified by removing IdeS via His Mag Sepharose excel magnetic beads (Cytiva, 17371221) according to manufacturer’s instructions.

Binding of bAbs was verified as previously described in the section “Production of human monoclonal antibodies binding to *S. aureus*”.

Phagocytosis mediated by bAbs was verified with phagocytes in suspension as previously described in the section “Phagocytosis of *S. aureus* by neutrophils and macrophages” with few changes to the protocol. To distinguish neutrophils from macrophages once combined in the same well with *S. aureus*, neutrophils were

fluorescently labelled with Celltrace Violet (Invitrogen, C34557) and macrophages with Celltrace Yellow (Invitrogen, C34573) cell proliferation kit for flow cytometry diluted in Hanks balanced salt solution (HBSS) according to the manufacturer's instructions for labelling cells in suspension. Phagocytes and bacteria were combined at a cell-to-bacterium ratio of 1-10 and samples were measured with MACSquant VYB flow cytometer and data analyzed with FlowJo. Gating strategy is summarized in Supplementary figure 1 as previously described for the phagocytosis assays with the additional selection from the total population of the two phagocytes based on their fluorescence on the violet (neutrophils) and yellow (macrophages) channels (**Supplementary figure 1B**).

Results

mAb-mediated phagocytosis of *S. aureus* by neutrophils and macrophages

We produced three full-length human IgG1 and IgG3 antibodies targeting different epitopes on *S. aureus* cell wall. These subclasses of antibodies were selected based on their high binding affinity to FcγRs and efficient activation of the complement system via the classical pathway (6), essential steps driving bacterial opsonophagocytosis by neutrophils and macrophages. Previous studies determined the binding affinity of these antibodies towards different staphylococcal strains (10,20,39). We confirmed that all mAbs efficiently recognized *S. aureus* Newman strain with similar binding levels, regardless of antibody subclass or epitope (**Figure 1**).

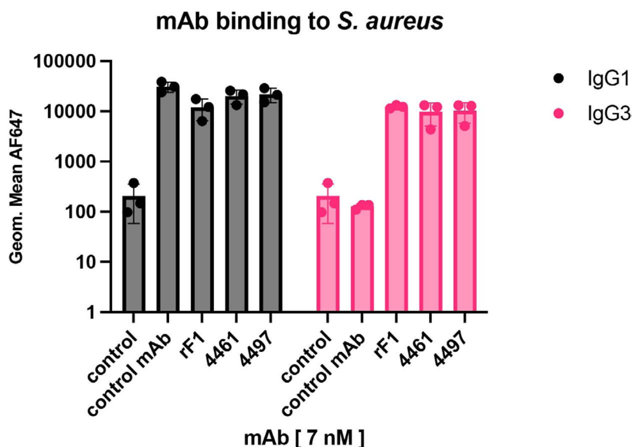


Figure 1. mAbs binding to *S. aureus* Newman strain. Bacteria were incubated with 7 nM of IgG1- and IgG3-mAbs rF1, 4461, and 4497 for 30 min at 4°C. Antibody binding was verified by incubation for 30 min at 4°C with a secondary antibody AF647-conjugated goat anti-human kappa F(ab)2. Control is bacteria incubated with only the secondary antibody, and control mAb is IgG1- or IgG3-DNP. Data are represented as geometric mean of AF647 in a log10 scale. (n=3).

We previously showed that the antibacterial functions of neutrophils and macrophages change according to *in vitro* conditions (26). When phagocytosis was studied with cells in suspension, bacterial recognition and uptake by phagocytes was exclusively dependent on the combined opsonization with complement proteins and polyclonal antibodies present in NHS. Lack of one of these components resulted in limited or no phagocytosis. On the contrary, when phagocytosis was studied in adhesion assays, opsonin-independent mechanisms were also involved in bacterial uptake by both phagocytes.

Here, we showed that the use of mAbs alone was sufficient to drive bacterial recognition and uptake by both phagocytes in suspension assays, even without the complement system (**Figure 2A**). Regardless of the specific antibody subclass, phagocytes recognized mainly *S. aureus* osponized with rF1 rather than 4461 or 4497. Almost 30-times lower IgG3 concentrations were needed to induce stronger phagocytosis than IgG1 by both neutrophils and macrophages. Addition of serum further enhanced the contribution of IgG3 but not IgG1 to *S. aureus* recognition and uptake by neutrophils. On the contrary, macrophages phagocytosis was not influenced using IgG3 alone or in combination with 1% ΔNHS (**Figure 2B**). In all conditions tested, neutrophils phagocytosed more bacteria than macrophages.

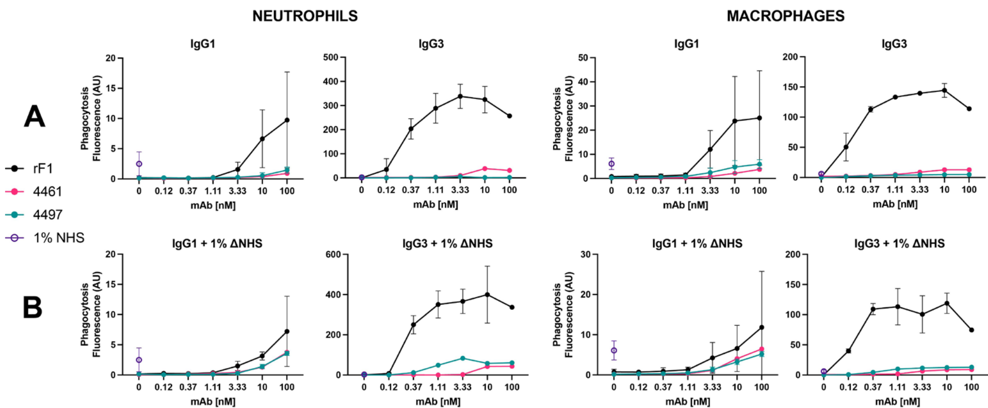


Figure 2. mAb-mediated phagocytosis of *S. aureus* by neutrophils and macrophages in suspension. Phagocytes were incubated with bacteria opsonized with several concentrations of IgG1- and IgG3-mAbs alone (**A**) or in combination with 1% ΔNHS (**B**) at a cell-to-bacterium ratio of 1-10. As a positive control, bacteria were opsonized with 1% NHS. Phagocytosis of *S. aureus* by neutrophils and macrophages was evaluated after 15 min incubation. The number of bacteria engulfed by phagocytes was measured by the intensity of the GFP signal, expressed in arbitrary units (AU) as values were compensated for cell type's autofluorescence. (n=2). Error bars represent SEM.

When antibody-mediated phagocytosis was studied with cells in adhesion, we could detect an enhancement of the phagocytic capacities of neutrophils and macrophages only after opsonization with IgG3 (**Figure 3A**). Compared to suspension assays, higher antibody concentrations were necessary to induce phagocytosis by adhering cells. In the presence of the complement system, both IgG1 and IgG3 increased bacterial uptake in comparison to NHS in both phagocytes (**Figure 3B**). Combined with complement proteins, 3-times lower antibody concentrations were required to induce phagocytosis than mAbs alone. In contrast to suspension assays, the difference in bacterial uptake between adhering phagocytes was less noticeable. Macrophages seemed to uptake slightly more pathogens than neutrophils, however a similar contribution to phagocytosis was observed by IgG1 and IgG3 in the presence of serum. Stronger bacterial uptake was observed after opsonization with rF1 and 4497 in neutrophils, and rF1 and 4461 in macrophages.

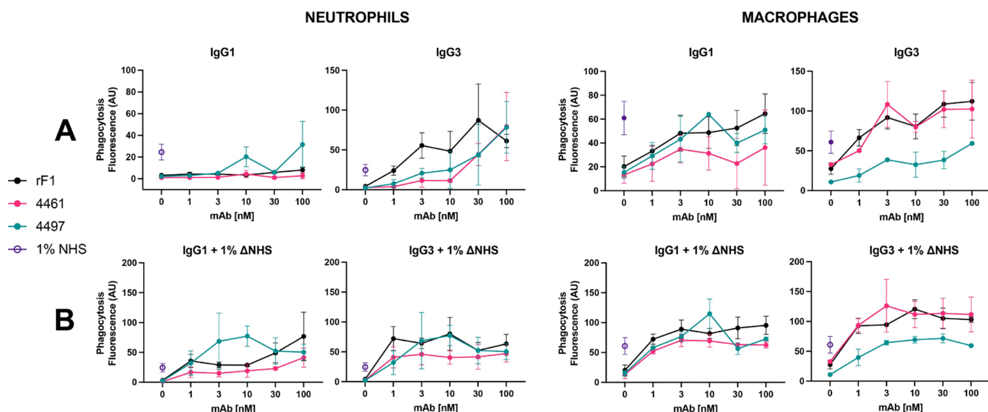


Figure 3. mAb-mediated phagocytosis of *S. aureus* by neutrophils and macrophages in adhesion. Phagocytes were incubated with bacteria opsonized with several concentrations of IgG1- and IgG3-mAbs alone (**A**) or in combination with 1% ΔNHS (**B**) at a cell-to-bacterium ratio of 1-10. As a positive control, bacteria were opsonized with 1% NHS. Phagocytosis of *S. aureus* by neutrophils and macrophages was evaluated after 1 hour incubation. The number of bacteria engulfed by phagocytes was measured by the intensity of the GFP signal, expressed in arbitrary units (AU) as values were compensated for cell type's autofluorescence. (n=2). Error bars represent SEM.

Despite similar binding affinities to *S. aureus*, IgG1 mAbs did not induce phagocytosis as efficiently as IgG3. This is caused by the expression on the surface of *S. aureus* of staphylococcal protein A (SpA) and *S. aureus* binder of IgG (Sbi). The binding of both proteins to the Fc region of IgG1 inhibited FcγR-mediated uptake by phagocytes (36). This effect was not observed when bacteria were opsonized with IgG3, as this antibody subclass does is not bound by SpA and Sbi (8,40,41).

Although expression of SpA and Sbi does not interfere with the mAbs binding levels (**Supplementary Figure 2A**), the absence of these virulence factors restored IgG1-mediated phagocytosis of *S. aureus* KO by both phagocytes (**Supplementary Figure 2B**). Even so, we could confirm that opsonization with rF1 induced stronger phagocytosis by neutrophils and macrophages than 4461 and 4497.

We could observe that mAbs enhanced bacterial recognition and uptake by phagocytes in suspension and adherent to the cell-culture plate. However, in adhesion assays it was not possible to clearly define differences in bacterial uptake between the two phagocytes. Moreover, we previously described an opsonin-independent uptake of bacteria by both phagocytes in adhesion (26), which might explain the similar contribution to phagocytosis of IgG1 and IgG3 in the presence of the complement system, regardless of expression of SpA and Sbi by *S. aureus*. For these reasons, the characterization of mAbs contribution to the antibacterial functions of phagocytes was continued only with assays in suspension where phagocytosis was exclusively opsonin-dependent.

mAb-mediated activation of neutrophils and macrophages.

Pathogen-presentation via mAbs did not induce a stronger activation of phagocytes as indicated by their expression of surface markers. Higher surface expression of CD11b and reduced levels of CD62L are considered typical features of primed neutrophils (42). Here, we observed that *S. aureus* opsonization with NHS, IgG1, or IgG3, alone or in combination with complement proteins, exert a similar priming action on neutrophils (**Figure 4A**). Activation of macrophages towards a pro-inflammatory phenotype is characterized by an increased expression of CD38 and CD64, while CD206 is usually upregulated within macrophages polarized toward an anti-inflammatory phenotype (43,44). Here, we observed a slightly increased expression of CD38 after opsonization of *S. aureus* with IgG3 alone or all mAbs in the presence of complement. In contrast, all conditions downregulated CD206. On the other hand, pathogen presentation via mAbs strongly reduced CD64 surface expression (**Figure 4B**). However, this surface marker codes for FcγRI, which plays an essential role in antibody-mediated phagocytosis. The lower signal detected might be due to internalization of the receptor after binding to the Fc tail of mAbs. This leaves a lower number of proteins exposed for detection.

While the use of mAb did not appear to enhance phagocytes activation against pathogens, it improved their antibacterial activity as measured by ROS release. When assessing the contribution to phagocytosis, opsonization with IgG3 led to the uptake of more bacteria than IgG1. Here, we showed that the higher IgG3-mediated bacterial load in neutrophils correlated with an increased release of ROS. Use of IgG3 alone induced ROS production similar to NHS, which was increased by complement proteins. Instead, lower IgG1-mediated uptake of pathogens resulted in no ROS generation by neutrophils, with or without complement (**Figure 4C**). Although rapid ROS generation is a distinctive feature of neutrophils bactericidal activity, we could observe a smaller mAb-mediated ROS release also by macrophages. Production

of ROS by macrophages was induced at slightly higher levels than NHS only in the presence of IgG1 or IgG3 and complement. No effect was observed using mAbs alone (**Figure 4D**).

Finally, in contrast to the phagocytosis assays, we did not observe any relevant difference among rF1, 4461, and 4497 in priming phagocytes or enhancing their ROS production.

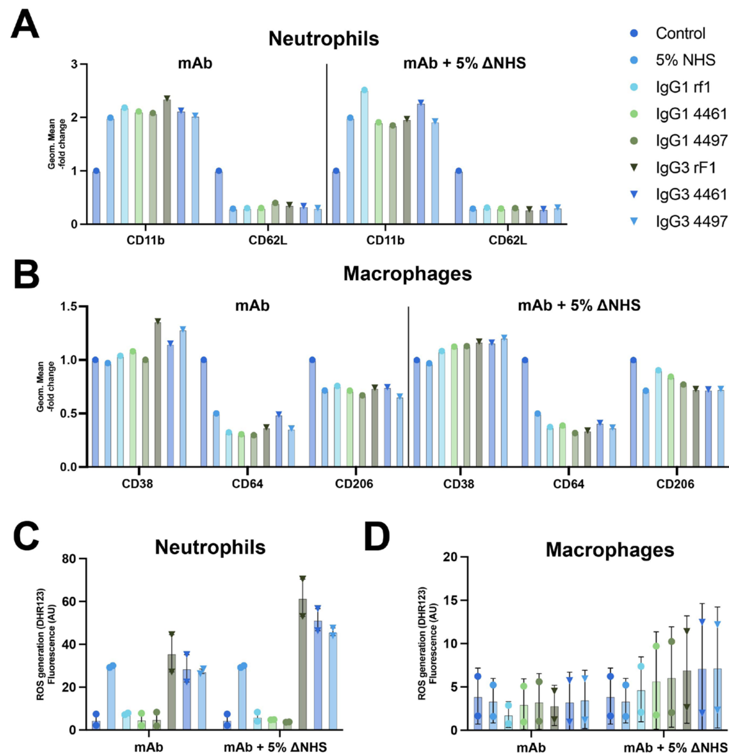


Figure 4. mAb-mediated priming against pathogens of neutrophils and macrophages. Phagocytes were incubated with mCherry (ROS) or gamma-irradiated (markers expression) *S. aureus* opsonized with 10 nM of IgG1- and IgG3-mAbs alone or in combination with 5% ΔNHS, at a cell-to-bacterium ratio of 1-10. In neutrophils (**A**), expression of CD11b and CD62L was verified after 30 min incubation with bacteria. In macrophages (**B**), expression of CD38, CD64, and CD206 was verified after 4 h incubation with bacteria. Values are represented as fold change of the geometric mean of the antibody-conjugated signal compared to control cells incubated without bacteria. In both neutrophils (**C**) and macrophages (**D**), ROS production was determined after 30 min incubation with bacteria by measuring the geometric mean of the DHR123 signal. Fluorescence values were normalized based on the values of the control group without bacteria and DHR123, which defines cell type's autofluorescence, and expressed in arbitrary units (AU). (n=2). Error bars represent SEM.

mAb-mediated killing of *S. aureus* by neutrophils and macrophages.

Although pathogen-presentation via mAbs increased bacterial uptake and ROS generation of phagocytes, this did not correlate with enhanced bactericidal functions.

Without mAbs, neutrophils killed about half of the bacteria internalized while almost no reduction in intracellular bacteria was observed in macrophages. Use of IgG1 alone or in combination with complement resulted in higher proportions of bacteria surviving within neutrophils but slightly lower within macrophages (**Figure 5A**). Similarly, the use of IgG3 did not improve the bactericidal functions of phagocytes, except under a few conditions. In the presence of IgG3-rF1, with or without complement, and IgG3-4497 with complement a similar proportion of bacteria surviving inside neutrophils was observed compared to the control group with NHS (**Figure 5B**). While only IgG3 greatly enhanced the phagocytic capacities of phagocytes, both subclasses of antibodies had a similar impact on the killing ability of neutrophils and macrophages.

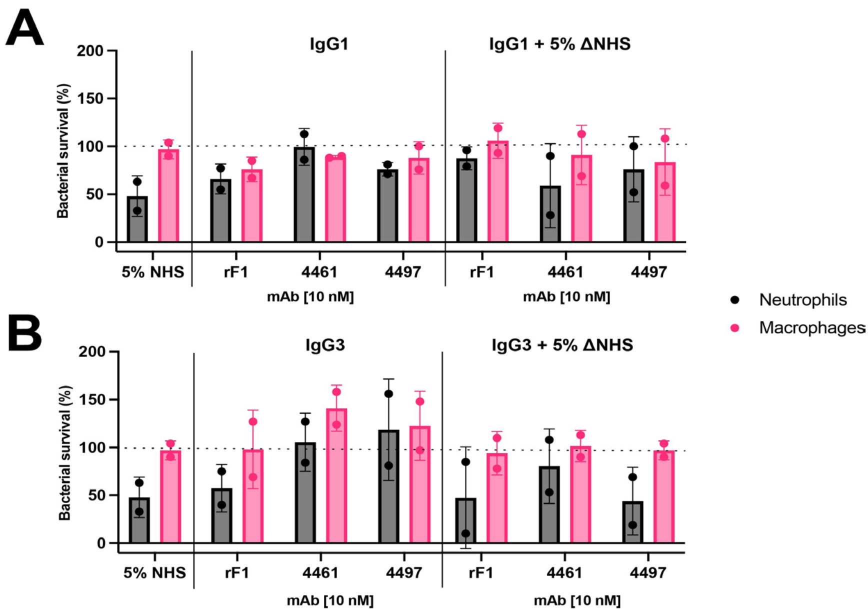


Figure 5. mAb-mediated killing of *S. aureus* by neutrophils and macrophages. Phagocytes were incubated with bacteria opsonized with 10 nM IgG1 (**A**) and IgG3 (**B**) mAbs alone or in combination with 5% ΔNHS at a cell-to-bacterium ratio of 1-1. As a positive control, bacteria were opsonized with 5% NHS. Killing of *S. aureus* by neutrophils and macrophages was evaluated after 90 min incubation at 37°C, 5% CO2. After lysis of the cell membranes of the phagocytes, the number of viable bacteria per each condition was normalized by the number of the bacteria counted at time 0. Data are depicted as percentage of bacterial survival. (n=2). Error bars represent SEM.

Selective bAb-mediated phagocytosis of *S. aureus* by neutrophils or macrophages.

We showed that mAb successfully enhanced the antibacterial functions against *S. aureus* of neutrophils and macrophages. However, in a realistic scenario, the two phagocytes coexist at the infection site and collaborate in the elimination of invading pathogens (22,45). As a proof of concept, we investigated the use of bAbs to selectively manipulate the phagocytic capacities of neutrophils and macrophages in the presence of *S. aureus*.

For this purpose, we generated IgG1 antibodies modified to prevent SpA and Sbi binding and allow recombination into bispecific antibodies. The targets selected were SDR proteins for *S. aureus* (brF1), CD66b for neutrophils (bCD66b) (46), and DC-SIGN for macrophages (bDC-SIGN) (47). Finally, we generated two bAbs, bCD66b+brF1 against neutrophils and *S. aureus*; and bDC-SIGN+brF1 against macrophages and *S. aureus*, plus the F(ab)2 portions of each bAb (**Figure 6A**). To verify the feasibility of our strategy, bAbs alone must influence bacterial uptake by phagocytes. Therefore, the experiments were run at high bAbs concentrations and without complement.

Both bAbs efficiently recognized *S. aureus* via the brF1 portion. Although bAbs recognized bacteria only via one arm, their binding levels were similar to those of the mAbs IgG1- and IgG3-rF1. However, limited or no binding to phagocytes was detected by both bAbs produced (**Figure 6B**). Despite their lack of specific binding to phagocytes, we investigated whether the bAbs could still influence the phagocytic capacities of neutrophils and macrophages.

We measured the number of bacteria phagocytosed after incubation with neutrophils, macrophages, and antibody-opsonized *S. aureus* (**Figure 6C**). In the presence of 1% NHS, neutrophils and macrophages engulfed a similar number of bacteria. Among the three antibodies employed in constructing the bAbs, only brF1 induced phagocytosis by both neutrophils and macrophages. As these antibodies were modified to not bind SpA and Sbi, they exhibited phagocytosis levels comparable to the mAb IgG3-rF1. Similarly, only brF1 contributed to bacterial uptake as observed when *S. aureus* was incubated with the control reactions bCD66b / brF1 and bDC-SIGN / brF1, where antibodies did not react to form a bAb. The bAb specific for neutrophils (bCD66b+brF1) did not prevent macrophages from taking up *S. aureus*. Similarly, phagocytosis by neutrophils was not inhibited by the bAb specific for macrophages (bDC-SIGN+brF1). However, a similar number of intracellular bacteria was detected in both phagocytes, suggesting a weaker interaction of neutrophils with bDC-SIGN+brF1.

To increase bAbs-mediated specific uptake, we isolated from each bAb the F(ab)2 region that determines the binding affinity of the antibodies. However, the use of the F(ab)2 fragments of the two bAbs did not induce bacterial uptake by neutrophils or macrophages. This might suggest that a functional Fc tail of the antibody is required for phagocytosis to occur.

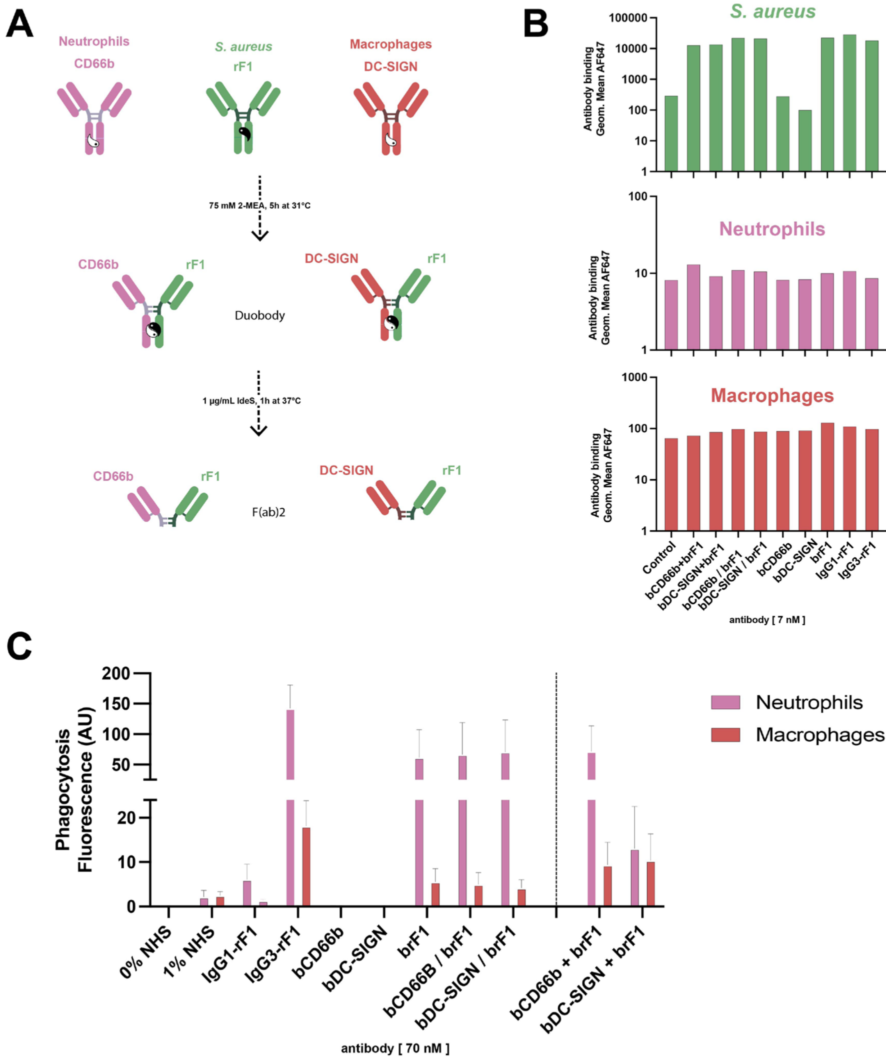


Figure 6. Selective bAb-mediated phagocytosis of *S. aureus* by neutrophils or macrophages. (**A**) Schematic representation of generation of bAbs. Antibodies targeting *S. aureus* and neutrophils, or *S. aureus* and macrophages were incubated with 2-MEA. This reducing agent broke the disulfide bonds the antibodies to allow generation of dCD66b+drF1 targeting neutrophils and *S. aureus*, dDC-SIGN+drF1 targeting macrophages and *S. aureus*. Next, the Fc portion of each bAb was cleaved by IdeS and F(ab)2 fragments of dCD66b+drF1 and dDC-SIGN+drF1 were isolated. Images created with Biorender.com. (**B**) Antibody binding to *S. aureus*, neutrophils, and macrophages was verified by incubation with 7 nM of each antibody followed by the secondary antibody AF647-conjugated goat anti-human kappa F(ab)2. Control is bacteria or phagocytes incubated with only the secondary antibody. Data are represented as geometric mean of AF647 in a logarithmic scale. (**C**) To assess the

contribution to phagocytosis, bacteria were opsonized with 70 nM of antibodies and added in the same well with neutrophils and macrophages at a cell-to-bacterium ratio of 1-10. Each phagocyte was identified based on the fluorescence in the PE channel (neutrophils) and Violet channel (macrophages). The amount of bacteria engulfed by each phagocyte, was measured by the intensity of the GFP signal, expressed in arbitrary units (AU) as values were compensated for cell type's autofluorescence. (n=2). Error bars represent SEM.

Discussion

In this study we compared the contribution of mAbs to the elimination of *S. aureus* by neutrophils and macrophages. When phagocytosis was studied in suspension, phagocytes without complement and polyclonal antibodies found in NHS were not capable to uptake *S. aureus* (26). On the contrary, the presence of mAbs alone was already sufficient to observe bacterial uptake by both neutrophils and macrophages. Addition of the complement system further enhanced mAb-mediated phagocytosis of *S. aureus*. However, this effect was more pronounced in neutrophils than macrophages. These findings suggest that phagocytosis by macrophages is mostly FcγR-dependent, while activation of both FcγRs and CRs drives bacterial uptake by neutrophils. While neutrophils do not express FcγRI, both phagocytes share the same type of CRs (26), which complicates the explanation for the negligible role covered by CR-mediated phagocytosis in macrophages. From one side, FcγR-mediated phagocytosis might induce a stronger macrophages activation (48). Conversely, macrophages might exhibit on their membranes a lower number of copies of CR3, the main receptor involved in the uptake of complement-opsonized bacteria (49,50). Nevertheless, further studies are required to investigate and validate these hypotheses.

Among the mAbs studied, rF1 provided a stronger phagocytosis and killing effect than 4461 and 4497. We hypothesize that the higher abundance of GlcNac residues on SDR proteins might increase the binding sites for rF1 (13), while the combined presence of GlcNac and D-alanine residues on the WTA backbone may disrupt protein charge and affect the binding of 4461 and 4497 (51,52). Nevertheless, further studies are needed to verify the interaction between mAbs and their targets.

All three mAbs equally promoted phagocytes activation and ROS production. By comparison with IgG1, IgG3 induced stronger phagocytosis (**Figure 2** and **3**), ROS production (**Figure 4C** and **4D**), and partially killing (**Figure 5**). In fact, IgG3 has higher affinity for FcγRs and induce a stronger activation of the complement system via the classical pathway than IgG1 (6). Moreover, expression of SpA and Sbi by *S. aureus* inhibits IgG1- but not IgG3-mediated phagocytosis, thanks to an amino acid substitution in the Fc region that prevents binding of these virulence factors to IgG3 (8,40,41). However, from a therapeutic perspective, IgG1 offers several advantages compared to IgG3, such as a longer half-life (6), easier production and purification (53), and its safety for clinical use has already been established for oncological and immunological diseases (54).

Use of mAbs enhanced the functions of both phagocytes, but neutrophils always showed stronger phagocytic and killing abilities than macrophages. Due to their longer life span and weaker bactericidal functions, macrophages might represent an ideal hub for the intracellular survival of *S. aureus*, entailing a higher risk of recalcitrant infections. To prevent this, a possible therapeutic approach might exploit the stronger antibacterial functions of neutrophils, rerouting bacterial uptake exclusively towards those phagocytes. This strategy was explored as a proof of concept via generation of bAbs targeting both pathogens and phagocytes. To improve their therapeutic value, these antibodies were generated from a IgG1 backbone modified to prevent binding of SpA and Sbi. According to a “piggyback” approach (55), one arm of the bAb binds *S. aureus* while the other arm presents the opsonized pathogen exclusively to one phagocyte via the specificity of the second arm.

Although bAbs efficiently enhanced the phagocytic capacities of both neutrophils and macrophages, they failed in driving phagocyte-specific uptake of *S. aureus* (**Figure 6**). The use of either of the bAbs did not prevent the other phagocyte from engulfing bacteria. Antibody-mediated phagocytosis relies on the interaction between FcγRs and the Fc region of antibodies, which is common in both generated bAbs. Therefore, we generated F(ab)2 fragments of the bAbs. With no Fc part of the antibody, only the crosslinking of opposite antigens on bacteria and white blood cells can be responsible for the observed phagocytosis. Unfortunately for these bAbs-constructs it was evident that this mechanis was insufficient to elicit phagocytosis.

Despite the undesired outcome of our results, the bispecific antibody strategy might still hold therapeutic value after some improvements in its design (55). Extra attention should be paid to the selection of the targets on phagocytes. Besides expression levels on the cell surface, the size, mobility in the membrane, and distance of the antigen from the target surface play a critical role in antibody efficacy (56–58). For example, Bakalar *et al.*, showed that distances longer than 10 nm between the antibody-opsonized antigen and the cell membrane negatively impact phagocytosis by macrophages (59). Alternatively, pathogens presentation to phagocytes might be enhanced by using bAbs binding two different virulence factors on the bacterial membrane (60), or generating high affinity F(ab)2 fragments against two epitopes on the same antigen (61). This approach might improve antibody therapy efficacy against a broad range of bacterial strains. On the other hand, our strategy of directing all pathogens against one cell type might be counterproductive for infection resolution. For instance, in a septic *in vitro* model, substantial exposure to *S. aureus* supernatant led to reduced phagocytic and killing capacities of neutrophils (46). As a solution, bAbs might be designed to bind intracellular targets, such as CD63 (or LAMP3) involved in lysosomal trafficking (62), to enhance the antibacterial functions of both phagocytes instead of only one.

As a different option from bAbs, phagocytes might be selectively stimulated based on their distinct response to opsonins. As previously described (63), we showed that macrophages primarily rely on antibodies for bacterial uptake, whereas the introduction of complement enhances phagocytosis in neutrophils. Therefore,

mAbs might be used to enhance complement activation and consequently stimulate primarily the antibacterial functions of neutrophils. For example, the IgG1 Fc domain can be modified to enhance Fc-dependent hexamerization of target-bound antibodies, resulting in stronger complement activation and deposition on the bacterial surface (9,10). Similarly, complement-mediated phagocytosis might be enhanced by switching the antibody subclass to IgM, which induces more efficient complement activation (64).

Conclusion

In conclusion, we confirmed that *in vitro* assay conditions affect the antibacterial functions of neutrophils and macrophages. When phagocytosis was studied in adhesion, no clear difference was observed between neutrophils and macrophages. In the presence of complement we could observe a similar contribution of IgG1- and IgG3-mediated phagocytosis, despite the expression of SpA and Sbi by *S. aureus*. On the contrary, assays in suspension clearly demonstrated the distinct roles of opsonins in promoting bacterial uptake, revealing a marked difference in phagocytic capabilities between neutrophils and macrophages. For instance, the addition of complement besides mAbs further enhanced bacterial uptake by neutrophils but not macrophages. Based on these observations, future *in vitro* tests aimed at screening and investigating the role of mAb should be run with phagocytes in suspension. Finally, our findings suggest that targeting SDR proteins, rather than WTA, on *S. aureus* may serve as a successful mAb therapeutic strategy.

Acknowledgements

The collaboration project is cofounded by the PPP Allowance made available by Health~Holland, Top Sector Life Sciences & Health, to stimulate public-private partnerships. This research also received co-funding from the EU's H2020 research and innovation program under Marie S. Curie cofund RESCUE grant agreement No 801540. This publication is also part of the project DARTBAC (with project number NWA.1292.19.354) of the research program NWA-ORC which is (partly) financed by the Dutch Research Council (NWO).

References

1. Murray CJ, Ikuta KS, Sharara F, Swetschinski L, Robles Aguilar G, Gray A, Han C, Bisignano C, Rao P, Wool E, *et al.* Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. The Lancet (2022) 399:629–655. doi: 10.1016/S0140-6736(21)02724-0
2. Pietrocola G, Nobile G, Rindi S, Speziale P. *Staphylococcus aureus* manipulates innate immunity through own and host-expressed proteases. Front Cell Infect Microbiol (2017) 7:1–15. doi: 10.3389/fcimb.2017.00166
3. Josse J, Valour F, Maali Y, Diot A, Batailler C, Ferry T, Laurent F. Interaction between staphylococcal biofilm and bone: How does the presence of biofilm promote prosthesis loosening? Front Microbiol (2019) 10: doi: 10.3389/fmicb.2019.01602
4. Jahantigh HR, Faezi S, Habibi M, Mahdavi M, Stufano A, Lovreglio P, Ahmadi K. The Candidate Antigens to Achieving an Effective Vaccine against *Staphylococcus aureus*. Vaccines (Basel) (2022) 10: doi: 10.3390/vaccines10020199
5. Pelegrin M, Naranjo-Gomez M, Piechaczyk M. Antiviral Monoclonal Antibodies: Can They Be More Than Simple Neutralizing Agents? Trends Microbiol (2015) 23:653–665. doi: 10.1016/j.tim.2015.07.005
6. Vidarsson G, Dekkers G, Rispens T. IgG subclasses and allotypes: From structure to effector functions. Front Immunol (2014) 5: doi: 10.3389/fimmu.2014.00520
7. van Kessel KPM, Bestebroer J, van Strijp JAG. Neutrophil-mediated phagocytosis of *Staphylococcus aureus*. Front Immunol (2014) 5: doi: 10.3389/fimmu.2014.00467
8. Cruz AR, den Boer MA, Strasser J, Zwarthoff SA, Beurskens FJ, de Haas CJC, Aerts PC, Wang G, de Jong RN, Bagnoli F, *et al.* Staphylococcal protein A inhibits complement activation by interfering with IgG hexamer formation. Proc Natl Acad Sci U S A (2021) doi: 10.1073/pnas.2016772118/-/DCSupplemental
9. Aguinagalde Salazar L, den Boer MA, Castenmiller SM, Zwarthoff SA, de Haas C, Aerts PC, Beurskens FJ, Schuurman J, Heck AJ, van Kessel K, *et al.* Promoting Fc-Fc interactions between anti-capsular antibodies provides strong immune protection against Streptococcus pneumoniae. Elife (2023) 12: doi: 10.7554/eLife.80669
10. de Vor L, Beudeker CR, Flier A, Scheepmaker LM, Aerts PC, Vijlbrief DC, Bekker MN, Beurskens FJ, van Kessel KPM, de Haas CJC, *et al.* Monoclonal antibodies effectively potentiate complement activation and phagocytosis of Staphylococcus epidermidis in neonatal human plasma. Front Immunol (2022) 13: doi: 10.3389/fimmu.2022.933251
11. Fong R, Kajihara K, Chen M, Hotzel I, Mariathasan S, Hazenbos WLW, Lupardus PJ. Structural investigation of human *S. aureus*-targeting antibodies that bind wall teichoic acid. MAbs (2018) 10:979–991. doi: 10.1080/19420862.2018.1501252
12. Lehar SM, Pillow T, Xu M, Staben L, Kajihara KK, Vandlen R, DePalatis L, Raab H, Hazenbos WL, Hiroshi Morisaki J, *et al.* Novel antibody-antibiotic conjugate eliminates intracellular *S. aureus*. Nature (2015) 527:323–328. doi: 10.1038/nature16057
13. Hazenbos WLW, Kajihara KK, Vandlen R, Morisaki JH, Lehar SM, Kwakkenbos MJ, Beaumont T, Bakker AQ, Phung Q, Swem LR, *et al.* Novel Staphylococcal Glycosyltransferases SdgA and SdgB Mediate Immunogenicity and Protection of Virulence-Associated Cell Wall Proteins. PLoS Pathog (2013) 9: doi: 10.1371/journal.ppat.1003653
14. Neuhaus FC, Baddiley J. A Continuum of Anionic Charge: Structures and Functions of d -Alanyl-Teichoic Acids in Gram-Positive Bacteria . Microbiology and Molecular Biology

Reviews (2003) 67:686–723. doi: 10.1128/mmbr.67.4.686-723.2003
15. Weidenmaier C, Peschel A. Teichoic acids and related cell-wall glycopolymers in Gram-positive physiology and host interactions. Nat Rev Microbiol (2008) 6:276–287. doi: 10.1038/nrmicro1861
16. Mistretta N, Brossaud M, Telles F, Sanchez V, Talaga P, Rokbi B. Glycosylation of *Staphylococcus aureus* cell wall teichoic acid is influenced by environmental conditions. Sci Rep (2019) 9: doi: 10.1038/s41598-019-39929-1
17. van Dalen R, Peschel A, van Sorge NM. Wall Teichoic Acid in *Staphylococcus aureus* Host Interaction. Trends Microbiol (2020) 28:985–998. doi: 10.1016/j.tim.2020.05.017
18. Cheng AG, Hwan KK, Burts ML, Krausz T, Schneewind O, Missiakas DM. Genetic requirements for *Staphylococcus aureus* abscess formation and persistence in host tissues. FASEB Journal (2009) 23:3393–3404. doi: 10.1096/fj.09-135467
19. Foster TJ. Colonization and infection of the human host by staphylococci: Adhesion, survival and immune evasion. Vet Dermatol (2009) 20:456–470. doi: 10.1111/j.1365-3164.2009.00825.x
20. de Vor L, van Dijk B, van Kessel K, Kavanaugh JS, de Haas C, Aerts PC, Viveen MC, Boel EC, Fluit AC, Kwiecinski JM, *et al.* Human monoclonal antibodies against *Staphylococcus aureus* surface antigens recognize *in vitro* and *in vivo* biofilm. Elife (2022) 11: doi: 10.7554/eLife.67301
21. Zwarthoff SA, Widmer K, Kuipers A, Strasser J, Ruyken M, Aerts PC, C de Haas CJ, Ugurlar D, den Boer MA, Vidarsson G, *et al.* C1q binding to surface-bound IgG is stabilized by C1r 2 s 2 proteases. Proc Natl Acad Sci U S A (2021) 118: doi: 10.1073/pnas.2102787118/-/DCSupplemental
22. Rosowski EE. Determining macrophage versus neutrophil contributions to innate immunity using larval zebrafish. DMM Disease Models and Mechanisms (2020) 13: doi: 10.1242/dmm.041889
23. Silva MT. When two is better than one: macrophages and neutrophils work in concert in innate immunity as complementary and cooperative partners of a myeloid phagocyte system. J Leukoc Biol (2010) 87:93–106. doi: 10.1189/jlb.0809549
24. Rosales C. Neutrophil: A cell with many roles in inflammation or several cell types? Front Physiol (2018) 9:1–17. doi: 10.3389/fphys.2018.00113
25. Pidwill GR, Gibson JF, Cole J, Renshaw SA, Foster SJ. The Role of Macrophages in *Staphylococcus aureus* Infection. Front Immunol (2021) 11:1–30. doi: 10.3389/fimmu.2020.620339
26. Cecotto L, van Kessel K, de Vor L, Passerini C, Vogely HC, van der Wal B, Weinans H, Rooijakkers S, Amin Yavari S, van Strijp J. The relative role of human neutrophils and macrophages in phagocytosis and killing of *Staphylococcus aureus*. Front Immunol (2023)
27. Surewaard BGJ, van Strijp JAG, Nijland R. Studying interactions of *Staphylococcus aureus* with neutrophils by flow cytometry and time lapse microscopy. J Vis Exp (2013)1–5. doi: 10.3791/50788
28. Boero E, Brinkman I, Juliet T, van Yperen E, van Strijp JAG, Rooijakkers SHM, van Kessel KPM. Use of Flow Cytometry to Evaluate Phagocytosis of *Staphylococcus aureus* by Human Neutrophils. Front Immunol (2021) 12:1–15. doi: 10.3389/fimmu.2021.635825
29. Sibbald MJJB, Winter T, Van Der Kooi-Pol MM, Buist G, Tsompanidou E, Bosma T, Schäfer T, Ohlsen K, Hecker M, Antelmann H, *et al.* Synthetic effects of secG and secY2 mutations on exoproteome biogenesis in *Staphylococcus aureus*. J Bacteriol (2010) 192:3788–3800. doi: 10.1128/JB.01452-09

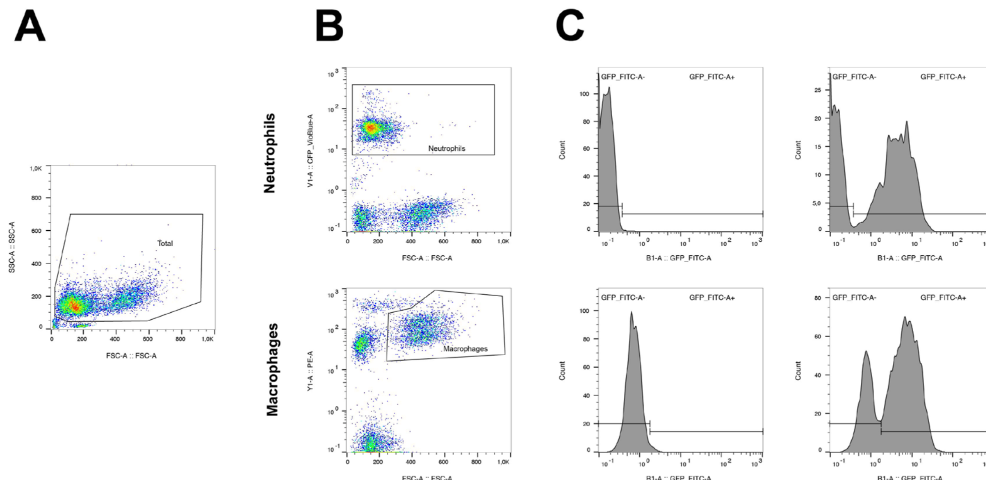
30. Gonzalez ML, Frank MB, Ramsland PA, Hanas JS, Waxman FJ. Structural analysis of IgG2A monoclonal antibodies in relation to complement deposition and renal immune complex deposition. Mol Immunol (2003) 40:307–317. doi: 10.1016/S0161-5890(03)00167-6
31. Reinhard NR, Mastop M, Yin T, Wu Y, Bosma EK, Gadella TWJ, Goedhart J, Hordijk PL. The balance between Gαi-Cdc42/Rac and Gα1/2/13-RhoA pathways determines endothelial barrier regulation by sphingosine-1-phosphate. Mol Biol Cell (2017) 28:3371–3382. doi: 10.1091/mbc.E17-03-0136
32. Postma M, Roelofs J, Goedhart J, Gadella TWJ, Visser AJWG, Van Haastert PJM. Uniform cAMP Stimulation of Dictyostelium Cells Induces Localized Patches of Signal Transduction and Pseudopodia. Mol Biol Cell (2003) 14:5019–5027. doi: 10.1091/mbc.E03-08
33. Purcu DU, Korkmaz A, Gunalp S, Helvaci DG, Erdal Y, Dogan Y, Suner A, Wingender G, Sag D. Effect of stimulation time on the expression of human macrophage polarization markers. PLoS One (2022) 17: doi: 10.1371/journal.pone.0265196
34. Schuurman J, Graus YF, Labrijn AF, Ruuls SR, Parren PW. Opening the door to innovation. MAbs (2014) 6:812–819. doi: 10.4161/mabs.29004
35. Ling WL, Lua WH, Poh JJ, Yeo JY, Lane DP, Gan SKE. Effect of VH-VL families in Pertuzumab and Trastuzumab recombinant production, Her2 and FcγIIA binding. Front Immunol (2018) 9: doi: 10.3389/fimmu.2018.00469
36. Cruz AR, Bentlage AEH, Blonk R, de Haas CJC, Aerts PC, Scheepmaker LM, Bouwmeester IG, Lux A, van Strijp JAG, Nimmerjahn F, *et al.* Toward Understanding How Staphylococcal Protein A Inhibits IgG-Mediated Phagocytosis. The Journal of Immunology (2022) 209:1146–1155. doi: 10.4049/jimmunol.2200080
37. Labrijn AF, Meesters JI, De Goeij BECG, Van Den Bremer ETJ, Neijssen J, Van Kampen MD, Strumane K, Verploegen S, Kundu A, Gramer MJ, *et al.* Efficient generation of stable bispecific IgG1 by controlled Fab-arm exchange. Proc Natl Acad Sci U S A (2013) 110:5145–5150. doi: 10.1073/pnas.1220145110
38. Novarra S, Grinberg L, Rickert KW, Barnes A, Wilson S, Baca M. A hingeless Fc fusion system for site-specific cleavage by IdeS. MAbs (2016) 8:1118–1125. doi: 10.1080/19420862.2016.1186321
39. Di Carluccio C, Soriano-Maldonado P, Berni F, De Haas CJC, Temming AR, Hendriks A, Ali S, Molinaro A, Silipo A, Van Sorge NM, *et al.* Antibody Recognition of Different *Staphylococcus aureus* Wall Teichoic Acid Glycoforms. ACS Cent Sci (2022) 8:1383–1392. doi: 10.1021/acscentsci.2c00125
40. de Taeye SW, Bentlage AEH, Mebius MM, Meesters JI, Lissenberg-Thunnissen S, Falck D, Sénard T, Salehi N, Wuhrer M, Schuurman J, *et al.* FcγR Binding and ADCC Activity of Human IgG Allotypes. Front Immunol (2020) 11: doi: 10.3389/fimmu.2020.00740
41. Jendeberg L, Nilsson P, Larsson A, Uhlen M, Nilsson B, Nygren´¨ A, Nygren´¨ N. Engineering of Fc and Fc from human immunoglobulin G to 1 3 analyse subclass specificity for staphylococcal protein A 1. (1997). 25–34 p.
42. Stockfelt M, Christenson K, Andersson A, Björkman L, Padra M, Brundin B, Ganguly K, Asgeirsdottir H, Lindén S, Qvarfordt I, *et al.* Increased CD11b and Decreased CD62L in Blood and Airway Neutrophils from Long-Term Smokers with and without COPD. J Innate Immun (2020) 12:480–489. doi: 10.1159/000509715
43. Li W, Li Y, Jin X, Liao Q, Chen Z, Peng H, Zhou Y. CD38: A Significant Regulator of Macrophage Function. Front Oncol (2022) 12: doi: 10.3389/fonc.2022.775649
44. Ambarus CA, Krausz S, van Eijk M, Hamann J, Radstake TRDJ, Reedquist KA, Tak PP,

Baeten DLP. Systematic validation of specific phenotypic markers for *in vitro* polarized human macrophages. J Immunol Methods (2012) 375:196–206. doi: 10.1016/j.jim.2011.10.013

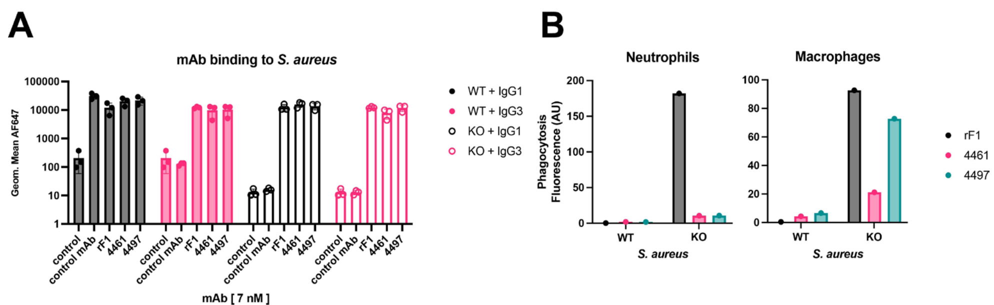
45. Silva MT, Correia-Neves M. Neutrophils and macrophages: The main partners of phagocyte cell systems. Front Immunol (2012) 3:2008–2013. doi: 10.3389/fimmu.2012.00174
46. Schmidt T, Brodesser A, Schnitzler N, Grüger T, Brandenburg K, Zinserling J, Zündorf J. CD66b overexpression and loss of C5a receptors as surface markers for *Staphylococcus aureus*-induced neutrophil dysfunction. PLoS One (2015) 10: doi: 10.1371/journal.pone.0132703
47. Njiri OA, Zhang X, Zhang Y, Wu B, Jiang L, Li Q, Liu W, Chen T. CD209 C-Type Lectins Promote Host Invasion, Dissemination, and Infection of Toxoplasma gondii. Front Immunol (2020) 11: doi: 10.3389/fimmu.2020.00656
48. Acharya D, Li XR, Heineman RES, Harrison RE. Complement Receptor-Mediated Phagocytosis Induces Proinflammatory Cytokine Production in Murine Macrophages. Front Immunol (2020) 10: doi: 10.3389/fimmu.2019.03049
49. Lukácsi S, Nagy-Baló Z, Erdei A, Sándor N, Bajtay Z. The role of CR3 (CD11b/CD18) and CR4 (CD11c/CD18) in complement-mediated phagocytosis and podosome formation by human phagocytes. Immunol Lett (2017) 189:64–72. doi: 10.1016/j.imlet.2017.05.014
50. Lukácsi S, Gerecsei T, Balázs K, Francz B, Szabó B, Erdei A, Bajtay Z. The differential role of CR3 (CD11b/CD18) and CR4 (CD11c/CD18) in the adherence, migration and podosome formation of human macrophages and dendritic cells under inflammatory conditions. PLoS One (2020) 15:1–21. doi: 10.1371/journal.pone.0232432
51. Gerlach D, Guo Y, De Castro C, Kim SH, Schlatterer K, Xu FF, Pereira C, Seeberger PH, Ali S, Codée J, *et al.* Methicillin-resistant *Staphylococcus aureus* alters cell wall glycosylation to evade immunity. Nature (2018) 563:705–709. doi: 10.1038/s41586-018-0730-x
52. Hendriks A, Van Dalen R, Ali S, Gerlach D, Van Der Marel GA, Fuchsberger FF, Aerts PC, De Haas CJC, Peschel A, Rademacher C, *et al.* Impact of Glycan Linkage to *Staphylococcus aureus* Wall Teichoic Acid on Langerin Recognition and Langerhans Cell Activation. ACS Infect Dis (2021) 7:624–635. doi: 10.1021/acsinfecdis.0c00822
53. Chu TH, Patz EF, Ackerman ME. Coming together at the hinges: Therapeutic prospects of IgG3. MAbs (2021) 13: doi: 10.1080/19420862.2021.1882028
54. Castelli MS, McGonigle P, Hornby PJ. The pharmacology and therapeutic applications of monoclonal antibodies. Pharmacol Res Perspect (2019) 7:e00535. doi: 10.1002/prp2.535
55. Labrijn AF, Janmaat ML, Reichert JM, Parren PWHI. Bispecific antibodies: a mechanistic review of the pipeline. Nat Rev Drug Discov (2019) 18:585–608. doi: 10.1038/s41573-019-0028-1
56. Li J, Stagg NJ, Johnston J, Harris MJ, Menzies SA, DiCara D, Clark V, Hristopoulos M, Cook R, Slaga D, *et al.* Membrane-Proximal Epitope Facilitates Efficient T Cell Synapse Formation by Anti-FcRH5/CD3 and Is a Requirement for Myeloma Cell Killing. Cancer Cell (2017) 31:383–395. doi: 10.1016/j.ccell.2017.02.001
57. Bluemel C, Hausmann S, Fluhr P, Sriskandarajah M, Stallcup WB, Baeuerle PA, Kufer P. Epitope distance to the target cell membrane and antigen size determine the potency of T cell-mediated lysis by BiTE antibodies specific for a large melanoma surface antigen. Cancer Immunology, Immunotherapy (2010) 59:1197–1209. doi: 10.1007/s00262-010-0844-y

58. Pfosser A, Brandl M, Salih H, Grosse-Hovest L, Jung G. Role of target antigen in bispecific-antibody-mediated killing of human glioblastoma cells: A pre-clinical study. Int J Cancer (1999) 80:612–616. doi: 10.1002/(SICI)1097-0215(19990209)80:4<612::AID-IJC21>3.0.CO;2-K
59. Bakalar MH, Joffe AM, Schmid EM, Son S, Podolski M, Fletcher DA. Size-Dependent Segregation Controls Macrophage Phagocytosis of Antibody-Opsonized Targets. Cell (2018) 174:131-142.e13. doi: 10.1016/j.cell.2018.05.059
60. Tkaczyk C, Kasturirangan S, Minola A, Jones-Nelson O, Gunter V, Shi YY, Rosenthal K, Aleti V, Semenova E, Warrener P, *et al.* Multimechanistic Monoclonal Antibodies (MAbs) Targeting *Staphylococcus aureus* Alpha-Toxin and Clumping Factor A: Activity and Efficacy Comparisons of a MAb Combination and an Engineered Bispecific Antibody Approach. Antimicrob Agents Chemother (2017) doi: 10.1128/AAC
61. Bahnan W, Happonen L, Khakzad H, Kumra Ahnlide V, de Neergaard T, Wrighton S, André O, Bratanis E, Tang D, Hellmark T, *et al.* A human monoclonal antibody bivalently binding two different epitopes in streptococcal M protein mediates immune function. EMBO Mol Med (2023) 15: doi: 10.15252/emmm.202216208
62. De Goeij BECG, Vink T, Ten Napel H, Breij ECW, Satijn D, Wubbolts R, Miao D, Parren PWHI. Efficient payload delivery by a bispecific antibody-drug conjugate targeting HER2 and CD63. Mol Cancer Ther (2016) 15:2688–2697. doi: 10.1158/1535-7163.MCT-16-0364
63. Berger M, Norvell TM, Tosi MF, Emancipator SN, Konstan MW, Schreiber JR. Tissue-Specific Fc γ and Complement Receptor Expression by Alveolar Macrophages Determines Relative Importance of IgG and Complement in Promoting Phagocytosis of Pseudomonas aeruginosa. Pediatr Res (1994) 35:68–77.
64. Schroeder HW, Cavacini L. Structure and function of immunoglobulins. Journal of Allergy and Clinical Immunology (2010) 125: doi: 10.1016/j.jaci.2009.09.046

Supplementary Figures



Supplementary Figure 1. Gating strategy to compare neutrophils (upper row) and macrophages (lower row) phagocytosis by flow cytometry. (**A**) Selection of cells population (Total gate) in the linear FSC and SSC. (**B**) Selection of the two phagocytes within the total population based on their fluorescence in the violet (neutrophils) and yellow (macrophages) channel. (**C**) Histogram setting GFP fluorescence baseline for cells incubated without bacteria (left) or with bacteria (right). Same gating strategy was applied for both neutrophils and macrophages.



Supplementary Figure 2. Expression of Spa and Sbi on the surface of *S. aureus* inhibits phagocytosis by neutrophils and macrophages. (**A**) IgG1 and IgG3 mAb binding to *S. aureus* WT (full bars) and *S. aureus* KO (empty bars). Data are represented as geometric mean of AF647 in a log10 scale. (n=3). (**B**) Bacteria WT and KO were opsonized with 10 nM of Ig1-mAbs. Then, opsonized bacteria were incubated at a cell-to-bacterium ratio of 1-10 with phagocytes in suspension. Phagocytosis of *S. aureus* WT and KO was evaluated after 15 min and expressed as fluorescence in arbitrary units (AU) of the GFP signal.

CHAPTER
6

General discussion

“Someday we might look back on this and decide that saving Private [Macrophage] was the one decent thing we were able to pull out of this whole […] mess”

Numerous studies, including this thesis, emphasize the ongoing challenges posed by *S. aureus* which consistently manages to outpace and outsmart our efforts to protect orthopedic implants from bacterial colonization. Despite limited success in therapeutic approaches, bacterial mutation rate surpasses scientific progress. On average, it takes approximately 13-14 years to discover and bring a new therapeutic compound to the market (1). Under optimal conditions, a single *S. aureus* cell can accumulate 30 different mutations in every single base pair of its genome within 30 hours (2). Historically, bacterial resistance emerged shortly after the introduction of new antibiotics in clinical applications, resulting in a notable decline in the discovery and development of antibiotic-based compounds over the past three decades (3). Nonetheless, improvements in the use of available resources still provide effective relief from *S. aureus* infections. One example consists of switching the administration of a combination of antibiotics to effectively clear infections and prevent resistance. For instance, strains resistant to an antibiotic become susceptible to a second class of drugs (4). Another approach is to use the implant itself as a vehicle to deliver locally antibacterial agents at higher concentrations. To further improve the efficiency of this strategy, considerable efforts have been devoted to modifying the surface of implants with antibacterial coatings capable of delivering a diverse range of compounds. These coatings may involve various elements, including antibiotics, low-molecular proteins, metal ions and nanoparticles, up to natural and synthetic polymers (5). Despite the wide range of strategies employed, antibacterial coatings have achieved limited success in preclinical and clinical studies and none of these coating strategies have been successfully implemented in clinical care yet.

Need for reliable *in vitro* models

One possible factor contributing to this failure is the unavailability of reliable *in vitro* models. Most studies have assessed the efficacy of newly developed modifications to implant surface primarily through simple *in vitro* assays. These tests typically focus on separately evaluating the antibacterial efficiency on bacterial cultures, and toxicity on host cells, without combining pathogens and host cells in the same assay. From those studies, host-host or host-pathogen interactions mimicking the complexity of the *in vivo* scenario were often ignored. However, this was not without consequences. Chen *et al.*, demonstrated the pro-osteogenic properties of a coating after *in vitro* tests with only MSCs, but the implant yielded opposite results *in vivo*, causing inflammation and bone destruction. Afterwards, the research group showed that a preliminary *in vitro* evaluation of the response of macrophages to the coating might have anticipated negative outcomes without sacrificing any animals (6). Similarly, coatings with significant bactericidal effects *in vitro* turned out to be toxic and not antibacterial *in vivo*. Once more, the use of more comprehensive *in vitro* models could have predicted the side effects of the implant (7). In fact, complex *in vitro* models involving multiple cell types can help assessing treatment effects. Luan

et al., showed that without the presence of a contaminated surface, macrophages assumed a pro-inflammatory phenotype resulting in a reaction against the implant. On the contrary, in the presence of a contaminated surface, macrophages adopted an anti-inflammatory phenotype which favored osteoblasts adhesion to the implant (8).

Furthermore, *in vitro* tests can be further improved by selection of appropriate cell models. Numerous studies employed cell lines, often derived from non-human sources, or combined cell types from different species in culture. However, these approaches fail to consider the potential variations in behavior between primary cells and cell lines from different species (9–12). Additionally, both *in vitro* and *in vivo* studies overlooked *S. aureus* host-specificity. In fact, the coevolution of pathogen and host has led to the development of highly host-specific virulence factors. This made *S. aureus* strains specialized to infect specific host species rather than all hosts equally. Therefore, animal studies, particularly those run in murine models, may inadequately predict the pathogenicity of *S. aureus* or the therapeutic efficacy of novel antibacterial compounds (13–15).

As we aim to define new alternative antibacterial compounds for the prevention of *S. aureus* implant-associated infections (IAI) in humans, to enhance the clinical relevance and applicability of our findings all studies presented in this thesis employed human tissue-derived cells.

In **Chapter 2** we designed a multicellular *in vitro* model that mimics the implant-associated infection (IAI) environment *in vivo*, with macrophages (immune system), MSCs (bone tissue), and *S. aureus* (pathogen) interacting with each other on the surface of an implant. This setup allowed us to study host-host interactions, such as the anti-inflammatory effect generated by culturing MSCs with macrophages, host-pathogen interactions, including the ability of *S. aureus* to survive intracellularly in both cell types over time, and host-implant interactions, including the influence of biomaterials on immune cells antibacterial functions. Moreover, to enhance the accuracy and clinical relevance of our results, we used primary human cells isolated from tissues. This is in contrast to other studies that employed non-human cell lines to evaluate antibacterial coatings efficacy. Our model represents a valuable screening tool to predict the possible benefits and pitfalls derived from the use of therapeutic compounds or antibacterial coatings, as we did in **chapter 2** for silver and in **chapter 3** for host defense peptides.

The use of metallic silver as an antibacterial agent in clinical practice is controversial. While silver-coated implants have been successfully introduced in clinical settings to reduce infection rates among oncologic patients, their use has also raised concerns due to alarming side effects reported. With our multicellular model we could exclude the existence of a therapeutic window where silver, both in free-ion ($AgNO_3$) and nanoparticle (AgNP) form, retained its antibacterial properties while not compromising the viability and functions of host cells. For instance, all silver formulations that were non-toxic to host cells failed to kill both extracellular

and intracellular *S. aureus*. Moreover, to reduce silver-associated toxicity, larger nanoparticles are generally preferred over free silver ions ($AgNO_3$). However, our multicellular model showed that 100 nm but not 20 nm silver nanoparticles (AgNP) triggered an inflammatory response when macrophages and MSCs were cultured together. Interestingly, this side effect would have been missed using monoculture assays.

Our results support the idea that increasing the complexity of *in vitro* models may provide deeper insights into the therapeutic efficacy of novel compounds. Nonetheless, different *in vitro* models have been proposed to approach a more accurate representation of an *in vivo* scenario. For example, addition of a flow system could simulate *in vivo*-like conditions such as shear stress and exchange of nutrients and molecules among cells in culture (16,17). Likewise, organ-on-chip microfluidic devices provide a viable alternative to animal models by reproducing pathophysiological features of human microbial infections (18). For example, Deinhardt-Emmer *et al.*, could monitor the spatiotemporal spread of *S. aureus* in a human alveolus-on-chip consisting of vascular and epithelial cell structures with tissue-resident macrophages (19). The same research group advanced the hypothesis to further expand the use of this model to study the role of staphylococcal toxins in immune cells. Regrettably, this technology still falls short on accurately replicating the intricate complexity of various cell types and the structural composition of the bone environment (20).

While organ-on-chips could mimic the spreading of bacterial infections to different tissues, organoids provide a more patient-specific screening and prediction model for treatment response. Although multiple methods have been established to study infectious diseases using organoids, the inclusion of components from the innate immune system has not yet been incorporated into these models (21–23).

Learning from nature

Nature provided means to combat pathogenic bacteria before antibiotic discovery. For example, several plants and insects evolved specific patterns on their surfaces to protect themselves against bacteria. By reproducing similar patterns into the surface of implants, several researchers developed nanostructured bactericidal surfaces that could kill bacteria through physico-mechanical rupture of the cell wall (24). Fungi naturally produce a bactericidal molecule from which we extract penicillin. Through thousands of years of evolution, our organism evolved a complex immune system that efficiently protects us from external threats quite well, without modern drugs. Therefore, if we enhance the intrinsic antibacterial functions of our immune system, we might be able to replace antibiotics as an antibacterial treatment. Accordingly, in **chapter 3** and **chapter 5** we tried to enhance the antibacterial functions of macrophages via host defense peptides (HDPs) and human monoclonal antibodies (mAbs).

HDPs are naturally occurring molecules expressed by a diverse range of species, from plants to mammals, and employed as a defense mechanism by several cell types. Thanks to their positive charge, cationic HDPs can interact with negatively charged membranes of both pathogens and host cells. This characteristic confers them the therapeutic potential to address bacterial infections through two mechanisms: direct antimicrobial activity and modulation of the immune response. Among the numerous natural and synthetic HDPs described in literature, three stood out for their antibacterial and immunomodulatory properties. CATH-2 exhibited broad-spectrum antibacterial activity (25), while IDR-1018 and LL-37 demonstrated potent anti-biofilm effects (26,27). Additionally, IDR-1018 and LL-37 promoted neutrophils activation and enhanced phagocytes bactericidal functions (28,29).

In **Chapter 3** we aimed to characterize and compare the antibacterial and immunomodulatory properties of these three peptides under the same *in vitro* conditions. It is important to note that variations in culture conditions across studies significantly impact HDPs functions, thereby generating contrasting data. In line with previous findings in literature, we found that all three peptides exhibited a strong anti-inflammatory action on macrophages, and no toxic effects were observed at the concentrations examined. However, contrary to previous studies, only CATH-2 demonstrated a direct antibacterial effect in our assays. Additionally, only IDR-1018 inhibited phagocytosis, while none of the HDPs enhanced the bactericidal functions of human macrophages.

Although HDPs have shown superior performances compared to silver, their application in clinical settings is still far. On the one hand, their non-specific interaction with bacterial membranes suggests that insurgence of resistance is unlikely (30). However, this interaction also raises concerns regarding their potential toxicity towards host cells (31). To avoid safety issues and bypass their instability *in vitro* and *in vivo*, the majority of HDPs currently undergoing preclinical and clinical development are formulated for topical administration (32,33), with ongoing research to apply HDPs as coatings for medical devices (34).

In contrast to HDPs, mAbs are characterized by high target specificity, long half-life, and low immunogenicity, making them particularly suitable for systemic administration (35). However, HDPs highlighted the importance of the *in vitro* conditions when testing new compounds. Therefore, before screening different mAbs for their efficacy, we optimized in **Chapter 4** the optimal *in vitro* conditions to study and compare the role of neutrophils and macrophages in the fight against *S. aureus*.

Classically, neutrophils functions are studied in suspension assays, while macrophages are adherent cells. We showed that neutrophils can be studied as adherent cells and vice versa macrophages under suspension conditions. However, this change in *in vitro* setups affected their response to *S. aureus*. Both phagocytes engulfed a higher number of bacteria when the assay was performed in suspension rather than in adhesion. Moreover, bacterial recognition and uptake in suspension

was exclusively dependent on the opsonization with serum factors. In contrast, other non-opsonic mechanisms were also involved when phagocytosis was studied in adhesion. Plus, neutrophils engulfed and killed more bacteria than macrophages in suspension assays, while the difference between the two cell types was less evident in adhesion assays.

In **Chapter 5** we confirmed that suspension assays represent the ideal *in vitro* platform to study the role of mAbs against *S. aureus* on phagocytes. We observed that mAbs targeting proteins on the cell wall of *S. aureus* improved bacterial recognition, uptake, and partially killing by both phagocytes, with neutrophils outperforming macrophages. However, expression of the virulence factors SpA and Sbi by *S. aureus* inhibited IgG1-mediated phagocytosis, while IgG3 strongly enhanced phagocytes' functions. From a therapeutic perspective, IgG1 offers several advantages over IgG3, such as longer half-life, easier production, and purification. Fortunately, antibody engineering technology provides solutions to enhance IgG1 functionalities to levels comparable to IgG3. For instance, the Fc tail of IgG1 can be modified to prevent binding by SpA and Sbi (36), and increase Fc-dependent hexamerization of target-bound antibodies to enhance complement activation and deposition on the bacterial surface (37,38).

Among the antibodies tested, the best performances were recorded in the presence of antibodies directed against SDR proteins (mAb rF1) rather than WTA (mAbs 4461 and 4497). One possible explanation could be attributed to a higher abundance of N-acetylglucosamine (GlcNac) residues on the SDR proteins, thereby increasing the binding sites for rF1 (39). On the other hand, the WTA backbone can be modified with GlcNac and D-alanine residues. However, D-alanylation might interfere with the protein charge given by the addition of glycosyl groups, thereby affecting 4461 and 4497 binding (40,41). Nonetheless, further studies are needed to verify antibodies interaction with their targets.

Considering the longer life span but weaker bactericidal functions highlighted in **chapters 4** and **5**, macrophages might represent an ideal hub for *S. aureus* intracellular survival, entailing a higher risk of recalcitrant infections. As a possible solution, in Chapter 5 we suggested the use of bispecific antibodies (bAbs) targeting both pathogens and phagocytes. Once bound to the bacterial surface, these bAbs are expected to selectively drive phagocytosis by either one of the phagocytes. Ideally, due to their stronger antibacterial functions, bacterial uptake should be redirected towards neutrophils. Unfortunately, the bAbs designed failed to induce phagocyte-specific uptake of *S. aureus*.

Future directions to prevent implant-associated infections

Overall, we showed that a one-for-all approach is not sufficient to prevent *S. aureus*-mediated IAI. While the therapeutic compounds investigated in this thesis offered some support, none proved capable of eliminating *S. aureus* on their own. A combinatorial approach could potentially hold the key to the future of antibacterial therapies to prevent IAI.

The combination of HDPs with antibiotics (42,43) or other HDPs (44) already showed improved antibacterial efficiency compared to the compounds used alone. Alternatively, HDPs can be immobilized on the implant surface and released locally to promote tissue regeneration (34). Likewise, to enhance therapeutic efficacy, mAbs should be engineered to target epitopes that are less susceptible to alterations or modifications by the pathogen. Additionally, they should also be capable of binding to epitopes shared by various strains within a single species or across several species of interest. Therefore, administration of a cocktail of multiple mAbs might ensure a broader coverage of the therapy while minimizing the risk of resistance development (45). As an alternative to our concept, multiple bispecific antibodies have been designed for various applications other than infectious diseases (46,47). For example, bAbs might be designed to bind intracellular targets of phagocytes to enhance their antibacterial functions (48), or conjugated to antibiotics or other antibacterial compounds to directly target and kill pathogens, both intracellular and extracellular.

In our hands, a possible therapeutic approach to preventing IAI might involve a combination of different antibacterial compounds (**Figure 1**).

On the one hand, the implant surface can be coated with different HDPs. Their release into the environment can attract and modulate macrophage functions, polarizing them towards an anti-inflammatory phenotype. This promotes bone regeneration via interaction with MSCs and osteoblasts. On the other hand, the administration of different antibodies prompts the presence of invading pathogens to immune cells, enhancing their antibacterial functions. Antibody-opsonized bacteria should be phagocytosed and killed mainly by neutrophils. This might effectively reduce the risk of intracellular survival of *S. aureus* within host cells, especially macrophages that can focus on the coordination of the healing process. Furthermore, the systemic administration of antibiotics or their release from the coated surface of the implant provides additional support to the immune system. This helps to prevent bacterial colonization of the implant.

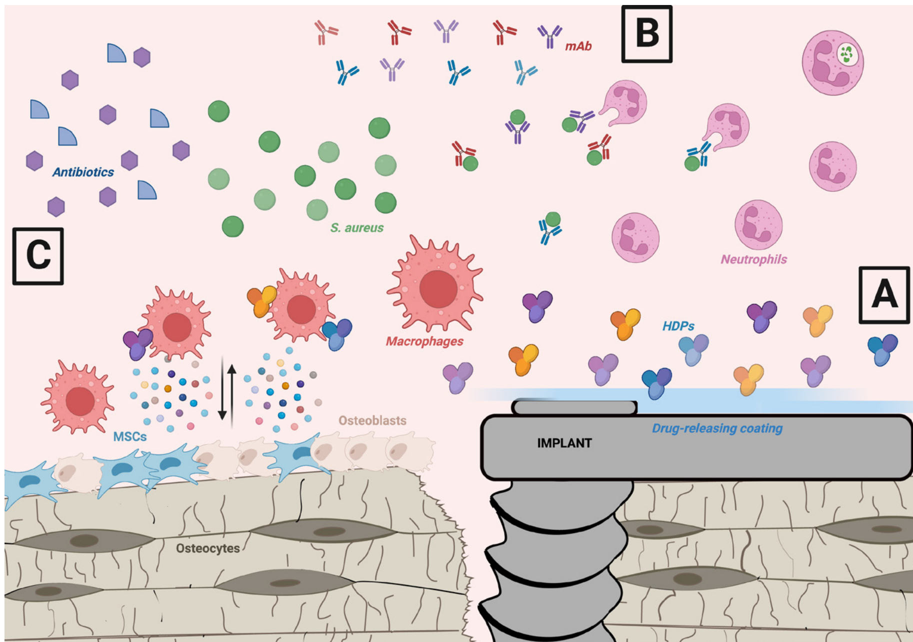


Figure 1. Concept of combining multiple antibacterial compounds to prevent IAI. This therapeutic strategy includes: (**A**) release of HDPs from the surface of the implant to enhance macrophage-mediated healing; (**B**) administration of mAb to enhance bacterial recognition and elimination by immune cells, specifically neutrophils; (**C**) use of antibiotics to support immune system protection of the surface of the implant.

In conclusion, IAI constitutes a complex environment where multiple factors are involved. Finding the therapeutic Holy Grail, one single compound able to prevent bacterial colonization of the implant while supporting tissue healing seems not possible at this stage. Likewise, despite their key role in protection against invading pathogens and coordination of the healing process, macrophages alone cannot completely resolve such a complicated clinical scenario. Nonetheless, to paraphrase the title of chapter 6 taken from the movie “Saving Private Ryan” (49), rescuing at least one cell type could bring us one step closer to victory in this ongoing war against *S. aureus*.

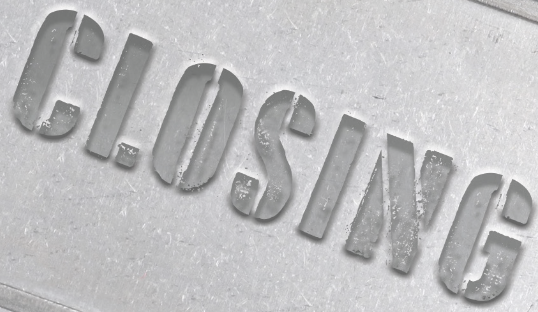
References

1. Miethke M, Pieroni M, Weber T, Brönstrup M, Hammann P, Halby L, Arimondo PB, Glaser P, Aigle B, Bode HB, et al. Towards the sustainable discovery and development of new antibiotics. *Nat Rev Chem* (2021) 5:726–749. doi: 10.1038/s41570-021-00313-1
2. Pray L. Antibiotic resistance, mutation rates and MRSA. *Nature Education* (2008) 1:
3. Li B, Webster TJ. Bacteria antibiotic resistance: New challenges and opportunities for implant-associated orthopedic infections. *Journal of Orthopaedic Research* (2018) 36:22–32. doi: 10.1002/jor.23656
4. Yoshida M, Reyes SG, Tsuda S, Horinouchi T, Furusawa C, Cronin L. Time-programmable drug dosing allows the manipulation, suppression and reversal of antibiotic drug resistance in vitro. *Nat Commun* (2017) 8: doi: 10.1038/ncomms15589
5. Chen X, Zhou J, Qian Y, Zhao LZ. Antibacterial coatings on orthopedic implants. *Mater Today Bio* (2023) 19: doi: 10.1016/j.mtbio.2023.100586
6. Chen Z, Yuen J, Crawford R, Chang J, Wu C, Xiao Y. The effect of osteoimmunomodulation on the osteogenic effects of cobalt incorporated β-tricalcium phosphate. *Biomaterials* (2015) 61:126–138. doi: 10.1016/j.biomaterials.2015.04.044
7. Croes M, Bakhshandeh S, van Hengel IAJ, Lietaert K, van Kessel KPM, Pouran B, van der Wal BCH, Vogely HC, Van Hecke W, Fluit AC, et al. Antibacterial and immunogenic behavior of silver coatings on additively manufactured porous titanium. *Acta Biomater* (2018) 81:315–327. doi: 10.1016/j.actbio.2018.09.051
8. Luan Y, Van Der Mei HC, Dijk M, Geertsema-Doornbusch GI, Atema-Smit J, Ren Y, Chen H, Busscher HJ. Polarization of Macrophages, Cellular Adhesion, and Spreading on Bacterially Contaminated Gold Nanoparticle-Coatings in Vitro. *ACS Biomater Sci Eng* (2020) 6:933–945. doi: 10.1021/acsbiomaterials.9b01518
9. Andreu N, Phelan J, De Sessions PF, Cliff JM, Clark TG, Hibberd ML. Primary macrophages and J774 cells respond differently to infection with Mycobacterium tuberculosis. *Sci Rep* (2017) 7:1–12. doi: 10.1038/srep42225
10. Fransen L, Ruysschaert MR, Van der Heyden J, Fiers W. Recombinant tumor necrosis factor: Species specificity for a variety of human and murine transformed cell lines. *Cell Immunol* (1986) 100:260–267. doi: 10.1016/0008-8749(86)90025-0
11. McCarron EP, Williams DP, Antoine DJ, Kipar A, Lemm J, Stehr S, Welters ID. Exploring the translational disconnect between the murine and human inflammatory response: Analysis of LPS dose–response relationship in murine versus human cell lines and implications for translation into murine models of sepsis. *J Inflamm Res* (2015) 8:201–209. doi: 10.2147/JIR.S89097
12. Mestas J, Hughes CCW. Of Mice and Not Men: Differences between Mouse and Human Immunology. *The Journal of Immunology* (2004) 172:2731–2738. doi: 10.4049/jimmunol.172.5.2731
13. Matuszewska M, Murray GGR, Harrison EM, Holmes MA, Weinert LA. The Evolutionary Genomics of Host Specificity in Staphylococcus aureus. *Trends Microbiol* (2020) 28:465–477. doi: 10.1016/j.tim.2019.12.007
14. Koymans KJ, Vrieling M, Gorham RD, van Strijp JAG. "Staphylococcal immune evasion proteins: Structure, function, and host adaptation.," *Current Topics in Microbiology and Immunology*. Springer Verlag (2017). p. 441–489 doi: 10.1007/82_2015_5017
15. Spaan AN, Van Strijp JAG, Torres VJ. Leukocidins: Staphylococcal bi-component pore-forming toxins find their receptors. *Nat Rev Microbiol* (2017) 15:435–447. doi: 10.1038/

nrmicro.2017.27
16. Da Silva Domingues JF, Roest S, Wang Y, Van Der Mei HC, Libera M, Van Kooten TG, Busscher HJ. Macrophage phagocytic activity toward adhering staphylococci on cationic and patterned hydrogel coatings versus common biomaterials. *Acta Biomater* (2015) 18:1–8. doi: 10.1016/j.actbio.2015.02.028
17. Subbiahdoss G, Fernández ICS, da Silva Domingues JF, Kuijer R, van der Mei HC, Busscher HJ. In vitro interactions between bacteria, Osteoblast-Like cells and macrophages in the pathogenesis of Biomaterial-Associated infections. *PLoS One* (2011) 6: doi: 10.1371/journal.pone.0024827
18. Baddal B, Marrazzo P. Refining host-pathogen interactions: Organ-on-chip side of the coin. *Pathogens* (2021) 10:1–13. doi: 10.3390/pathogens10020203
19. Deinhardt-Emmer S, Rennert K, Schicke E, Cseresnyés Z, Windolph M, Nietzsche S, Heller R, Siwczak F, Haupt KF, Carlstedt S, et al. Co-infection with Staphylococcus aureus after primary influenza virus infection leads to damage of the endothelium in a human alveolus-on-a-chip model. *Biofabrication* (2020) 12: doi: 10.1088/1758-5090/ab7073
20. Mansoorifar A, Gordon R, Bergan RC, Bertassoni LE. Bone-on-a-Chip: Microfluidic Technologies and Microphysiologic Models of Bone Tissue. *Adv Funct Mater* (2021) 31: doi: 10.1002/adfm.202006796
21. Wu B (Catherine), Haney EF, Akhoundsadegh N, Pletzer D, Trimble MJ, Adriaans AE, Nibbering PH, Hancock REW. Human organoid biofilm model for assessing antibiofilm activity of novel agents. *NPJ Biofilms Microbiomes* (2021) 7: doi: 10.1038/s41522-020-00182-4
22. Blutt SE, Estes MK. Organoid Models for Infectious Disease. *Annu Rev Med* (2021) doi: 10.1146/annurev-med-042320
23. Xie X, Tong X, Li Z, Cheng Q, Wang X, Long Y, Liu F, Wang Y, Wang J, Liu L. Use of mouse primary epidermal organoids for USA300 infection modeling and drug screening. *Cell Death Dis* (2023) 14: doi: 10.1038/s41419-022-05525-x
24. Tripathy A, Sen P, Su B, Briscoe WH. Natural and bioinspired nanostructured bactericidal surfaces. *Adv Colloid Interface Sci* (2017) 248:85–104. doi: 10.1016/j.cis.2017.07.030
25. Coorens M, Scheenstra MR, Veldhuizen EJA, Haagsman HP. Interspecies cathelicidin comparison reveals divergence in antimicrobial activity, TLR modulation, chemokine induction and regulation of phagocytosis. *Sci Rep* (2017) 7:1–11. doi: 10.1038/srep40874
26. Reffuveille F, De La Fuente-Nũnez C, Mansour S, Hancock REW. A broad-spectrum antibiofilm peptide enhances antibiotic action against bacterial biofilms. *Antimicrob Agents Chemother* (2014) 58:5363–5371. doi: 10.1128/AAC.03163-14
27. Kang J, Dietz MJ, Li B. Antimicrobial peptide LL-37 is bactericidal against Staphylococcus aureus biofilms. *PLoS One* (2019) 14:1–13. doi: 10.1371/journal.pone.0216676
28. Alalwani SM, Sierigk J, Herr C, Pinkenburg O, Gallo R, Vogelmeier C, Bals R. The antimicrobial peptide LL-37 modulates the inflammatory and host defense response of human neutrophils. *Eur J Immunol* (2010) 40:1118–1126. doi: 10.1002/eji.200939275
29. Niyonsaba F, Madera L, Afacan N, Okumura K, Ogawa H, Hancock REW. The innate defense regulator peptides IDR-HH2, IDR-1002, and IDR-1018 modulate human neutrophil functions. *J Leukoc Biol* (2013) 94:159–170. doi: 10.1189/jlb.1012497
30. Mookherjee N, Anderson MA, Haagsman HP, Davidson DJ. Antimicrobial host defence peptides: functions and clinical potential. *Nat Rev Drug Discov* (2020) 19:311–332. doi: 10.1038/s41573-019-0058-8
31. Hancock REW, Sahl HG. Antimicrobial and host-defense peptides as new anti-infective

therapeutic strategies. *Nat Biotechnol* (2006) 24:1551–1557. doi: 10.1038/nbt1267
32. Magana M, Pushpanathan M, Santos AL, Leanse L, Fernandez M, Ioannidis A, Giulianotti MA, Apidianakis Y, Bradfute S, Ferguson AL, et al. The value of antimicrobial peptides in the age of resistance. *Lancet Infect Dis* (2020) 20:e216–e230. doi: 10.1016/S1473-3099(20)30327-3
33. Ting DSJ, Beuerman RW, Dua HS, Lakshminarayanan R, Mohammed I. Strategies in Translating the Therapeutic Potentials of Host Defense Peptides. *Front Immunol* (2020) 11: doi: 10.3389/fimmu.2020.00983
34. Hao Z, Chen R, Chai C, Wang Y, Chen T, Li H, Hu Y, Feng Q, Li J. Antimicrobial peptides for bone tissue engineering: Diversity, effects and applications. *Front Bioeng Biotechnol* (2022) 10: doi: 10.3389/fbioe.2022.1030162
35. Hansel TT, Kropshofer H, Singer T, Mitchell JA, George AJT. The safety and side effects of monoclonal antibodies. *Nat Rev Drug Discov* (2010) 9:325–338. doi: 10.1038/nrd3003
36. Cruz AR, Bentlage AEH, Blonk R, de Haas CJC, Aerts PC, Scheepmaker LM, Bouwmeester IG, Lux A, van Strijp JAG, Nimmerjahn F, et al. Toward Understanding How Staphylococcal Protein A Inhibits IgG-Mediated Phagocytosis. *The Journal of Immunology* (2022) 209:1146–1155. doi: 10.4049/jimmunol.2200080
37. Aguinagalde Salazar L, den Boer MA, Castenmiller SM, Zwarthoff SA, de Haas C, Aerts PC, Beurskens FJ, Schuurman J, Heck AJ, van Kessel K, et al. Promoting Fc-Fc interactions between anti-capsular antibodies provides strong immune protection against Streptococcus pneumoniae. *Elife* (2023) 12: doi: 10.7554/eLife.80669
38. de Vor L, Beudeker CR, Flier A, Scheepmaker LM, Aerts PC, Vijlbrief DC, Bekker MN, Beurskens FJ, van Kessel KPM, de Haas CJC, et al. Monoclonal antibodies effectively potentiate complement activation and phagocytosis of Staphylococcus epidermidis in neonatal human plasma. *Front Immunol* (2022) 13: doi: 10.3389/fimmu.2022.933251
39. Hazenbos WLW, Kajihara KK, Vandlen R, Morisaki JH, Lehar SM, Kwakkenbos MJ, Beaumont T, Bakker AQ, Phung Q, Swem LR, et al. Novel Staphylococcal Glycosyltransferases SdgA and SdgB Mediate Immunogenicity and Protection of Virulence-Associated Cell Wall Proteins. *PLoS Pathog* (2013) 9: doi: 10.1371/journal.ppat.1003653
40. Gerlach D, Guo Y, De Castro C, Kim SH, Schlatterer K, Xu FF, Pereira C, Seeberger PH, Ali S, Codée J, et al. Methicillin-resistant Staphylococcus aureus alters cell wall glycosylation to evade immunity. *Nature* (2018) 563:705–709. doi: 10.1038/s41586-018-0730-x
41. Hendriks A, Van Dalen R, Ali S, Gerlach D, Van Der Marel GA, Fuchsberger FF, Aerts PC, De Haas CJC, Peschel A, Rademacher C, et al. Impact of Glycan Linkage to Staphylococcus aureus Wall Teichoic Acid on Langerin Recognition and Langerhans Cell Activation. *ACS Infect Dis* (2021) 7:624–635. doi: 10.1021/acsinfecdis.0c00822
42. Pletzer D, Hancock REW. Antibiofilm peptides: Potential as broadspectrum agents. *J Bacteriol* (2016) 198:2572–2578. doi: 10.1128/JB.00017-16
43. Zharkova MS, Orlov DS, Golubeva OY, Chakchir OB, Eliseev IE, Grinchuk TM, Shamova O V. Application of antimicrobial peptides of the innate immune system in combination with conventional antibiotics-a novel way to combat antibiotic resistance? *Front Cell Infect Microbiol* (2019) 9: doi: 10.3389/fcimb.2019.00128
44. Yu G, Baeder DY, Regoes RR, Rolff J. Combination effects of antimicrobial peptides. *Antimicrob Agents Chemother* (2016) 60:1717–1724. doi: 10.1128/AAC.02434-15
45. Frandsen TP, Næsted H, Rasmussen SK, Hauptig P, Wiberg FC, Rasmussen LK, Jensen AMV, Persson P, Wikén M, Engström A, et al. Consistent manufacturing and

quality control of a highly complex recombinant polyclonal antibody product for human therapeutic use. *Biotechnol Bioeng* (2011) 108:2171–2181. doi: 10.1002/bit.23166
46. Ma J, Mo Y, Tang M, Shen J, Qi Y, Zhao W, Huang Y, Xu Y, Qian C. Bispecific Antibodies: From Research to Clinical Application. *Front Immunol* (2021) 12: doi: 10.3389/fimmu.2021.626616
47. Labrijn AF, Janmaat ML, Reichert JM, Parren PWHI. Bispecific antibodies: a mechanistic review of the pipeline. *Nat Rev Drug Discov* (2019) 18:585–608. doi: 10.1038/s41573-019-0028-1
48. De Goeij BECG, Vink T, Ten Napel H, Breij ECW, Satijn D, Wubbolts R, Miao D, Parren PWHI. Efficient payload delivery by a bispecific antibody-drug conjugate targeting HER2 and CD63. *Mol Cancer Ther* (2016) 15:2688–2697. doi: 10.1158/1535-7163.MCT-16-0364
49. Spielberg S. Saving Private Ryan. (1998).


CLOSING
PAGES

Summary in English

Samenvatting in het Nederlands

Riassunto in Italiano

Acknowledgements

About the author

List of publications

Summary in English

We are at war! From the patient's point of view, orthopedic surgeries might be challenging, particularly when implants are involved. Although their presence is crucial to restoring tissue functionality, implants are perceived as foreign bodies by our organism, and therefore they are considered enemies that are being attacked by our immune system. On top of that, most orthopedic surgeries employ non-biodegradable implants (e.g., made in titanium) designed to stay in our body for the long-term or permanently, resulting in stronger and prolonged immune system activation. Although stressful for the patient and the organism, this reaction is an essential component of the healing process, ultimately leading to proper integration of bone and implant.

In this context, macrophages play a key role. This type of white blood cells, from one side coordinate the activity of bone cells to deposit new bone around the newly inserted implant. On the other hand, macrophages defend our organism from external threats, such as foreign substances, dead cells, or microbes, by eating and digesting them in a process called phagocytosis. During and after surgery, macrophages work tirelessly to clean the area around the implant surface to favor healing and new bone deposition. However, this leaves little to no energy available to fulfill their other tasks, such as protecting the organism from invading pathogens. In 1 to 5% of patients undergoing joint replacement surgeries, pathogens present in the operating theatre, e.g., on surgical staff or patients themselves, can find their way through the open wound and proliferate undisturbed on the surface of the implant.

As traditional treatments with systemic antibiotics administration are not always effective against orthopedic implant-associated infections, new approaches have been suggested to improve the therapeutic outcome. According to orthopedic surgeons, a promising therapeutic approach involves the modification of the implants surface to deliver drugs locally. For example, antibiotic-loaded implants may create at the surgical site an inhospitable environment for bacterial growth, so that the immune system can efficiently clear the infection while coordinating the wound healing process. However, the success of these strategies depends on the pathogen causing the infection.

Orthopedic implant-associated infections are mostly caused by *Staphylococcus aureus*. This pathogen represents a worldwide threat to human health due to its remarkable efficacy in surviving human drugs and clinical treatments, while sustaining the attacks from our immune system. Historically, bacterial resistance to antibiotics emerged shortly after the introduction of new drugs in clinical applications, resulting in a notable decline in the discovery and development of antibiotic-based compounds

over the past three decades. On average, it takes approximately 13-14 years to discover and bring a new therapeutic compound to the market, while under optimal conditions, it takes only a few hours for a single *S. aureus* cell to completely change its genome and characteristics. Moreover, *S. aureus* evolved multiple strategies to counteract the activation, recognition, and elimination by the immune system. One such strategy includes the ability to invade, survive, and proliferate inside host cells, including macrophages, which are supposedly responsible for killing pathogens. In fact, *S. aureus* can even use immune cells as a “Trojan horse” to survive and spread infection. Once inside our bodies, traditional antibacterial treatments and host defense mechanisms fail to solve the infection, making *S. aureus* able to prolong, almost indefinitely, its stay within the host.

With this thesis we aim to characterize the therapeutic potential of alternative compounds to antibiotics for preventing implant-associated infections. As *S. aureus* survival within host cells, especially macrophages, has been identified as a risk factor for recalcitrant infections, we have investigated the efficacy of several therapeutic compounds in reducing the number of intracellular pathogens, either by direct killing or modulation of immune cells’ functions.

Metallic silver has been used as an antibacterial agent even before the introduction of antibiotics. However, its use in clinical practice is controversial. Despite its strong antibacterial action, the use of silver is counterbalanced by equally strong toxic effects on the organism. In **chapter 2**, we excluded the existence of a therapeutic window where silver retained its antibacterial properties while not compromising the viability and functions of macrophages. To support this conclusion, we developed a new multicellular *in vitro* model that mimics implant-associated infections. In other words, we were able to replicate the complex interactions at the implant-bone interface in the laboratory using different cell types such as macrophages and bone marrow mesenchymal stem cells (the precursors of osteoblasts).

Moving on from the use of silver, nature provided means to combat pathogenic bacteria before antibiotic discovery. Through thousands of years of evolution, our organism evolved a complex immune system that efficiently protects us from external threats, without modern drugs. Therefore, if we enhance the intrinsic antibacterial functions of our immune system, we might be able to replace antibiotics as an antibacterial treatment. In **chapter 3**, we explored the use of host defense peptides which are naturally occurring molecules employed as a defense mechanism by several cell types. Thanks to their chemical properties, these molecules can tackle bacterial infections via two mechanisms: direct antibacterial activity and modulation of the immune response. Although one host defense peptide did efficiently kill *S. aureus*, all other two peptides that were tested failed to enhance the antibacterial functions of macrophages.

Nonetheless, macrophages are not the only immune cells responsible for the protection against *S. aureus*. Close collaboration between macrophages and neutrophils, one of the most abundant types of immune cell, is essential in clearing bacterial infections. Absence of one or the other cell type would negatively impact our organism defenses against invading pathogens. Therefore, in **chapter 4** we compared the contribution of neutrophils and macrophages to *S. aureus* elimination of. We observed that change in the *in vitro* testing conditions affected immune cells response to pathogens. Specifically, when assays were performed with cells floating, their recognition and phagocytosis of bacteria required the presence of antibodies and complement proteins normally circulating in our bloodstream. Absence of one of these elements, involved the loss of phagocytic capacity by both immune cells when in a floating state, but not when adhering to a surface. In both assay conditions, neutrophils could eat and kill more bacteria than macrophages. These observations were further expanded in **chapter 5**. There, as a basis for the development of a vaccine therapy against *S. aureus*, we observed that pathogen-specific monoclonal antibodies enhanced the ability of both neutrophils and macrophages to recognize, eat, and kill pathogens. Once more, neutrophils outperformed macrophages in terms of ability to clear the infection. Based on these results, we suggested as a possible therapeutic strategy the use bispecific antibodies. These are antibodies able to bind two targets at the same time. We hypothesized that antibodies bound to the bacterial surface could force pathogen's phagocytosis only by neutrophils, given their stronger antibacterial functions, and not by macrophages. Unfortunately, the bispecific antibodies designed failed to induce immune cell-specific phagocytosis of *S. aureus*.

In conclusion, we showed that a one-for-all approach is not sufficient to eradicate *S. aureus* infections. The single compounds studied in this thesis were not sufficient by themselves to kill bacteria or enhance macrophages antibacterial functions. However, a combination of multiple molecules might help preventing infection while promoting the bone healing process. Likewise, although playing a crucial role, macrophages alone are unable to fully resolve intricate clinical scenarios like implant-associated infections. *S. aureus* can negatively affect multiple cell types and tissues surrounding the implant. To effectively overcome this challenge, therapeutic compounds must offer comprehensive protection to all the elements involved in the process. Nevertheless, even rescuing one cell type could lead us one step closer to victory in the ongoing war against *S. aureus*.

Samenvatting in het Nederlands

We zijn in oorlog! Vanuit het oogpunt van de patiënt zijn orthopedische operaties uitdagend, vooral wanneer er implantaten bij betrokken zijn. Hoewel hun aanwezigheid cruciaal is voor het herstel van de weefselfunctionaliteit, worden implantaten door ons organisme gezien als vreemde lichamen en daarom worden ze beschouwd als vijanden die worden aangevallen door ons immuunsysteem. Bovendien gebruiken de meeste orthopedische operaties niet-biologisch afbreekbare implantaten (bijvoorbeeld gemaakt van titanium) die zijn ontworpen om langdurig of permanent in ons lichaam te blijven, wat resulteert in een sterkere en langdurige immuunsysteem activatie. Hoewel stressvol voor de patiënt en het organisme, is deze reactie een essentieel onderdeel van het genezingsproces, dat uiteindelijk leidt tot een goede integratie van bot en implantaat.

Hierbij spelen macrofagen een sleutelrol. Dit type witte bloedcel coördineert aan de ene kant de activiteit van botcellen om nieuw bot af te zetten rond het nieuw ingebrachte implantaat. Aan de andere kant verdedigen macrofagen ons organisme tegen externe bedreigingen, zoals vreemde stoffen, dode cellen of microben, door ze op te eten en te verteren in een proces dat fagocytose wordt genoemd. Tijdens en na de operatie werken macrofagen onvermoeibaar om het gebied rond het implantaatoppervlak schoon te maken om genezing en nieuwe botafzetting te bevorderen. Hierdoor blijft er echter weinig tot geen energie over om hun andere taken te vervullen, zoals het beschermen van het organisme tegen binnendringende ziekteverwekkers. Bij 1 tot 5% van de patiënten die gewrichtsvervangingsoperaties ondergaan, kunnen ziekteverwekkers die aanwezig zijn in de operatiekamer, bijvoorbeeld bij chirurgisch personeel of patiënten zelf, hun weg vinden door de open wond en zich ongestoord vermenigvuldigen op het oppervlak van het implantaat.

Aangezien traditionele behandelingen met systemische toediening van antibiotica niet altijd effectief zijn tegen orthopedische implantaatgerelateerde infecties, zijn nieuwe behandelingen voorgesteld om de therapeutische uitkomst te verbeteren. Volgens orthopedisch chirurgen houdt een veelbelovende therapeutische benadering in dat het oppervlak van het implantaat wordt aangepast om lokaal medicijnen af te geven. Antibiotica geladen implantaten kunnen bijvoorbeeld op de plaats van de operatie een onherbergzame omgeving voor bacteriegroei creëren, zodat het immuunsysteem de infectie efficiënt kan opruimen en tegelijkertijd het wondgenezingsproces coördineert. Het succes van deze strategieën hangt echter af van de ziekteverwekker die de infectie veroorzaakt.

Orthopedisch implantaatgerelateerde infecties worden meestal veroorzaakt door *Staphylococcus aureus*. Deze ziekteverwekker vormt een wereldwijde bedreiging

voor de menselijke gezondheid vanwege zijn opmerkelijke vaardigheid om menselijke medicijnen en klinische behandelingen te overleven, terwijl het de aanvallen van ons immuunsysteem verdraagt. Historisch gezien ontstond bacteriële resistentie tegen antibiotica kort na de introductie van nieuwe geneesmiddelen in klinische toepassingen, wat resulteerde in een opmerkelijke afname in de ontdekking en ontwikkeling van nieuwe antibiotica in de afgelopen drie decennia. Gemiddeld duurt het ongeveer 13-14 jaar om een nieuw geneesmiddel te ontdekken en op de markt te brengen, terwijl het onder optimale omstandigheden slechts enkele uren kost voor een enkele *S. aureus*-cel om zijn genoom en kenmerken volledig te veranderen. Bovendien ontwikkelde *S. aureus* meerdere strategieën om de activering, herkenning en eliminatie door het immuunsysteem tegen te gaan. Eén zo'n strategie omvat het vermogen om gastheercellen binnen te dringen, daar te overleven en zich te vermenigvuldigen, inclusief in macrofagen, die zogenaamd verantwoordelijk zijn voor het doden van pathogenen. *S. aureus* kan zelfs immuuncellen gebruiken als een "Trojaans paard" om te overleven en de infectiete verspreiden. Eenmaal in ons lichaam kunnen traditionele antibacteriële behandelingen en verdedigingsmechanismen van de gastheer de infectie niet oplossen, waardoor *S. aureus* zijn verblijf in de gastheer voor bijna onbepaalde tijd kan verlengen.

Met dit proefschrift willen we het therapeutisch potentieel karakteriseren van alternatieve middelen voor antibiotica voor het voorkomen van implantaat-geassocieerde infecties. Aangezien de overleving van *S. aureus* in gastheercellen, met name macrofagen, is geïdentificeerd als een risicofactor voor chronisch infecties, hebben we de werkzaamheid onderzocht van verschillende therapeutische verbindingen bij het verminderen van het aantal intracellulaire pathogenen, hetzij door directe doding of modulatie van de functies van immuuncellen.

Metaalzilver werd al vóór de introductie van antibiotica als antibacterieel middel gebruikt. Het gebruik ervan in de klinische praktijk is echter controversieel. Ondanks zijn sterke antibacteriële werking wordt het gebruik van zilver gecompenseerd door even sterke toxische effecten op het organisme. In **hoofdstuk 2** hebben we het bestaan van een therapeutisch venster uitgesloten waar zilver zijn antibacteriële eigenschappen behield zonder de levensvatbaarheid en functies van macrofagen in gevaar te brengen. Om deze conclusie te ondersteunen, ontwikkelden we een nieuw meercellig *in vitro* model dat implantaat-geassocieerde infecties nabootst. In andere woorden, we konden de complexe interacties van het implantaat-bot grensvlak repliceren in het laboratorium door middel van verschillende celtypen zoals macrofagen en mesenchymale stamcellen van het beenmerg (de voorlopers van osteoblasten).

Voortbouwend op het gebruik van zilver, bood de natuur middelen om pathogene bacteriën te bestrijden voordat antibiotica werden ontdekt. Door duizenden jaren van evolutie heeft ons organisme een complex immuunsysteem ontwikkeld dat ons

beschermt tegen bedreigingen van buitenaf, zonder moderne medicijnen. Daarom, als we de intrinsieke antibacteriële functies van ons immuunsysteem versterken, kunnen we misschien antibiotica als antibacteriële behandeling vervangen. In **hoofdstuk 3** onderzochten we het gebruik van afweerpeptiden van de gastheer. Dit zijn van nature voorkomende moleculen die door verschillende celtypen als verdedigingsmechanisme worden gebruikt. Dankzij hun chemische eigenschappen kunnen deze moleculen bacteriële infecties aanpakken via twee mechanismen: directe antibacteriële activiteit en modulatie van de immuunrespons. Hoewel één gastheerverdedigingspeptide *S. aureus* efficiënt doodde, konden alle andere drie geteste peptiden de antibacteriële functies van macrofagen niet versterken.

Toch zijn macrofagen niet de enige immuuncellen die verantwoordelijk zijn voor de bescherming tegen *S. aureus*. Nauwe samenwerking tussen macrofagen en neutrofielen, een van de meest voorkomende soorten immuuncellen, is essentieel bij het opruimen van bacteriële infecties. Afwezigheid van het ene of het andere celtype zou een negatieve invloed hebben op de afweer van ons organisme tegen binnendringende ziekteverwekkers. Daarom hebben we in **hoofdstuk 4** de bijdrage van neutrofielen en macrofagen aan *S. aureus* eliminatie vergeleken. We zagen dat verandering in de *in vitro* testomstandigheden de reactie van immuuncellen op ziekteverwekkers beïnvloedde. Specifiek, wanneer assays werden uitgevoerd met drijvende cellen, hun herkenning en fagocytose van bacteriën vereisten de aanwezigheid van antilichamen en complementeiwitten die normaal in onze bloedbaan circuleren. Afwezigheid van een van deze elementen betekende het verlies van fagocytische capaciteit door beide immuuncellen wanneer ze in een drijvende toestand waren, maar niet wanneer ze zich aan een oppervlak hechtten. In beide testomstandigheden konden neutrofielen meer bacteriën eten en doden dan macrofagen. Deze observaties werden verder uitgebreid in **hoofdstuk 5**. Daar, als basis voor de ontwikkeling van een vaccintherapie tegen *S. aureus*, zagen we dat pathogeen-specifieke monoklonale antilichamen het vermogen van zowel neutrofielen als macrofagen verbeterden om ziekteverwekkers te herkennen, op te eten en te doden. Eens te meer presteerden neutrofielen beter dan macrofagen wat betreft het vermogen om de infectie op te ruimen. Op basis van deze resultaten stelden we als mogelijke therapeutische strategie het gebruik van bispecifieke antilichamen voor. Dit zijn antilichamen die twee doelwitten tegelijkertijd kunnen binden. Onze hypothese was dat antilichamen gebonden aan het bacteriële oppervlak de fagocytose van de ziekteverwekker alleen door neutrofielen zouden kunnen forceren, gezien hun sterkere antibacteriële functies, en niet door macrofagen. Helaas konden de ontworpen bispecifieke antilichamen geen immuuncel-specifieke fagocytose van *S. aureus* induceren.

We hebben aangetoond dat een “one-for-all”-aanpak niet voldoende is om *S. aureus*-infecties uit te roeien. De afzonderlijke medicijnen die in dit proefschrift zijn bestudeerd, waren op zichzelf niet voldoende om bacteriën te doden of antibacteriële

functies van macrofagen te versterken. Een combinatie van meerdere moleculen kan echter infectie helpen voorkomen en tegelijkertijd het botgenezingsproces bevorderen. Evenzo, hoewel macrofagen een cruciale rol spelen, zijn ze niet in staat om ingewikkelde klinische scenario's zoals implantaat-geassocieerde infecties volledig op te lossen. *S. aureus* kan meerdere celtypen en weefsels rond het implantaat negatief beïnvloeden. Om deze uitdaging effectief te overwinnen, moeten therapeutische medicijnen uitgebreide bescherming bieden aan alle elementen die bij het proces betrokken zijn. Niettemin kan zelfs het redden van één celtype ons een stap dichter bij de overwinning brengen in de voortdurende oorlog tegen *S. aureus*.

Riassunto in Italiano

Siamo in guerra! Dal punto di vista del paziente, sottoporsi a interventi chirurgici ortopedici potrebbe essere un'esperienza difficoltosa, soprattutto quando coinvolge l'uso di un impianto. Sebbene la sua presenza sia cruciale per ripristinare la funzionalità dei tessuti, qualsiasi impianto viene prima percepito come un corpo estraneo dal nostro organismo e perciò considerato nemico e attaccato dal nostro sistema immunitario. Inoltre, la maggior parte delle operazioni ortopediche utilizza impianti non biodegradabili (realizzati ad esempio in titanio) progettati per rimanere nel nostro corpo a lungo termine o permanentemente, provocando una forte e prolungata attivazione del sistema immunitario. Anche se stressante per il paziente e l'organismo, questa reazione è una fase essenziale del processo di guarigione, che alla fine porta a una corretta integrazione tra osso e impianto.

In questo contesto, i macrofagi giocano un ruolo chiave. Questo tipo di globuli bianchi, da un lato, coordina l'attività delle cellule ossee per depositare nuovo osso attorno all'impianto appena inserito. Dall'altro lato, i macrofagi difendono il nostro organismo da minacce esterne, quali sostanze estranee, cellule morte o microbi, mangiandoli e digerendoli in un processo chiamato fagocitosi. Durante e dopo l'intervento chirurgico, i macrofagi lavorano instancabilmente per pulire l'area intorno alla superficie dell'impianto per favorire la guarigione e la deposizione di nuovo osso. Tuttavia, questo lascia molta poca energia disponibile per svolgere gli altri loro compiti, come proteggere l'organismo dagli agenti patogeni. I microbi presenti in sala operatoria, ad esempio sul personale chirurgico o sui pazienti stessi, possono trovare una via di accesso al nostro organismo attraverso la ferita aperta, e proliferare indisturbati sulla superficie dell'impianto nell'1% fino al 5% dei casi di pazienti sottoposti a interventi chirurgici di sostituzione.

La pratica comune per il trattamento di infezioni associate agli impianti ortopedici prevede la somministrazione sistemica di antibiotici. Tuttavia, questa pratica non è sempre efficace. Per questo motivo, sono state suggerite nuove pratiche per migliorare l'esito terapeutico. Secondo i chirurghi ortopedici, un approccio promettente comporta la modifica della superficie degli impianti per somministrare i farmaci localmente. Ad esempio, l'uso di impianti ricoperti con antibiotici può creare nel sito chirurgico un ambiente inospitale per la crescita batterica, così da favorire il lavoro del sistema immunitario nell'eliminare efficacemente l'infezione e allo stesso tempo coordinare il processo di guarigione della ferita. Tuttavia, il successo di queste strategie terapeutiche dipende dall'agente patogeno che causa l'infezione.

La maggior parte delle infezioni associate agli impianti ortopedici è causata da *Staphylococcus aureus*. Questo patogeno rappresenta una minaccia a livello

globale per la salute dell'uomo. *S. aureus* è noto per la sua incredibile resistenza a trattamenti farmacologici e clinici dell'uomo, sopravvivendo allo stesso tempo agli attacchi del nostro sistema immunitario. Storicamente, la resistenza batterica agli antibiotici è emersa poco dopo l'introduzione di nuovi farmaci nella pratica medica, determinando un notevole declino nella scoperta e nello sviluppo di nuovi farmaci negli ultimi tre decenni. In media, occorrono circa 13-14 anni per scoprire e portare sul mercato un nuovo farmaco, mentre in condizioni ottimali, bastano poche ore a una singola cellula di *S. aureus* per cambiare completamente il suo genoma e le sue caratteristiche. Inoltre, *S. aureus* ha sviluppato molteplici strategie per contrastare l'attivazione, il riconoscimento e l'eliminazione da parte del sistema immunitario. Una di queste strategie include la capacità di invadere, sopravvivere e proliferare all'interno delle cellule del nostro organismo, inclusi i macrofagi, il cui ruolo sarebbe proprio quello di eliminare i patogeni. Inoltre, *S. aureus* può persino utilizzare le cellule immunitarie come un "cavallo di Troia" per sopravvivere e diffondere l'infezione nel nostro organismo. Una volta all'interno del nostro corpo, i tradizionali trattamenti antibatterici e i meccanismi di difesa del nostro organismo non riescono a risolvere l'infezione, rendendo *S. aureus* in grado di prolungare, quasi indefinitamente, la sua permanenza all'interno del nostro corpo.

Con questa tesi ci proponiamo di caratterizzare il potenziale terapeutico di composti diversi dagli antibiotici per la prevenzione delle infezioni associate all'impianto. La sopravvivenza di *S. aureus* all'interno delle cellule del nostro organismo, soprattutto nei macrofagi, è stata identificata come un fattore di rischio per le infezioni persistenti. Per questo motivo, ci proponiamo di studiare l'efficacia di diversi composti terapeutici nel ridurre il numero di batteri sia uccidendoli direttamente, sia tramite stimolazione delle cellule immunitarie.

L'argento metallico è stato utilizzato come agente antibatterico anche prima dell'introduzione degli antibiotici. Tuttavia, il suo utilizzo clinico è controverso. Nonostante una forte azione antibatterica, l'uso dell'argento è controbilanciato da altrettanto forti effetti tossici per l'organismo. Nel **capitolo 2**, abbiamo escluso l'esistenza di una finestra terapeutica in cui l'argento mantenesse le sue proprietà antibatteriche senza compromettere la vitalità e le funzioni dei macrofagi. Per supportare questa conclusione, abbiamo sviluppato un nuovo modello multicellulare *in vitro* che imita l'ambiente delle infezioni associate all'impianto. In altre parole, abbiamo replicato le complesse interazioni all'interfaccia tra impianto e osso in laboratorio, usando macrofagi e cellule staminali mesenchimali del midollo osseo (i precursori degli osteoblasti).

Lasciando da parte l'uso dell'argento, la natura ci ha fornito di mezzi per combattere le infezioni batteriche ancora prima della scoperta degli antibiotici. Attraverso migliaia di anni di evoluzione, il nostro organismo ha sviluppato un complesso sistema immunitario che ci protegge efficacemente dalle minacce esterne, senza ricorrere

ai farmaci moderni. Pertanto, potremmo trovare un'alternativa agli antibiotici se riuscissimo ad aumentare le intrinseche funzioni antibatteriche del nostro sistema immunitario. Nel **capitolo 3**, abbiamo studiato l'uso di peptidi antibatterici: molecole presenti in natura impiegate come meccanismo di difesa da diversi tipi di cellule. Grazie alle loro proprietà chimiche, queste molecole possono contrastare le infezioni batteriche attraverso due meccanismi: diretta attività antibatterica e modulazione della risposta immunitaria. Un solo peptide antibatterico è risultato efficace contro *S. aureus*, mentre gli altri 2 peptidi testati non hanno potenziato le funzioni antibatteriche dei macrofagi.

Tuttavia, i macrofagi non sono le uniche cellule immunitarie responsabili della protezione contro *S. aureus*. Una stretta collaborazione tra macrofagi e neutrofili, uno dei tipi più abbondanti di cellule immunitarie presenti nel nostro organismo, è essenziale per contrastare le infezioni batteriche. L'assenza di uno o dell'altro tipo cellulare avrebbe un impatto negativo sulle difese del nostro organismo contro i batteri. Nel **capitolo 4** abbiamo confrontato il contributo di neutrofili e macrofagi all'eliminazione di *S. aureus*. Abbiamo osservato che il cambiamento delle condizioni di esperimenti *in vitro* influenza la risposta antibatterica delle cellule immunitarie. Nello specifico, quando i test sono stati eseguiti con cellule fluttuanti, il loro riconoscimento e la fagocitosi dei batteri richiedevano la presenza di anticorpi e proteine del complemento normalmente presenti nel nostro sangue. L'assenza di uno di questi elementi comportava la perdita della capacità di fagocitosi da parte di entrambe le cellule immunitarie. Evento non registrato se invece le cellule aderivano a una superficie. In entrambe le condizioni testate, i neutrofili hanno mangiato e ucciso più batteri rispetto ai macrofagi. Queste osservazioni sono state ulteriormente ampliate nel **capitolo 5**. Qui, come base per lo sviluppo di un vaccino contro *S. aureus*, abbiamo osservato che gli anticorpi monoclonali specifici per il batterio miglioravano la capacità sia dei neutrofili che dei macrofagi di riconoscere, mangiare e uccidere gli agenti patogeni. Ancora una volta, i neutrofili hanno superato i macrofagi in termini di capacità di risolvere l'infezione. Sulla base di questi risultati, come possibile strategia terapeutica abbiamo suggerito l'utilizzo di anticorpi bispecifici. Si tratta di anticorpi in grado di legare due bersagli contemporaneamente. Abbiamo ipotizzato che gli anticorpi legati alla superficie batterica potessero forzare la fagocitosi del batterio solo da parte dei neutrofili, date le loro migliori prestazioni antibatteriche, e non dai macrofagi. Sfortunatamente, gli anticorpi bispecifici progettati non sono riusciti a indurre la fagocitosi di *S. aureus* specificamente da una delle cellule immunitarie.

In conclusione, abbiamo dimostrato che un approccio "uno per tutti" non è sufficiente per debellare le infezioni provocate da *S. aureus*. I singoli composti studiati in questa tesi non erano sufficienti da soli né per uccidere i batteri né per potenziare le funzioni antibatteriche dei macrofagi. Tuttavia, una combinazione di più agenti terapeutici potrebbe aiutare a prevenire l'infezione e promuovere al contempo il processo di guarigione ossea. Allo stesso modo, pur ricoprendo un ruolo

molto importante, i macrofagi da soli non sono in grado di risolvere completamente scenari clinici complicati come le infezioni associate all'impianto. *S. aureus* può colpire e danneggiare diversi tipi di cellule e tessuti che circondano l'impianto. Per superare questa sfida, i trattamenti dovrebbero offrire una protezione completa a tutti gli elementi coinvolti nel processo. Tuttavia, anche salvare un solo tipo di cellula potrebbe portarci un passo avanti verso la vittoria nella guerra in corso contro *S. aureus*.

Acknowledgements

Grazie di cuore a tutti quelli che mi sono stati di supporto in questi anni molto intensi!
Thank you from the bottom of the hearth to all those who supported me during these very intense years!

And honestly, I would like to end here this section. Those who know me are well aware of how much I'm struggling to write this section. I'd rather start over the PhD than write this section.
Mmmh…actually, never mind.
Let's write the acknowledgements!

Keep in mind that I might forget someone, and you might be highly disappointed. If you fall into these categories, I hope I already made it up in person. If not, are you sure? Come talk to me, I might have an "I'm sorry"-jar of Pesticello for you. In case, please blame those who refused to be my ghost writers and forced me to write this section!

Jos, you have been an amazing support throughout this journey, scientifically and personally. I learnt a lot and incredibly enjoyed hearing all your anecdotes about scientific, lab, and life discoveries. You were the first one that made me realize I should maybe think about what I want to do after the PhD and help me in that direction. I hope I can give you an answer soon.

Harrie, your enthusiasm for science has been a great inspiration. Every time I shared my results with you, your excitement and genuine interest were amazing, I always saw you as the most enthusiastic person in the room. Your ideas and great feedback have been invaluable to improve my work and reaching this point.

Saber, without you this journey would have never started in the first place. Thank you for being the first one to believe in me. Despite all the challenges encountered, like a pandemic or losing support in the lab, just to mention a few, you have always been there to support me.

Bart, despite our different academic backgrounds, you always supported me. Your curiosity and enthusiasm to engage with my research and progress have been incredibly encouraging. And let's not forget that your health recommendations saved my dislocated shoulder! Both me and shoulder thank you for that!

Daphne, **Kok**, and **Michiel**. Let's be honest, the help from the people above was essential, but without your constant guidance and help in the lab, none of the experiments of this thesis would have been possible. Thank you for all the teaching and fruitful discussions we had over these years. Too bad that you had to leave before the end of my journey but thank you for sticking around nonetheless!

Talking about support for the experiments in this thesis, thank you **Maartje**, **Carla**, **Piet**, **Lisette**, and **Erik** for being the very backbone of the lab. Same feelings but from a different lab, thank you **Mattie** for always helping me finding stuff at the RMCU. Ah! Piet, sooner or later I will find a white Chianti to drink with you! While you're waiting, I can recommend you a nice red (sweet) wine: Ramandolo.

Last but not least, an incredible amount of help in shaping this thesis came from **Zeldali**, **Claudio**, and **Elisabeth**. It was an absolute pleasure working with you. I hope I managed to teach you something with no traumas! I definitely learnt a lot from you.

A big thanks goes to my two families (or groups), conveniently separated in two buildings so everyone always doubted if they were real, or I was just taking long breaks when saying "I'm going to the other building".

Thanks to the orthopedic group: **Azin**, **Pardis**, **Zhengquing**, **Kelly** (you are the only one who truly understands the suffering behind working with macrophages), **Jaqueline**, **Zhiming**, **Zijan**, **Ruben**, **Anneli**, **Paree**, **Nada**. Although we all work on different topics, we always tried to give feedback and ideas for our projects. I learnt a lot from all of you!

Big thanks to the MMB group! **Suzan**, thank you for welcoming me in your (amazing) research group and meetings. It was an amazing opportunity to learn and grow as a scientist. Also, thanks to **Bart**, **Dani**, **Priscilla**, **Magda**, **Pieter-Jan**, **András** for your valuable insights, questions, and feedbacks in and outside the meetings.

Sweet little **G.04.527**. For the past years you have been like my second home, and what an amazing place you were with all the amazing scientists that came in and out. I'm sorry if **Coco** (read with your perfect Italian pronunciation) didn't appreciate you enough and abandoned you, maybe he was offended that you couldn't keep cold his second lunch. At least, **Julian** moved office for a good reason, but always came back to motivate his young wolves! Or maybe they didn't want to be part of the memes? **Jesse**, this is my official retirement as office meme generator. You've proven worthy of this role, go meme around, and embarrass everyone. I can already share material on **Barath**, **Ruben**, and **Tristan** (thanks Luv for all the plants). I hope you can find another muse as amazing as **Jiannan** was for all of us! If you're reading, I miss you Jiannan!

Last but not least, **Gijs**, you really fought hard to earn your spot in this section. You would have made it anyway, just for the good series recommendations, but you wanted to overdo it by being my official Dutch translator, dank je wel!

Moving out from the office, I would miss hearing my name shout out in the corridors or in the lab, in the best Italian pronunciation. Thank you, **Leeeeire**! Hope to meet you again tutto presto!

Surprisingly, even more amazing people could also be found in other offices. **Remy**, I'm not sure about your qualities as a supervisor in the lab, but you were definitely an amazing skiing teacher. I'm slow on keeping that promise, I know, but one day I hope we can finally do a photography day! I don't have energies to write stories about all of you, so please just make it work with a big thank you **Lisanne**, **Sjors**, **Eva**, **Coco**, **Marije**, **Kulsum**, **Lotte**, **Ninée**, **Marieke** for all the breaks, outings, vrijmibo, skiing trips, and all the rest!

If this was a powerpoint presentation, then **Frerich** you are the Sleeping Beauty of animations to move from the acknowledgments of people from work to those outside work. Ragazzo, la mia vita sociale e salute mentale ti hanno amato con tutto il cuore; il mio fegato invece ti ha odiato con tutte le sue (ora molto poche) cellule sane.

Thanks to all the **RESCUE people**!! Sorry, you're too many to write all your names, just know that you've been the very first family that I met here in The Netherlands. I feel very lucky to have met all of you.

For example, talking about luck, what were the odds of going out for dinner and coming back home with a packed lunch for the day after?! Thanks **Paree**, for feeding me delicious Indian food, and I'm still waiting on that monkey you promised for me.

Thank you, **Paul** and **Koen**, for always hearing our complaints and let us organize many nice events with the RM committee!

I'm very proud that I could share with all of you many lunches, BBQs, dinners, parties, and sleepovers in my house. But what everyone should know, is that if it weren't for **Leanne**, none of this would have been possible, and I might still be homeless. To be fully honest, I should thank you and the countless bottles of Cava we drunk, before and after getting the house. Sorry if my first gratitude action was to cook for you the worst frozen pizza that ever existed in this world.

There is also someone that once invited, never wanted to leave the house. Even worse, that someone was the cause for most of the scars of the house. Any resemblance to actual persons, living or really really old, or actual events is purely coincidental, **TJ**. I couldn't expect better crazy Balkan vibes from you. Speaking of… let's raise a glass of rakija/whisky/any liquor ever produced, for my favorite girls, **Ali** and **Stefan**, who have massively supported me along these years. Cheers!!

Alessandro non dimentichiamoci che sei stato la primissima persona che ho conosciuto qui a Utrecht. E siamo ancora qui! Dopo essere riusciti a rompere

Lorenzo, la Cultura che abbiamo condiviso sarà per sempre conservata nel locus amoenus del mio cuore. Il tutto, con la consapevolezza che se da ragazzini avessimo avuto anche noi degli insegnanti così…oggi saremmo scienziati!

Bomber e **Carla**/**Jessica**, non vi darò la soddisfazione di diventare smielato e sentimentale in questi ringraziamenti. Per usare una delle mille citazioni raccolte nel mio telefono (e quelle dette da voi non possono essere scritte in una tesi), siamo un gruppo di persone che “viaggia con la mente ma non con le parole”. Beh, con voi è stato, e voglio che continui a essere, un viaggio fantastico. Grazie **Matteo** e **Ludo** per esserci! <3

A tutti voi che siete in Italia, **Fugu**, **Spola**, **Alby**, **Fede**, **Ricky**, **Vane**, **Ale**, **Ste**, **Thomas**, **Andrea**, **Mattia**, **Alen**, **Filip**, **Arianna**, e tanti altri, grazie di cuore per avermi sempre seguito in tutti i posti che ho cambiato in questi anni, ma soprattutto grazie per avermi sempre accolto quando torno a casa.

Lorenzo, che alchimista! (*cit.*). E direi che questa citazione dice già tutto nel modo più sincero possibile senza che mi impegni a scriverti dei ringraziamenti. Anzi a dirla proprio tutta, sei quello che ha contribuito più di tutti a questa tesi grazie alla parola d'ordine “Mandala in vacca!”. Spero di aver seguito il tuo insegnamento nel miglior modo possibile. E spero sia anche il messaggio chiave del nostro polo.

Julia, ours was kind of a rough start. You refused to write my acknowledgments, you never appreciated my presentations about saving Private MotherFucker…if I really have to point out a bonding moment, I should be thanking your zapatillas?! So, let's restart.

Julia's **zapatillas**, you are a wonderful person, your friendship means a lot to me (*cit.* Ron Swanson), you have been an amazing moral and fun support throughout these years. It was always difficult to get through your incomprehensibly busy agenda, but I'm happy that you did get out of your copertina sometimes!

Mamma e **Papà**, immagino che tutti questi anni vi siate sempre chiesti che cosa stessi facendo qui, e la mia consueta abitudine di non farmi sentire mai non credo abbia aiutato. Spero che questi giorni vi abbiano potuto dare un'idea di quale sia il mio mondo e le persone che mi circondano. Anche se ci sentiamo e vediamo poco, sappiate che tutti questi anni fuori da casa sono stati possibili perché sapevo di poter contare sul vostro supporto e affetto incondizionato. E ovviamente questo non vale solo per questi ultimi anni in Olanda, ma per tutti quelli passati e futuri! Grazie per non aver mai smesso di credere in me!

Ovviamente, tutto questo vale anche per **Marco** ed **Eleonora**, anzi, se non fosse stato per voi, questa tesi non esisterebbe nemmeno fisicamente! E ancor di più vale per **Agata**, sarei contentissimo se un giorno leggerai questa tesi.

About the author

Leonardo Cecotto was born on 17 November 1993 in San Dona' di Piave, Italy. In 2012, he graduated from the science-focused high school "XXV Aprile" and moved to Trieste to start his bachelor's studies in Biological Sciences and Technologies with focus in Biotechnology. During his bachelor, Leonardo ventured on his first experience abroad thanks to the Erasmus+ study program, studying for one semester at University of Rennes 1, in France. In 2016, he obtained his BSc and again, thanks to the Erasmus+ internship program, he traveled from Trieste to Bangor University, in Wales. There, he spent 5 months in the lab working on the discovery of genes coding for enzymes of industrial relevance from archaeal metagenomic library.

Back in Italy, he moved again to Parma to start in 2016 his master's studies in Medical, Veterinary, and Pharmaceutical Biotechnologies with focus in Regenerative Medicine. There he worked on the characterization of adult rat thyroid stem cells as a source of brown adipose tissue. To complete his master's internship thesis, he spent 2 months at Maastricht University, in The Netherlands. In retrospect, this brief experience combined to all his experiences abroad, profoundly influenced Leonardo's future decisions.

After receiving his MSc cum laude in 2018, he was strongly determined to stay in research and continue his education abroad. In February 2019, he moved to Utrecht to start his PhD on a project focused on finding alternative therapeutic options to prevent orthopedic implant-associated infections. the results of his research are described in this thesis and in peer-reviewed publications in scientific journals.

Aside his research activity, Leonardo has been actively involved in the RM PhD committee organizing events to foster social and working interactions among his fellow PhD students in the Regenerative Medicine program.

List of publications

Cecotto L, van Kessel KPM, Wolfert MA, Vogely HC, van der Wal BCH, Weinans H, van Strijp JAG, Amin Yavari S. Antibacterial and anti-inflammatory properties of host defense peptides against *Staphylococcus aureus*. iScience. 2022 Sep 24;25(10):105211. doi: 10.1016/j.isci.2022.105211. PMID: 36248729; PMCID: PMC9563556.

Cecotto L, Stapels DAC, van Kessel KPM, Croes M, Lourens Z, Vogely HC, van der Wal BCH, van Strijp JAG, Weinans H, Amin Yavari S. Evaluation of silver bio-functionality in a multicellular *in vitro* model: towards reduced animal usage in implant-associated infection research. Front Cell Infect Microbiol. 2023 Jun 5;13:1186936. doi: 10.3389/fcimb.2023.1186936. PMID: 37342248; PMCID: PMC10277478.

Cecotto L, van Kessel KPM, de Vor L, Passerini C, Vogely HC, van der Wal BCH, Weinans H, Rooijakkers SHM, Amin Yavari S, van Strijp JAG. The relative role of human neutrophils and macrophages in phagocytosis and killing of *Staphylococcus aureus*. Submitted.

PhD Training certificate - Graduate School of Life Sciences

Discipline-specific educational activities	# ECTS
(2019) Intro to regenerative medicine	1.5
(2019) FACS course/masterclass	0.25
(2019) Enabling technologies	1.5
(2020) Sophisticated laboratory techniques in cardiovascular research	3
(2020) Introduction to Stem Cells	2.4
(2020) Writing a scientific paper	1.5
(2020) Statistics in the lab (*in vivo* and *in vitro*)	1.5
(2021) Supervising Research of MSs students at the GSLS	1.2
General educational activities	
(2019) Dutch Language intro course	0.8
(2019) Adobe InDesign Essentials	0.6
(2020) Scientific Artwork – Data visualization and infographics with Adobe Illustrator	0.6
(2020) Research planning and Time Management	0.4
(2020) The art of presenting science	1
(2021) Science Communication: Relevant and Clear	3
(2022) Ethics in RM	0.3
Symposia/conferences (oral/poster presenter) and other activities	
(2019 / 2021 / 2022) RM Retreat	3
(2022) EMCHD	1
(2022) EBJIS	1
(2022 / 2023) Scientific spring meeting KNVM NVMM	2
(2019 / 2022) NBTE	2
(2022) PhD day	0.2
(2022 / 2023) RM PhD Networking and Poster event	1
TOTAL NUMBER OF ECTS	**29.75**