

**Alma Mater Studiorum – Università di Bologna**

**DOTTORATO DI RICERCA IN BIOLOGIA CELLULARE,  
MOLECOLARE E INDUSTRIALE**

Ciclo XXV

Settore Concorsuale di afferenza: 05/E2

Settore Scientifico disciplinare: BIO/11

**The silent survival of *Staphylococcus aureus*  
phagocytosed by HL-60 derived neutrophils**

**Presentata da: Sarah Nosari**

Coordinatore Dottorato

**Ch.mo Prof. Vincenzo Scarlato**

Relatore

**Ch.mo Prof. Vincenzo Scarlato**

**Dott.ssa Ilaria Ferlenghi**

Esame finale anno 2013

## GENERAL OVERVIEW

*Staphylococcus aureus* is a facultatively anaerobic, Gram-positive coccus.

*S. aureus* has been recognized as serious pathogen for over a century (1, 2) particularly in hospitals, they remain the most common cause of community- and health care-associated bacteremia. Of the approximately 2 million of patients who acquire a health care-associated infection annually in the United States, approximately 230.000 will have an infection associated with *S. aureus* (3,4).

The disease spectrum of the *S. aureus* includes abscesses, bacteremia, central nervous system infections, endocarditis, osteo-myelitis, pneumonia and a host of syndrome caused by endotoxins, including food poisoning and toxic shock syndrome.

The incidence of nosocomial infections is steadily increasing due to medical interventions and antibiotic resistance. Today, approximately half of all *S. aureus* strains isolated in hospitals worldwide are resistant to multiple antibiotics, such as methicillin, rendering disease management difficult. To date there are no approved prophylactic vaccines so, effective treatment and prevention strategies are urgently needed.

Although *S. aureus* is considered to be an extracellular, pyogenic pathogen recent in vitro studies have revealed that *S. aureus* can invade a variety of not professional phagocytes cells (5,6). Internalized bacteria reside in endosomal vacuoles or are diverted from the endosomal pathway to autophagosomes depending on the cell type invaded and/or the *S. aureus* strain (7).

Professional phagocytes such as neutrophils, macrophages and dendritic cells are designed to engulf microbes and kill them. Only a few types of microbial pathogen can survive phagocytosis by neutrophils and macrophages and they do so by using several distinct mechanisms to avoid destruction in phagolysosomes (8). Surprisingly, *S. aureus* also appears to be resistant to bactericidal attack inside the phagocytic vacuoles of neutrophils which can contain viable intracellular bacteria when isolated from sites of infections. Recent in vivo studies confirmed the high level of resistance to killing by neutrophils (9).

The objective of my PhD thesis is to study two aspects of the host-pathogen interaction of *S. aureus* with the host cells: firstly the recognition step and secondly the internal localization of the pathogen during the phagocytosis process. In particular the first part of this study is focused on the analysis of the binding between the HL-60 neutrophils receptors and *S. aureus* receptors expressed on the surface; while the second part is focused on the intracellular

localization of bacteria. For the first purpose CD1 mice were immunized with final vaccine protein formulations. The collected animal sera were tested in order to evaluate the functional activity of vaccine specific antibodies by opsonophagocytosis assay (OPKA). The data show a low level of killing of *S. aureus* cells suggesting that the pathogen is not undergoing to the phagocytic pathway normally activated by the infected cell. We also performed intracellular survival studies and obtained results showing that *S. aureus* persists inside phagocytes up to six hours followed by a burst of growth in the supernatant immediately after. These data suggest that the strong survival ability of *S. aureus* in the phagocytes can be the cause of the low killing level measured by OPKA. To gain a broader view of the internalization steps in which *S. aureus* occurs during his uptake in the host cell we decided to investigate its localization in HL-60 cells compartments by three different type of microscopy able to generate different kind of morphological information: confocal microscopy, scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Interestingly we observed that staphylococcus cells have an intracellular localization, more specifically inside the vacuoles, during their entire pathogenic life, and moreover that are able to maintain the integrity of their membrane indicating a preservation of their wellness. Evidence of this status is that many of the bacteria present in the vacuoles are not only alive but also even in the process of dividing inside vacuolar compartments. Finally

in order to generate 3D volume of whole bacteria when present inside neutrophilic vacuoles, we collected a series of tomographic two-dimensional (2D) images by using a transmission electron microscope. We generate 5 different tomograms and for each tomogram more than 120 images were kept, one for each tilt angle. After image analysis we produce a three-dimensional model of a specific volume of a cell containing the *S. aureus* in a vacuole compartment. The three-dimensional reconstruction reveals the presence of intact bacteria within neutrophil vacuoles. The *S. aureus* membrane appears completely undamaged and integral in contrast with the physiological process of phagocytosis through vacuoles progression. *S. aureus* bacteria show a homogenous distribution of the density in all the three dimensions (X, Y, Z). No preferential density distribution has been noticed and the cell turgor is maintained. All these evidences definitely explain the ability of the pathogen to survive inside the endosomal vacuoles and should be the cause of the low killing level.

## REFERENCES

- [1] Chambers, H. F. 2001. The changing epidemiology of *Staphylococcus aureus* Emerg. Infect. Dis. 7:178-182
- [2] Lowy, F.D. 1998. *Staphylococcus aureus* infections. N. Engl. J med. 339:520-532
- [3] Emori, T. G., and R.P. Gayenes. 1993. An overview of nosocomial infections including the role for the microbiology laboratory. Clin. Microbiol. Rev. 6:428-442
- [4] National Nosocomial Infections Surveillance System. 2003. National Nosocomial Infections Surveillance (NNIS) report, data summary from January 1992 through June 2003, issued August 2003. Am J. Infect. Control 31:481-498
- [5] Bayles KW, Wesson CA, Liou LE, Fox LK, Bohach GA, et al. (1998) Intracellular *Staphylococcus aureus* escapes the endosome and induces apoptosis in epithelial cells. Infect Immun 66:336-342
- [6] Peacock SJ, Foster TJ, Cameron BJ, Berendt AR (1999) Bacterial fibronectin binding proteins and endothelial cell surface fibronectin mediate adherence of *Staphylococcus aureus* to resting human endothelial cells. Microbiology 145: 347-3486
- [7] Schnaith A, Kashkar H, Leggio SA, Addicks K, Kronze M, et al. (1994) *Staphylococcus aureus* subvert autophagy for induction of caspase-independent host cell death. J Biol Chem 282: 2695-2706
- [8] Amer AO, Swanson MS (2002) A phagosome of one's own: a microbial guide to life in the macrophage. Curr Opin Microbiol 5:56-61
- [9] Voyich JM, Braughton KR, Sturdevant DE, Whitney AR, Said-Salim B, et al. (2005) Insights into mechanism used by *Staphylococcus aureus* to avoid destruction by human neutrophils. J Immunol 175: 3907-3919

# TABLE OF CONTENTS

<b>1. INTRODUCTION.....</b>	<b>10</b>
1.1 <i>Staphylococcus aureus</i> .....	10
1.2 The epidemiology of <i>Staphylococcus aureus</i> .....	11
1.3 Community acquired infections.....	12
○ Colonization and infection of skin and soft tissue.....	12
○ Skin Infections.....	13
○ Bacteremia and Endocarditis.....	13
○ Toxin-mediated Diseases.....	14
1.4 Health care- associated infections.....	15
1.5 Antimicrobial resistance.....	15
○ Antimicrobial resistance in the hospital setting.....	15
○ Penicillin resistance.....	16
○ Methicillin resistance.....	17
1.6 Neutrophils in innate host defense against <i>Staphylococcus aureus</i> infection.....	19
○ Neutrophils development.....	19
○ Neutrophils recruitment.....	20
○ Pathogen recognition and phagocytosis.....	22

1.7	<i>Staphylococcus aureus</i> and the complement system.....	23
1.7.1	The complement system.....	23
1.7.2	Immune evasion by <i>Staphylococcus aureus</i> .....	26
	○ Virulence factors of <i>S. aureus</i> .....	26
	○ Inhibition of neutrophil chemo taxis.....	27
	○ Resistance to phagocytes.....	28
1.7	<i>Staphylococcus aureus</i> - Eukaryotic Interactions.....	33
1.8	Intracellular survival of <i>Staphylococcus aureus</i> .....	35
	<b>➤ BIBLIOGRAPHY.....</b>	<b>36</b>
<b>2.</b>	<b>MATERIAL AND METHODS.....</b>	<b>46</b>
2.1	Bacterial strain and culture condition.....	46
2.2	Animal model.....	46
2.3	Cells culture.....	47
2.4	Opsonophagocytosis Killing Assay.....	47
2.5	Invasion assay.....	50
2.6	Bacterial labeling with PHrodo™.....	50
2.7	Fluorescence staining and confocal microscopy.....	52
2.8	Scanning electron microscopy.....	53

2.9	Transmission electron microscopy.....	53
2.10	Tomography electron microscopy.....	54
2.11	Image analysis.....	55

### **3. RESULTS**

3.1	OPKA on Newman wt strain shows a killing percentage around 50 %.....	56
3.2	Confocal microscopy analysis shows that <i>S. aureus</i> cell are engulfed by neutrophils after 1 hour of incubation.....	63
3.3	<i>S. aureus</i> persists inside neutrophils until the cells lyse.....	67
3.4	Ultrastructural microscopy analysis shows that survival of bacteria in neutrophils affect the viability of host cells.....	68
3.5	<i>S. aureus</i> perseveres in intracellular vacuoles of HI-60 cells.....	73
3.6	Electron tomography analysis of <i>S. aureus</i> bacteria in intracellular vacuoles of HI-60 cells.....	77

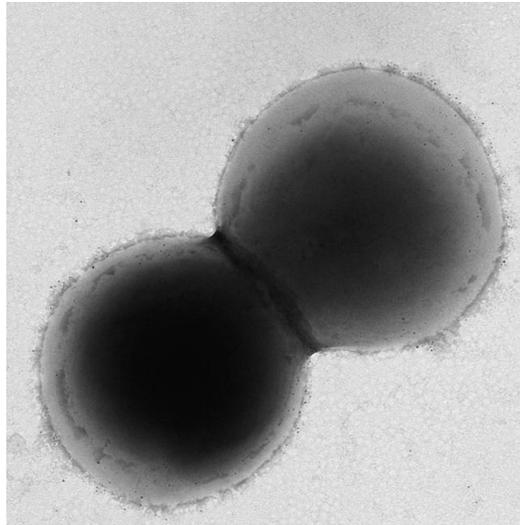
### **4. DISCUSSION.....84**

### **➤ BIBLIOGRAPHY.....87**

## 1. INTRODUCTION

### 1.1 *Staphylococcus aureus*

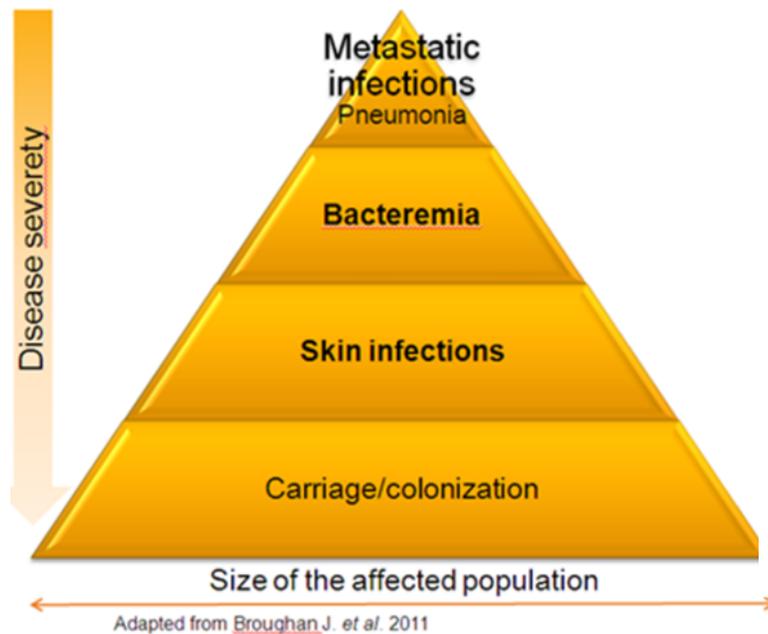
*Staphylococcus aureus* is a spherical (coccus) Gram-positive bacterium, about 1 micrometer in size, with thick cell wall and thin capsule. It does not form spores, but it can still survive outside the body, for example, on bed lining or computer keyboards from few days to several weeks. It is a facultative anaerobe – it grows in the air, but can also thrive in anaerobic conditions. It is an opportunistic pathogen – it may be present in the body without causing any harm, but in lowered immunity or injury, it may cause a disease. Staph bacteria have no flagella, so they are immobile. They grow in pairs, short chains or clusters. *S. aureus* bacteria are resistant to temperatures as high as 122 °F (50°C), to high salt concentrations (<10%), and to drying (11). Colonies are usually 6-8 mm in diameter, rounded and smooth, golden yellow or pale yellow to orange.



**Figure 1: Anti-surface protein Immune Gold Electron microscopy of fixed *S. aureus* Newman strain:** bacterium shows a size around 1 micrometer and typically grows in pairs.

## 1.2 The epidemiology of *Staphylococcus aureus*

*Staphylococcus aureus* causes a variety of diseases, ranging from innocuous skin infection to more serious and life-threatening diseases. Of the approximately 2 million patients who acquire a health care-associated infection annually in the United States, approximately 230,000 will have an infection associated with *Staphylococcus aureus*<sup>1,2</sup>. The disease spectrum of *S. aureus* includes abscesses, bacteremia, central nervous system infections, endocarditis, osteomyelitis, pneumonia, urinary tract infections, and a host of syndromes caused by exotoxins, including bullous impetigo and toxin shock syndrome. Remarkably, in addition to being the leading cause of bacteremia in the United States, *S. aureus* is the most common cause of food-borne illness and skin diseases<sup>3,4</sup> (Figure 1).



**Figure 1: Schematic overview of the main associated *S. aureus* diseases:** the graph has been made on the basis of disease severity and size of affected population. *S. aureus* is the most common cause of health care-associated infections, including pneumonia, bacteremia and skin infections. (Broughan J. et al., 2011)

### 1.3 Community acquired infections

#### Colonization and infection of skin and soft tissue

*S. aureus* is able to colonize a sizable portion of the human population: the colonization gives to the pathogen the possibility to access to skin sites, which, when infected, can serve as a source for more serious diseases such as bacteremia, endocarditis or toxemias. Approximately 30% of the population is stably colonized with *S. aureus* and as many as 30% to 50% of the population may show transient colonization of the nares, axilla, perineum, or vagina<sup>5-7</sup>. Diabetics, intravenous (i.v.) drug users, patients on dialysis, and patients with

AIDS have higher rates of *S. aureus* colonization<sup>8-12</sup>. Hospitalized patients and health care workers are also at higher risk of becoming colonized for extended period of time<sup>5</sup>.

## **Skin Infections**

Approximately one-half of all skin infections are caused by *S. aureus*<sup>13</sup>. Infections include carbuncles, cellulitis, folliculitis, hydradenitis suppurativa, impetigo, mastitis, pyodermas, and pyomyositis. Impetigo, which involves release of epidermolytic toxins, can range from mild, recurrent infections to the more serious bullous impetigo, characterized by blisters that continually break and become infected, to the potentially life-threatening scaled skin syndrome<sup>14</sup>.

## **Bacteremia and Endocarditis**

Virtually any *S. aureus* infection can lead to bacteremia. *S. aureus* causes about 11 to 38% of community- acquired bacteremia<sup>15,16</sup>. Mortality from *S. aureus* bacteremia ranges from 11 to 48%, a figure that has increased steadily from a number of years<sup>17</sup>.

Approximately 10 to 40% of community-acquired cases of *S. aureus* bacteremia progress to endocarditis<sup>17, 18</sup>. This figure is higher in i.v. drug users often because they are heavily colonized with *S. aureus* and have frequent breaches

of skin barriers, and is lower in patients with nosocomial bacteremia. *S. aureus* differs from many other pathogens in that it can cause infectious endocarditis on a normal, native heart valve.

### **Toxin-mediated Diseases**

Several staphylococcal diseases are mediated by toxins, including impetigo, food poisoning, necrotizing pneumonia, and toxic shock syndrome. Staphylococcal food poisoning is a result of ingesting one of several staphylococcal enterotoxins, the most ubiquitous of which is enterotoxin A<sup>19-21</sup>. The disease mediated by toxic shock syndrome 1 (TSST-1) was first described in 1978 and consists of fever above 39°C, hypotension, rash, usually followed by desquamation of the skin, and involvement of multiple organ system.

Panton-Valentine leukocidin (PVL) is a biocomponent synergomenotropic staphylococcal cytotoxin that causes leukocyte destruction and tissue necrosis<sup>22</sup>. Although its precise role in disease is unclear, it has been associated with necrotic lesions involving the skin and severe necrotizing pneumonia<sup>22</sup>.

## **1.4 Health care- associated infections**

Staphylococci are among the most common causes of health care-associated infections, including bacteremia, surgical site infections (SSIs), and pneumonia<sup>23</sup>. Data from the National Nosocomial Infection Surveillance (NNIS) system from intensive care units (ICUs) for 2000 to 2004 show *S. aureus* to be the most common cause of nosocomial pneumonia and surgical site infections, and the third most common cause of nosocomial bloodstream infection.

## **1.5 Antimicrobial resistance**

### **Antimicrobial resistance in the hospital setting**

Several reports suggest that the prevalence of *S. aureus* strains resistant to methicillin, oxacillin, or nafcillin is increasing in the United States and abroad<sup>24-</sup><sup>28</sup>. The ability of this pathogen could be due to its intrinsic virulence, and its capacity to adapt to environmental conditions<sup>14</sup>. *S. aureus* isolates from intensive care units across the country and from blood culture isolates worldwide are increasingly resistant to a greater number of antimicrobials agents<sup>29</sup>. As rapidly as new antibiotics are introduced, *S. aureus* has developed efficient mechanism to neutralize them (Table 1, Lowy F.D., 2003).

**Table 1**  
Mechanisms of *S. aureus* resistance to antimicrobials<sup>A</sup>

Antibiotic	Resistance gene(s)	Gene product(s)	Mechanism(s) of resistance	Location(s)
β-Lactams	1) <i>blaZ</i>	1) β-Lactamase	1) Enzymatic hydrolysis of β-lactam nucleus	1) Pl:Tn
	2) <i>mecA</i>	2) PBP2a	2) Reduced affinity for PBP	2) C:SCC <i>mec</i>
Glycopeptides	1) Unknown (VISA)	1) Altered peptidoglycan	1) Trapping of vancomycin in the cell wall	1) C
	2)	2) D-Ala-D-Lac	2) Synthesis of dipeptide with reduced affinity for vancomycin	2) Pl:Tn
Quinolones	1) <i>parC</i>	1) ParC (or GrlA) component of topoisomerase IV	1,2) Mutations in the QRDR region, reducing affinity of enzyme-DNA complex for quinolones	1) C
	2) <i>gyrA</i> or <i>gyrB</i>	2) GyrA or GyrB components of gyrase		2) C
Aminoglycosides (e.g., gentamicin)	Aminoglycoside-modifying enzymes (e.g., <i>aac</i> , <i>aph</i> )	Acetyltransferase, phosphotransferase	Acetylating and/or phosphorylating enzymes modify aminoglycosides	Pl, Pl:Tn
Trimethoprim-sulfamethoxazole (TMP-SMZ)	1) Sulfonamide: <i>sulA</i>	1) Dihydropteroate synthase	1) Overproduction of <i>p</i> -aminobenzoic acid by enzyme	1) C
	2) TMP: <i>dfrB</i>	2) Dihydrofolate reductase (DHFR)	2) Reduced affinity for DHFR	2) C
Oxazolidinones	<i>rrn</i>	23S rRNA	Mutations in domain V of 23S rRNA component of the 50S ribosome. Interferes with ribosomal binding	C
Quinupristin-dalfopristin (Q-D)	1) Q: <i>ermA</i> , <i>ermB</i> , <i>ermC</i>	1) Ribosomal methylases	1) Reduce binding to the 23S ribosomal subunit	1) Pl, C
	2) D: <i>vat</i> , <i>vatB</i>	2) Acetyltransferases	2) Enzymatic modification of dalfopristin	2) Pl

<sup>A</sup>Examples of several of the *S. aureus* mechanisms of resistance to selected antibiotics (77, 95-97). Pl, plasmid; C, chromosome; Tn, transposon; QRDR, quinolone resistance-determining region.

## Penicillin resistance

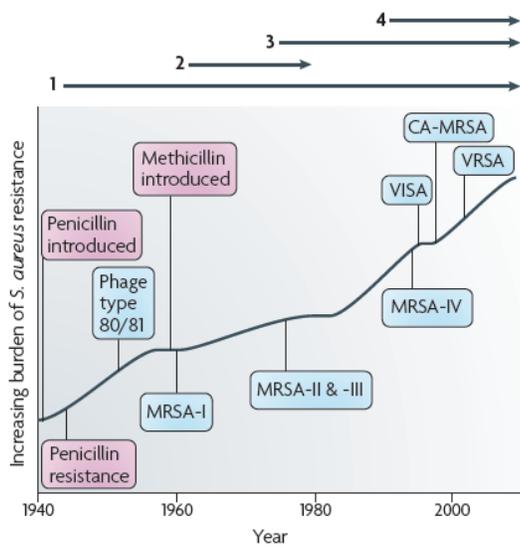
The mortality of patients with *S. aureus* bacteremia in the pre-antibiotic era exceeded 80% and over 70% developed metastatic infections. The introduction of penicillin in the early 1940s dramatically improved the prognosis of patients with staphylococcal infections. However, as early as 1942, penicillin-resistant staphylococci were recognized, first in hospitals and subsequently in the community. By the late 1960s, more than 80% of both community and hospital-acquired staphylococcal isolates were resistant to penicillin. The mechanism of resistance of *S. aureus* is mediated by *blaZ*, the gene that encodes β-lactamase. This predominantly extracellular enzyme, synthesized when staphylococci are

exposed to  $\beta$ -lactam antibiotics, hydrolyzes the  $\beta$ -lactam ring, rendering the  $\beta$ -lactam inactive<sup>30</sup>.

## **Methicillin resistance**

Methicillin, introduced in 1961, was the first of the semisynthetic penicillinase-resistant penicillins. Its introduction was rapidly followed by reports of methicillin-resistant isolates. The first reports of a *S. aureus* strain that was resistant to methicillin were published in 1961<sup>31</sup>. Although the specific gene responsible for methicillin resistance (*mecA*, which encodes the low-affinity penicillin-binding protein PBP2a (also known as PBP2')) was not identified until over 20 years later, it was appreciated early on that the resistance mechanism involved was different from penicillinase-mediated resistance because drug inactivation did not occur. Unlike penicillinase-mediated resistance, which is narrow in its spectrum of activity, methicillin resistance is broad, conferring resistance to the entire  $\beta$ -lactam class of antibiotics, which include penicillins, cephalosporins and carbapenems. Outbreaks of infections caused by MRSA strains were reported in hospitals in the United States in the late 1970s, and by the mid-1980s these strains were endemic<sup>32, 33</sup>, leading to the worldwide pandemic of MRSA in hospitals that continues to the present time. Although global in its distribution and impact, MRSA was still confined mainly to hospitals and other institutional health care settings, such as long-term care

facilities. The ever-increasing burden of MRSA infections in hospitals led to the increased use of vancomycin, the last remaining antibiotic to which MRSA strains were reliably susceptible. This intensive selective pressure resulted in the emergence of vancomycin-intermediate *S. aureus* (VISA) strains, which are not inhibited in vitro at vancomycin concentrations below 4–8 g ml<sup>-1</sup> <sup>34, 35</sup>, and vancomycin-resistant *S. aureus* (VRSA) strains, which are inhibited only at concentrations of 16 g ml<sup>-1</sup> or more <sup>36, 37</sup> (Figure 2).



**Figure 2: The four waves of antibiotic resistance in *Staphylococcus aureus*.** Wave 1 (indicated above the graph) started after the introduction of penicillin into clinical practice. After few years, the first penicillin resistant strain (Phage type 80/81) appeared. Wave 2 began almost immediately following the introduction of methicillin into clinical practice with the isolation of the first MRSA strain (an archaic clone), which contained staphylococcal chromosome cassette mec I (SCCmecI) (indicated on the graph as MRSA-I); this wave extended into the 1970s in the form of the Iberian clone. Wave 3 began in the mid to late 1970s with the emergence of new MRSA strains that contained the new SCCmec allotypes, SCCmecII and SCCmecIII (MRSA-II and MRSA-III), marking the ongoing worldwide pandemic of MRSA in hospitals and health care facilities. The increase in vancomycin use for the treatment of MRSA infections eventually led to the emergence of vancomycin-intermediate *S. aureus* (VISA) strains. Wave 4, which began in the mid to late 1990s, marks the emergence of MRSA strains in the community. Community-associated MRSA (CA-MRSA) strains were susceptible to most antibiotics other than  $\beta$ -lactams, were unrelated to hospital strains and contained a new, smaller, more mobile SCCmec allotype, SCCmecIV (MRSA-IV) and various virulence factors, including PVL. Vancomycin-resistant *S. aureus* (VRSA) strains, ten or so of which have been isolated exclusively in health care settings, were first identified in 2002. (Chambers et al., 2009)

## **1.6 Neutrophils in innate host defense against *Staphylococcus aureus* infections**

### **Neutrophils development**

Neutrophils are short-lived granulocytes derived from pluripotent hematopoietic stem cells in the bone marrow [72]. During the early step of differentiation, cells develop phagocytic capacity followed by development of oxygen-dependent microbial activity, increase adhesiveness, cell motility, chemotactic response and other cell type-specific traits, proceeding through a well-characterized progression into mature neutrophils [73, 74]. For many pathogens, like *Francisella*, the ability to survive and proliferate within mammalian cells is essential for their virulence [75]. In order to replicate within macrophages *Salmonella Enterica* must withstand or surmount bacteriostatic and bactericidal responses by the host cell, including the delivery of hydrolytic enzymes from lysosomes to the phagosome [76]. During maturation the number of mitochondria and ribosomes decrease, while glycogen granules, the main source of energy, fill the cytoplasm of mature neutrophils. There are two major populations of granules present in mature neutrophils. Primary or azurophilic granules, which are first to develop during granulopoiesis, contain myeloperoxidase (MPO) and a variety of proteolytic enzymes (cathepsins, proteinase-3, and elastase), antimicrobial defensins, and

bactericidal/permeability-increasing protein [73,[77-79]]. These microbicidal granules are considered unique from lysosomes, in that they lack traditional lysosomal membrane markers and traffic as regulated secretory granules [122–125]. The other major type of granules present are secondary or specific granules, which mature late during differentiation, and contain a number of functionally important membrane proteins including flavocytochrome b558, lactoferrin, collagenase, as well as receptors for chemotactic peptides, cytokines, opsonins, adhesion proteins, and extracellular matrix proteins [73]. Upon maturation, neutrophils are released into the bloodstream where they circulate for ~10–24 h before migrating into tissue where they may function for an additional 1–2 days before undergoing apoptosis and being cleared by macrophages [80]. Normal neutrophil turnover in an average adult is on the order of 10-11 cells per day [75]. In addition to maintaining steady-state levels of circulating neutrophils, the hematopoietic system has the remarkable ability to drive “emergency” granulopoiesis in response to the increased demand of infection, expanding the pool of neutrophils in circulation when necessary [81, 82].

### **Neutrophils recruitment**

A dynamic portion of circulating neutrophils rolls along the walls of post capillary venules for signs of tissue damage, inflammation or invading

microorganism. In response to damage or the presence of invading pathogens, a variety of host cells produce and secrete potent inflammatory mediators and neutrophil chemo-attractants like interleukin-8 (IL-8,CXCL8), GRO $\alpha$  (CXCL1), granulocyte chemotactic protein 2 (GCP2, CXCL6), and leukotriene B4 (LTB4) which bind and engage specific surface receptors on surveying neutrophils. Contemporary *S. aureus* surface components such as lipoteichoic acid (LTA) or capsular polysaccharide as well as secreted molecule such as toxic shock syndrome toxin (TSST)-1, staphylococcal enterotoxin A, and staphylococcal enterotoxin B have been shown to elicit IL-8 production by monocytes, epithelial cells, and endothelial cells [83-85]. Bacterial-derived products such as N-formyl peptides or the phenol-soluble modulins (PSMs) produced by *S. aureus* have the demonstrated ability to recruit neutrophils directly [86]. Corroborating evidence to date demonstrates that the more responsive state of the neutrophil is attributable to (a) partial assembly of the NADPH oxidase, (b) reorganization of the plasma membrane and redistribution of signaling molecules into lipid rafts, (c) modulation of intracellular signaling intermediates, (d) mobilization of secretory vesicles and enrichment of specific surface receptors (CD11b/CD18), (e) cytokine secretion, and (f) transcriptional regulation of several gene families. Although priming leads to observable phenotypic differences, it remains distinct from neutrophil activation in that it triggers neither release of azurophilic granules nor production of superoxide.

## **Pathogen recognition and phagocytosis**

Once at the site of infection, the real work for neutrophils begins, as they bind and ingest invading microorganisms by a process known as phagocytosis, a critical first step in removal of bacteria during infection. Neutrophils recognize numerous surface-bound and freely secreted bacterial products such as PGN, lipoproteins, LTA, lipopolysaccharide, CpG-containing DNA, and flagellin. Such conserved bacterial products are generally known as pathogen associated molecular patterns (PAMPs) and are recognized directly by pattern recognition receptors (PRRs) expressed on the surface of the neutrophil. Engagement of such receptors activates signal transduction pathways that prolong cell survival, facilitate adhesion and phagocytosis, induce release of cytokines and chemokines, elicit degranulation, and promote reactive oxygen species (ROS) production and release, ultimately contributing to microbicidal activity [87]. These molecules enhance recognition by surface receptors on neutrophils (opsonization via the lectin pathway of complement activation), activate other PRRs, facilitate efficient phagocytosis, and elicit release of cytokines and production of ROS [88-90]. Peptidoglycan recognition protein (PGRP) is a secreted host protein that binds PGN and Gram-positive bacteria [91, 92]. An isoform known as PGRP-short is produced by neutrophils and contributes directly to bactericidal activity rather than directing downstream signaling responses [93]. Unlike the unique phagocytic uptake induced by dectin-1,

phagocytosis of most microorganisms is promoted or at least markedly enhanced by opsonization with serum host factors, such as specific antibody, complement, and/or MBL (generally fungi) [94]. For this purpose, neutrophils express multiple antibody-Fc and serum complement receptors, including CD16 (FcγIIIb, low affinity IgG receptor)[95], CD23 (FcεRI, IgE receptor) [96], CD32 (FcγRIIa, low affinity IgG receptor) [97], CD64 (FcγRI, IgG receptor), CD89 (FcαR, IgA receptor) [98], ClqR [99], CD35 (CR1) [100, 101], CD11b/CD18 (CR3) [102, 103], and CD11c/CD18 (CR4) [104]. Activation of complement facilitates the deposition of complement components C3b, iC3b, and Clq on the surface of invading microorganisms, and serum complement is fixed readily on the surface of antibody-coated microbes. Taken together, the combined action of PRRs, and complement and antibody receptors maximizes recognition and phagocytosis of invading microorganisms.

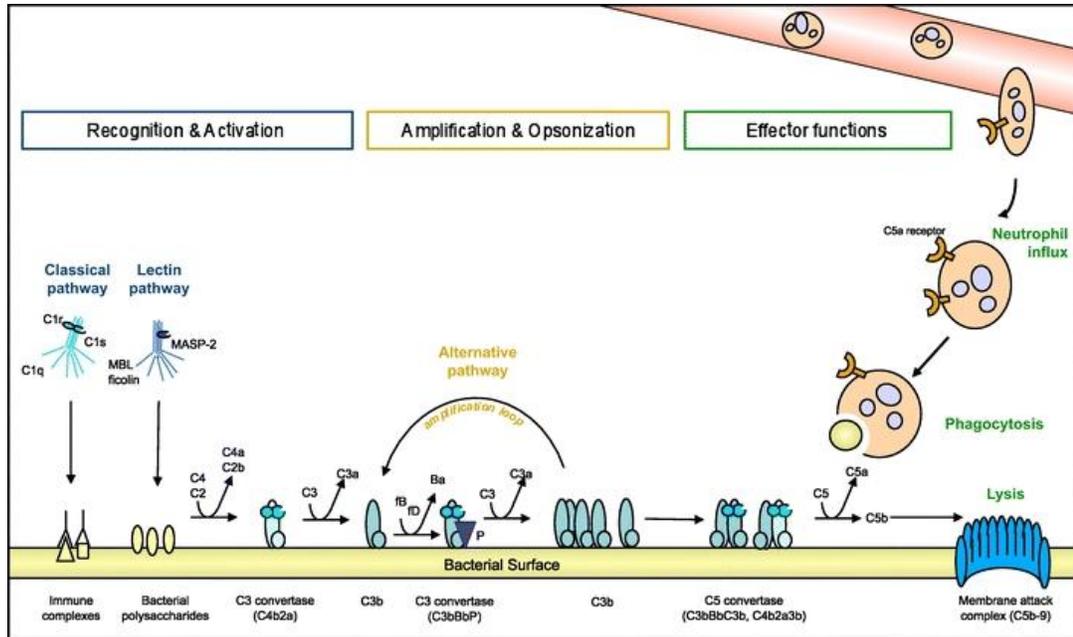
## **1.7 *Staphylococcus aureus* and the complement system**

### **1.7.1 The complement system**

The plasma proteins of the complement system are essential in the innate immune response against bacteria. Complement labels bacteria with opsonins to support phagocytosis and generates chemo-attractants to attract phagocytes to the site of infection. In turn, bacterial human pathogens have

evolved different strategies to specifically impair the complement response. The innate immune system plays a critical role in our host defense against invading pathogens. The complement system consists of three different parts: (a) the complement system, (b) phagocytes, and (c) antimicrobial peptides. The three major functions of the complement cascade in innate immunity are <sup>38</sup> to label pathogens or immunogenic particles with C3b and iC3b to facilitate phagocytic uptake via complement receptors, <sup>39</sup> to attract phagocytes by producing chemoattractant C5a, and <sup>40</sup> to directly lyse gram-negative bacteria through membrane attack complex (MAC; C5b-9) formation <sup>38</sup>. Complement is initiated by two specific recognition pathways, the classical and lectin pathway, which are amplified by the alternative pathway. All three pathways converge at the formation of the C3 convertases. These bimolecular surface-bound enzyme complexes catalyze the key reaction in complement activation: cleavage of complement protein C3 into C3a, a chemoattractant with bactericidal activity, and C3b <sup>41</sup>. Convertase formation is pivotal in complement activation since C3b and its inactive derivative iC3b facilitate phagocytosis. Furthermore, the deposited C3b can form new convertases, thereby amplifying the opsonization process. Subsequently, the high concentrations of locally deposited C3b induce a shift in substrate specificity of the convertase to complement protein C5. The cleavage products of C5 are C5a, a potent chemoattractant, and C5b that

initiates the lytic pathway. Due to the resulting C5a gradient, neutrophils migrate toward the site of infection and phagocytose the invaders.



**Figure 3:** Schematic overview of the complement system. The complement cascade is activated by recognition of microbe-bound antibodies or microbial sugars by the C1 complex (CP) or the MBL- and ficolin-MASP-2 complex (LP), respectively. The Alternative Pathway (AP) C3 convertase C3bBb is generated after binding of fB to surface-bound C3b and subsequent cleavage by fD. (Laarman et al., 2010)

## 1.7.2 Immune evasion by *Staphylococcus aureus*

### Virulence factors of *S.aureus*:

*S. aureus* secretes immunomodulatory protein that compromise both induced humoral and cell-mediated immunity. This pathogen expresses a wide array of secreted and cell-surface-associated virulence factors, including surface proteins that promote adhesion to damage tissue and to the surface of host cells. Moreover *S. aureus* expresses an array of extracellular enzymes such as proteases, a hyaluronidase, a lipase and a nuclease that facilitate tissue destruction and spreading, membrane-damaging toxins that cause cytolytic effects on host cells and tissue damage, and superantigens that contribute to the symptoms of septic shock<sup>42</sup>.

One major class of surface-located proteins comprises those that are covalently anchored to cell-wall peptidoglycan by sortase, a membrane-associated enzymes that recognizes and cleaves the C-terminal LPXTG motif in the sorting signal<sup>43, 44</sup>.

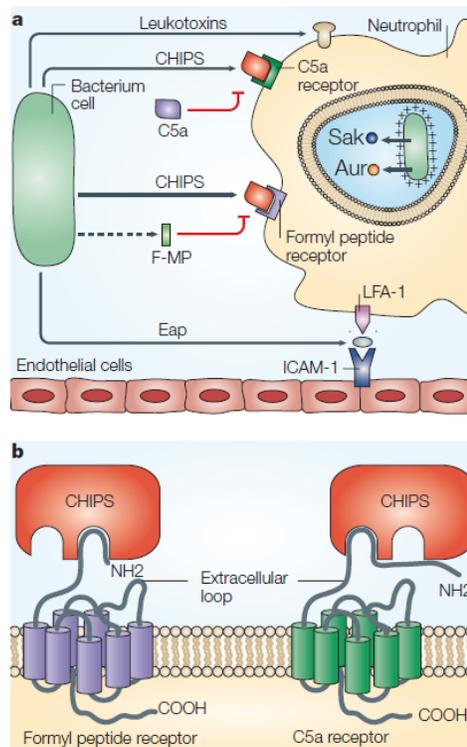
## **Inhibition of neutrophil chemo taxis**

Immediately as the bacterium gains a foothold in the host and starts to grow, several chemoattractants are liberated which are specifically recognized by neutrophils at low concentration, resulting in a strong chemotactic response. The peptide fragments C3a and C5a, released by complement activation, as well as formylated peptides secreted from growing bacteria cells, are recognized at high affinity by specific transmembrane G-protein-coupled receptors on the neutrophil surface <sup>45</sup>. These are stimulated and activate intracellular signaling cascades, resulting in migration of neutrophils from the blood to the site of inflammation.

About 60% of *S. aureus* strains secrete the chemotaxis inhibitory protein of staphylococci CHIPS that can bind avidly to both the formyl peptide receptor (FPR) and C5a receptor (C5aR) to block the cognate agonist from binding <sup>46</sup>. Recent studies identified a FPR- binding domain in the N terminus of CHIPS, and showed the Phe residues at position 1 and 3 are critical for activity.

Once of many ligands recognized by the extracellular adherence protein Eap is intracellular adhesion molecule-1 (ICAM-1) on the surface of endothelial cells <sup>47</sup>. Binding of Eap to ICAM-1 blocks binding of the lymphocyte-function-associated antigen LFA-1 on the surface of neutrophils and prevents leucocyte adhesion,

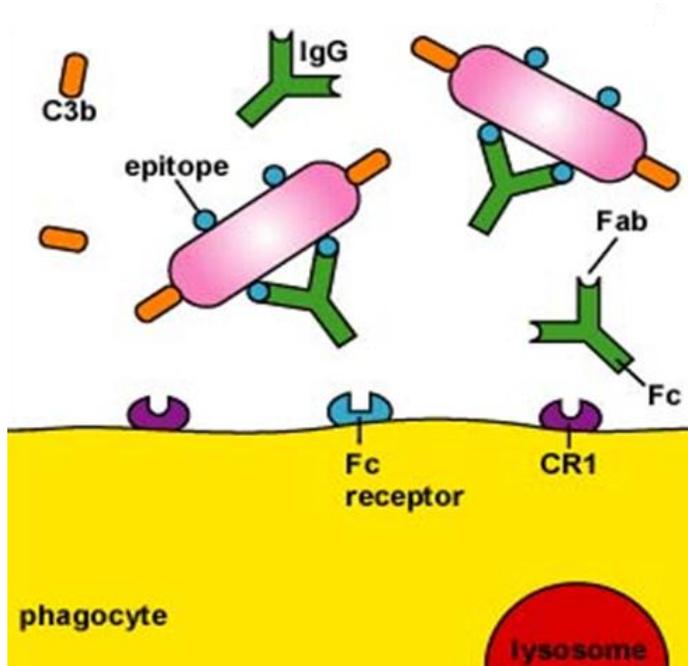
diapedesis and extravasation. The Eap protein will likely act in concert to CHIPS to inhibit neutrophil recruitment to the site of infection <sup>48</sup>.



**Figure 4: Inhibition of the neutrophils response to infection. a:** the chemotaxis inhibitory protein of staphylococci (CHIPS) and the extracellular adherence protein (Eap) interfere with neutrophil chemotaxis and extravasation. **b:** model for interaction between CHIPS and formyl peptide receptor (FPR) and C5a receptor. Two distinct but closely linked binding domains in CHIPS are indicated, one for extreme N terminus of FPR and involving residues F1 and F3, the second for a domain located between residues 10-20 of the c5a form. (Foster T.J.,2005)

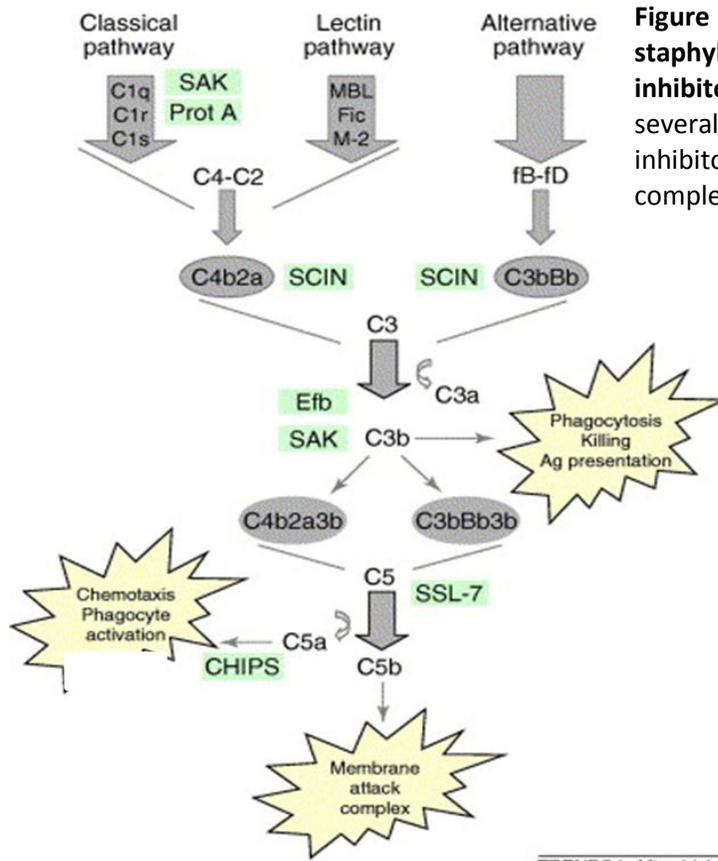
### Resistance to phagocytes:

The primary defense against *S. aureus* infection is the innate immunity mediated by neutrophils. During the phagocytic process, *S. aureus* bacteria are coated with antibodies and complement (serum opsonins) that engage, on phagocytic cells, receptor binding the Fc region (tail) of antibodies (Fc receptor) and complement receptors (RC1) triggering bacterial uptake. The presence of functional antibodies leads to an effective opsonization and recovery from infection (Figure 5).



**Figure 5: Schematic overview of opsonophagocytic process:** the first step is the recognition between bacterial antigens and antibodies. Subsequently the addition of complement leads to the binding receptors on phagocytic cell, triggering bacterial uptake.

The ability of *S. aureus* to avoid opsonins present in the normal serum is an important factor in the success of infection. *S. aureus* expressed surface-associated anti-opsonic proteins and a polysaccharide capsule that can both interfere with the deposition of antibodies and complement formation by classical and alternative pathway, or with their access to neutrophils complement receptor and Fc receptor. Therefore, efficient phagocytosis by neutrophils that requires recognition of bound complement and antibodies is compromised. Figure 6 illustrates the highly diverse strategies of staphylococcal complement inhibitors.



**Figure 6: Schematic overview of staphylococcal complement inhibitor.** *S. aureus* produces several proteins involved that act as inhibitors at different steps of complement pathway.

TRENDS in Microbiology

**Protein A** is well known for its capacity to bind the Fc part of IgG. Protein A amino acid sequence revealed a molecule comprised of five nearly identical IgG binding domains as well as the molecular elements involved in binding Ig<sup>49-51</sup>. The consequence of interaction between protein A and IgG is to coat the surface of the cell with IgG molecules that are in the incorrect orientation to be recognized by the neutrophils Fc receptor<sup>52</sup>. Mutations in the protein A gene (*spa*) cause significant defects in the pathogenesis of *S. aureus* infections.

**Staphylokinase (Sak)** is a potent activator of plasminogen (PLG), the precursor of the fibrinolytic protein plasmin. Surface-bound plasmin (PL) has the ability to cleave both IgG and C3b. the conversion of PLG by SAK at physiological concentrations, leads to the removal of important opsonic molecules necessary for recognition by immune cells <sup>53</sup> . PL cleaves human IgG, as well as human C3b and C3bi, from the bacterial cell wall leading to impaired phagocytosis by human neutrophils. PL cleaves IgG at position Lys 222 and thus removes the entire Fc fragment, including the glycosylation site (Asn 297) that is necessary for recognition by C1q, thereby inhibiting the activation of the classical pathway of complement. In addition, PL-activity created by PLG and SAK led to a decrease of the C3b and C3bi molecules at the staphylococcal surface. PLG+rSAK cleaved C3b in both the  $\alpha$ -chain as well as the  $\beta$ -chain. The decrease of C3b molecules will indirectly diminish C3 convertases as well as C5 convertases.

**Staphylococcal complement inhibitor (SCIN)** is the most efficient complement inhibitor <sup>54</sup>. SCIN is a 10 kDa, excreted protein that blocks all complement pathways: the lectin, classical and alternative pathway. SCIN efficiently prevents phagocytosis and killing of staphylococci and C5a production. SCIN acts specifically on surface-bound C3 convertases, stabilizing both C3bBb as

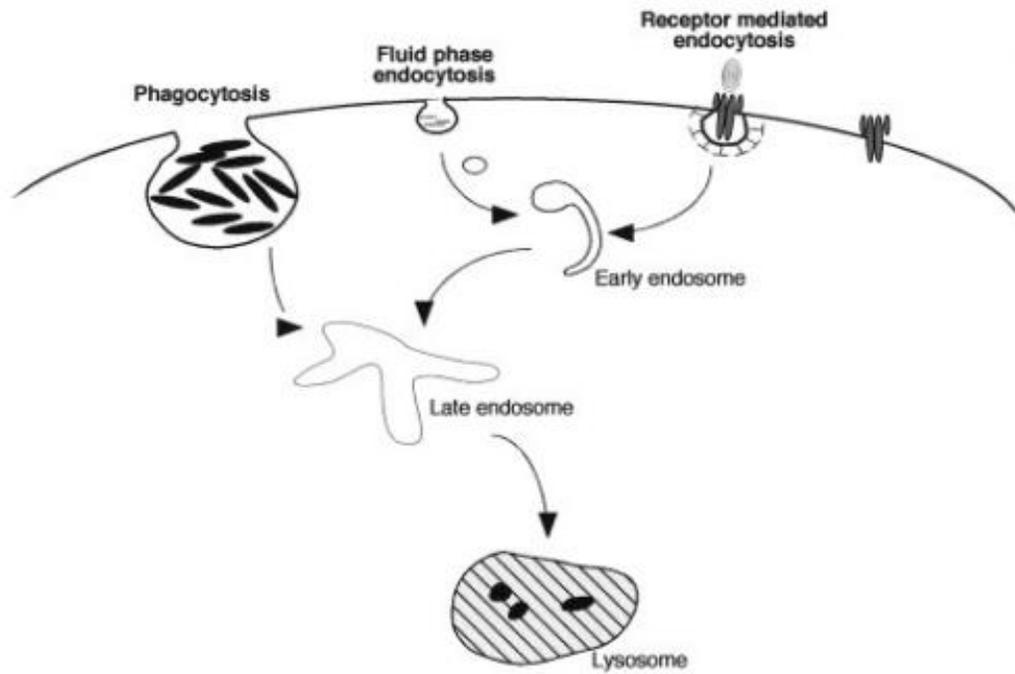
well as C4b2a at the surface of the bacterium and at, the same time, preventing generation of additional convertases.

**The staphylococcal superantigen-like proteins (SSLs)** are close relatives of the superantigens but are located on a separate gene cluster within a 19-kb region of the pathogenicity island SaPI<sub>n</sub>2. Staphylococcal superantigen-like 7 (SSL-7, 23.2 kDa) binds human IgA1 and IgA2 resulting in an impaired IgA binding to cell surface Fc $\alpha$ RI (CD89).

**Extracellular fibrinogen binding molecule (Efb)** is a 15.6 kDa excreted molecule that was described earlier to bind fibrinogen. Efb blocks classical pathway dependent opsonization and subsequent phagocytosis. However, for inhibition of the classical and alternative pathway and phagocytosis, high (microgram) concentrations of Efb were required. Although the present data on Efb do not demonstrate Efb binding to bacterium-bound C3d, a role for Efb in the modulation of C3d-mediated recognition by CR2 on B-cells cannot be excluded. The C3b-binding site of Efb is distinct from its fibrinogen binding site, in fact, Efb can bind both molecules simultaneously<sup>55, 56</sup>.

## 1.8 *Staphylococcus aureus*- Eukaryotic Interactions

Professional phagocytes like neutrophils and macrophages are designed to actively engulf microbes and kill them. Only a few type of microbial pathogen can survive phagocytosis by neutrophils and macrophages and they do so by a several distinct mechanism to avoid destruction in phagolysosomes<sup>57, 58</sup>. Surprisingly, *S. aureus* also appears to be resistant to bactericidal attack inside the phagocytic vacuoles of neutrophils which can contain viable intracellular bacteria.<sup>59</sup> During the first step on invasion process *S. aureus* bacterial cells adhere to the cell membrane by means of several surface proteins, including a group of surface adhesins called microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). Among these MSCRAMMs, the fibronectin binding proteins (FnBPs) are necessary and sufficient for internalization of bacteria<sup>60, 61</sup>. Internalization is an active process with respect to the host cell and a passive process with respect to the bacteria<sup>62</sup>. Internalized *S. aureus* bacteria reside in the endosomal vacuoles or are divided from the endosomal pathway to autophagosomes depending on the cell type invaded and/or the *S. aureus* strains<sup>63</sup>. Subsequently, *S. aureus* escapes into the cytoplasm where it eventually kills the host cell through the induction of apoptosis<sup>64, 65</sup>.



**Figure 7: schematic overview of internalization process.** The internalization can be mediated both by phagocytosis and receptor mediated endocytosis. Basically the entire process can be divided in three steps: early endosome, late endosome and lysosome formation respectively.

## **1.9 Intracellular survival of *Staphylococcus aureus***

*S. aureus* was considered for many years to be an extracellular pathogen: the fact that *S. aureus* infections frequently recur, coupled with the observation that this pathogen can survive within eukaryotic cells and led to speculation that *S. aureus* may be an intracellular pathogen [66]. Several studies show that *S. aureus* is internalized and survives in a variety of mammalian cells [64, 67-69]. In order to survive within the host cell, *S. aureus* must first escape from the encapsulating endosomal membrane and then multiply or at least survive in the host cell cytoplasm [70, 71]. Several observations suggest the hypothesis that the *agr* locus controls the expression of some of the genes necessary for escape from the endosome and further growth in the cytoplasm. After escaping the endosome some strains are able to survive within eukaryotic cytoplasm for long periods of time and therefore are potentially capable of causing persistence or recurrent infections.

## Bibliography

- [1] Deysine M, Isenberg HD, Steiner G. Chronic haematogenous osteomyelitis; studies on an experimental model. *Int Orthop* 1983;7:69-78.
- [2] Molne L, Tarkowski A. An experimental model of cutaneous infection induced by superantigen-producing *Staphylococcus aureus*. *J Invest Dermatol* 2000;114:1120-5.
- [3] Abdelnour A, Bremell T, Holmdahl R, Tarkowski A. Clonal expansion of T lymphocytes causes arthritis and mortality in mice infected with toxic shock syndrome toxin-1-producing staphylococci. *Eur J Immunol* 1994;24:1161-6.
- [4] Lee JC. The prospects for developing a vaccine against *Staphylococcus aureus*. *Trends Microbiol* 1996;4:162-6.
- [5] FEKETY FR, Jr. THE EPIDEMIOLOGY AND PREVENTION OF STAPHYLOCOCCAL INFECTION. *Medicine (Baltimore)* 1964;43:503-13.
- [6] Kluytmans J, van BA, Verbrugh H. Nasal carriage of *Staphylococcus aureus*: epidemiology, underlying mechanisms, and associated risks. *Clin Microbiol Rev* 1997;10:505-20.
- [7] Martin RR, Buttram V, Besch P, Kirkland JJ, Petty GP. Nasal and vaginal *Staphylococcus aureus* in young women: quantitative studies. *Ann Intern Med* 1982;96:951-3.
- [8] Frank U, Daschner FD, Schulgen G, Mills J. Incidence and epidemiology of nosocomial infections in patients infected with human immunodeficiency virus. *Clin Infect Dis* 1997;25:318-20.
- [9] Noble WC, Valkenburg HA, Wolters CH. Carriage of *Staphylococcus aureus* in random samples of a normal population. *J Hyg (Lond)* 1967;65:567-73.
- [10] Tuazon CU, Sheagren JN. Increased rate of carriage of *Staphylococcus aureus* among narcotic addicts. *J Infect Dis* 1974;129:725-7.
- [11] Yu VL, Goetz A, Wagener M, Smith PB, Rihs JD, Hanchett J et al. *Staphylococcus aureus* nasal carriage and infection in patients on hemodialysis. Efficacy of antibiotic prophylaxis. *N Engl J Med* 1986;315:91-6.
- [12] Zimakoff J, Bangsgaard PF, Bergen L, Baago-Nielsen J, Daldorph B, Espersen F et al. *Staphylococcus aureus* carriage and infections among patients

in four haemo- and peritoneal-dialysis centres in Denmark. The Danish Study Group of Peritonitis in Dialysis (DASPID). *J Hosp Infect* 1996;33:289-300.

**[13]** ELEK SD, CONEN PE. The virulence of *Staphylococcus pyogenes* for man; a study of the problems of wound infection. *Br J Exp Pathol* 1957;38:573-86.

**[14]** Lowy FD. *Staphylococcus aureus* infections. *N Engl J Med* 1998;339:520-32.

**[15]** Cockerill FR, III, Hughes JG, Vetter EA, Mueller RA, Weaver AL, Ilstrup DM et al. Analysis of 281,797 consecutive blood cultures performed over an eight-year period: trends in microorganisms isolated and the value of anaerobic culture of blood. *Clin Infect Dis* 1997;24:403-18.

**[16]** Weinstein MP, Towns ML, Quartey SM, Mirrett S, Reimer LG, Parmigiani G et al. The clinical significance of positive blood cultures in the 1990s: a prospective comprehensive evaluation of the microbiology, epidemiology, and outcome of bacteremia and fungemia in adults. *Clin Infect Dis* 1997;24:584-602.

**[17]** Mortara LA, Bayer AS. *Staphylococcus aureus* bacteremia and endocarditis. New diagnostic and therapeutic concepts. *Infect Dis Clin North Am* 1993;7:53-68.

**[18]** Dajani AS, Taubert KA, Wilson W, Bolger AF, Bayer A, Ferrieri P et al. Prevention of bacterial endocarditis: recommendations by the American Heart Association. *Clin Infect Dis* 1997;25:1448-58.

**[19]** Holmberg SD, Blake PA. Staphylococcal food poisoning in the United States. New facts and old misconceptions. *JAMA* 1984;251:487-9.

**[20]** Le LY, Baron F, Gautier M. *Staphylococcus aureus* and food poisoning. *Genet Mol Res* 2003;2:63-76.

**[21]** Wieneke AA, Roberts D, Gilbert RJ. Staphylococcal food poisoning in the United Kingdom, 1969-90. *Epidemiol Infect* 1993;110:519-31.

**[22]** Lina G, Piemont Y, Godail-Gamot F, Bes M, Peter MO, Gauduchon V et al. Involvement of Panton-Valentine leukocidin-producing *Staphylococcus aureus* in primary skin infections and pneumonia. *Clin Infect Dis* 1999;29:1128-32.

**[23]** Emori TG, Gaynes RP. An overview of nosocomial infections, including the role of the microbiology laboratory. *Clin Microbiol Rev* 1993;6:428-42.

- [24] Aubry-Damon H, Legrand P, Brun-Buisson C, Astier A, Soussy CJ, Leclercq R. Reemergence of gentamicin-susceptible strains of methicillin-resistant *Staphylococcus aureus*: roles of an infection control program and changes in aminoglycoside use. *Clin Infect Dis* 1997;25:647-53.
- [25] Barrett SP, Mummery RV, Chattopadhyay B. Trying to control MRSA causes more problems than it solves. *J Hosp Infect* 1998;39:85-93.
- [26] Irish D, Eltringham I, Teall A, Pickett H, Farelly H, Reith S et al. Control of an outbreak of an epidemic methicillin-resistant *Staphylococcus aureus* also resistant to mupirocin. *J Hosp Infect* 1998;39:19-26.
- [27] Panlilio AL, Culver DH, Gaynes RP, Banerjee S, Henderson TS, Tolson JS et al. Methicillin-resistant *Staphylococcus aureus* in U.S. hospitals, 1975-1991. *Infect Control Hosp Epidemiol* 1992;13:582-6.
- [28] Tiemersma EW, Bronzwaer SL, Lyytikainen O, Degener JE, Schrijnemakers P, Bruinsma N et al. Methicillin-resistant *Staphylococcus aureus* in Europe, 1999-2002. *Emerg Infect Dis* 2004;10:1627-34.
- [29] Diekema DJ, Pfaller MA, Schmitz FJ, Smayevsky J, Bell J, Jones RN et al. Survey of infections due to *Staphylococcus* species: frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, Latin America, Europe, and the Western Pacific region for the SENTRY Antimicrobial Surveillance Program, 1997-1999. *Clin Infect Dis* 2001;32 Suppl 2:S114-S132.
- [30] Lowy FD. Antimicrobial resistance: the example of *Staphylococcus aureus*. *J Clin Invest* 2003;111:1265-73.
- [31] BARBER M. Methicillin-resistant staphylococci. *J Clin Pathol* 1961;14:385-93.
- [32] Crossley K, Landesman B, Zaske D. An outbreak of infections caused by strains of *Staphylococcus aureus* resistant to methicillin and aminoglycosides. II. Epidemiologic studies. *J Infect Dis* 1979;139:280-7.
- [33] Peacock JE, Jr., Marsik FJ, Wenzel RP. Methicillin-resistant *Staphylococcus aureus*: introduction and spread within a hospital. *Ann Intern Med* 1980;93:526-32.
- [34] Hiramatsu K, Aritaka N, Hanaki H, Kawasaki S, Hosoda Y, Hori S et al. Dissemination in Japanese hospitals of strains of *Staphylococcus aureus* heterogeneously resistant to vancomycin. *Lancet* 1997;350:1670-3.

- [35] Weigel LM, Clewell DB, Gill SR, Clark NC, McDougal LK, Flannagan SE et al. Genetic analysis of a high-level vancomycin-resistant isolate of *Staphylococcus aureus*. *Science* 2003;302:1569-71.
- [36] O'Brien FG, Lim TT, Chong FN, Coombs GW, Enright MC, Robinson DA et al. Diversity among community isolates of methicillin-resistant *Staphylococcus aureus* in Australia. *J Clin Microbiol* 2004;42:3185-90.
- [37] Chambers HF, DeLeo FR. Waves of resistance: *Staphylococcus aureus* in the antibiotic era. *Nat Rev Microbiol* 2009;7:629-41.
- [38] Walport MJ. Complement. First of two parts. *N Engl J Med* 2001;344:1058-66.
- [39] Hartmann K, Henz BM, Kruger-Krasagakes S, Kohl J, Burger R, Guhl S et al. C3a and C5a stimulate chemotaxis of human mast cells. *Blood* 1997;89:2863-70.
- [40] Daffern PJ, Pfeifer PH, Ember JA, Hugli TE. C3a is a chemotaxin for human eosinophils but not for neutrophils. I. C3a stimulation of neutrophils is secondary to eosinophil activation. *J Exp Med* 1995;181:2119-27.
- [41] Gros P, Milder FJ, Janssen BJ. Complement driven by conformational changes. *Nat Rev Immunol* 2008;8:48-58.
- [42] Dinges MM, Orwin PM, Schlievert PM. Exotoxins of *Staphylococcus aureus*. *Clin Microbiol Rev* 2000;13:16-34, table.
- [43] Foster TJ, Hook M. Surface protein adhesins of *Staphylococcus aureus*. *Trends Microbiol* 1998;6:484-8.
- [44] Mazmanian SK, Ton-That H, Schneewind O. Sortase-catalysed anchoring of surface proteins to the cell wall of *Staphylococcus aureus*. *Mol Microbiol* 2001;40:1049-57.
- [45] Murdoch C, Finn A. Chemokine receptors and their role in inflammation and infectious diseases. *Blood* 2000;95:3032-43.
- [46] de Haas CJ, Veldkamp KE, Peschel A, Weerkamp F, van Wamel WJ, Heezius EC et al. Chemotaxis inhibitory protein of *Staphylococcus aureus*, a bacterial antiinflammatory agent. *J Exp Med* 2004;199:687-95.
- [47] Chavakis T, Hussain M, Kanse SM, Peters G, Bretzel RG, Flock JI et al. *Staphylococcus aureus* extracellular adherence protein serves as anti-

inflammatory factor by inhibiting the recruitment of host leukocytes. *Nat Med* 2002;8:687-93.

[48] Foster TJ. Immune evasion by staphylococci. *Nat Rev Microbiol* 2005;3:948-58.

[49] Boden MK, Flock JI. Cloning and characterization of a gene for a 19 kDa fibrinogen-binding protein from *Staphylococcus aureus*. *Mol Microbiol* 1994;12:599-606.

[50] Sjodahl J. Repetitive sequences in protein A from *Staphylococcus aureus*. Arrangement of five regions within the protein, four being highly homologous and Fc-binding. *Eur J Biochem* 1977;73:343-51.

[51] Uhlen M, Guss B, Nilsson B, Gatenbeck S, Philipson L, Lindberg M. Complete sequence of the staphylococcal gene encoding protein A. A gene evolved through multiple duplications. *J Biol Chem* 1984;259:1695-702.

[52] Foster TJ. Immune evasion by staphylococci. *Nat Rev Microbiol* 2005;3:948-58.

[53] Rooijackers SH, van Kessel KP, van Strijp JA. Staphylococcal innate immune evasion. *Trends Microbiol* 2005;13:596-601.

[54] Rooijackers SH, Ruyken M, Roos A, Daha MR, Presanis JS, Sim RB et al. Immune evasion by a staphylococcal complement inhibitor that acts on C3 convertases. *Nat Immunol* 2005;6:920-7.

[55] Lee LY, Hook M, Haviland D, Wetsel RA, Yonter EO, Syribeys P et al. Inhibition of complement activation by a secreted *Staphylococcus aureus* protein. *J Infect Dis* 2004;190:571-9.

[56] Lee LY, Liang X, Hook M, Brown EL. Identification and characterization of the C3 binding domain of the *Staphylococcus aureus* extracellular fibrinogen-binding protein (Efb). *J Biol Chem* 2004;279:50710-6.

[57] Amer AO, Swanson MS. A phagosome of one's own: a microbial guide to life in the macrophage. *Curr Opin Microbiol* 2002;5:56-61.

[58] Staali L, Bauer S, Morgelin M, Bjorck L, Tapper H. *Streptococcus pyogenes* bacteria modulate membrane traffic in human neutrophils and selectively inhibit azurophilic granule fusion with phagosomes. *Cell Microbiol* 2006;8:690-703.

- [59] Voyich JM, Braughton KR, Sturdevant DE, Whitney AR, Said-Salim B, Porcella SF et al. Insights into mechanisms used by *Staphylococcus aureus* to avoid destruction by human neutrophils. *J Immunol* 2005;175:3907-19.
- [60] Ahmed S, Meghji S, Williams RJ, Henderson B, Brock JH, Nair SP. *Staphylococcus aureus* fibronectin binding proteins are essential for internalization by osteoblasts but do not account for differences in intracellular levels of bacteria. *Infect Immun* 2001;69:2872-7.
- [61] Sinha B, Francois P, Que YA, Hussain M, Heilmann C, Moreillon P et al. Heterologously expressed *Staphylococcus aureus* fibronectin-binding proteins are sufficient for invasion of host cells. *Infect Immun* 2000;68:6871-8.
- [62] Ellington JK, Reilly SS, Ramp WK, Smeltzer MS, Kellam JF, Hudson MC. Mechanisms of *Staphylococcus aureus* invasion of cultured osteoblasts. *Microb Pathog* 1999;26:317-23.
- [63] Schnaith A, Kashkar H, Leggio SA, Addicks K, Kronke M, Krut O. *Staphylococcus aureus* subvert autophagy for induction of caspase-independent host cell death. *J Biol Chem* 2007;282:2695-706.
- [64] Bayles KW, Wesson CA, Liou LE, Fox LK, Bohach GA, Trumble WR. Intracellular *Staphylococcus aureus* escapes the endosome and induces apoptosis in epithelial cells. *Infect Immun* 1998;66:336-42.
- [65] Menzies BE, Kourteva I. Internalization of *Staphylococcus aureus* by endothelial cells induces apoptosis. *Infect Immun* 1998;66:5994-8.
- [66] Lowy FD. Is *Staphylococcus aureus* an intracellular pathogen? *Trends Microbiol* 2000;8:341-3.
- [67] Almeida RA, Matthews KR, Cifrian E, Guidry AJ, Oliver SP. *Staphylococcus aureus* invasion of bovine mammary epithelial cells. *J Dairy Sci* 1996;79:1021-6.
- [68] Hess DJ, Henry-Stanley MJ, Erickson EA, Wells CL. Intracellular survival of *Staphylococcus aureus* within cultured enterocytes. *J Surg Res* 2003;114:42-9.
- [69] Molinari G, Chhatwal GS. Streptococcal invasion. *Curr Opin Microbiol* 1999;2:56-61.

- [70] Gresham HD, Lowrance JH, Caver TE, Wilson BS, Cheung AL, Lindberg FP. Survival of *Staphylococcus aureus* inside neutrophils contributes to infection. *J Immunol* 2000;164:3713-22.
- [71] Hudson MC, Ramp WK, Nicholson NC, Williams AS, Nousiainen MT. Internalization of *Staphylococcus aureus* by cultured osteoblasts. *Microb Pathog* 1995;19:409-19.
- [72] Weissman IL, Anderson DJ, Gage F. Stem and progenitor cells: origins, phenotypes, lineage commitments, and transdifferentiations. *Annu Rev Cell Dev Biol* 2001;17:387-403.
- [73] Glasser L, Fiederlein RL. Functional differentiation of normal human neutrophils. *Blood* 1987;69:937-44.
- [74] Rosenbauer F, Tenen DG. Transcription factors in myeloid development: balancing differentiation with transformation. *Nat Rev Immunol* 2007;7:105-17.
- [75] Chong A, Celli J. The francisella intracellular life cycle: toward molecular mechanisms of intracellular survival and proliferation. *Front Microbiol* 2010;1:138.
- [76] Thompson JA, Liu M, Helaine S, Holden DW. Contribution of the PhoP/Q regulon to survival and replication of *Salmonella enterica* serovar Typhimurium in macrophages. *Microbiology* 2011;157:2084-93.
- [77] Cramer E, Pryzwansky KB, Villeval JL, Testa U, Breton-Gorius J. Ultrastructural localization of lactoferrin and myeloperoxidase in human neutrophils by immunogold. *Blood* 1985;65:423-32.
- [78] Csernok E, Ludemann J, Gross WL, Bainton DF. Ultrastructural localization of proteinase 3, the target antigen of anti-cytoplasmic antibodies circulating in Wegener's granulomatosis. *Am J Pathol* 1990;137:1113-20.
- [79] Ohlsson K, Olsson I. The neutral proteases of human granulocytes. Isolation and partial characterization of granulocyte elastases. *Eur J Biochem* 1974;42:519-27.
- [80] Calafat J, Kuijpers TW, Janssen H, Borregaard N, Verhoeven AJ, Roos D. Evidence for small intracellular vesicles in human blood phagocytes containing cytochrome b558 and the adhesion molecule CD11b/CD18. *Blood* 1993;81:3122-9.

- [81] Hirai H, Zhang P, Dayaram T, Hetherington CJ, Mizuno S, Imanishi J et al. C/EBPbeta is required for 'emergency' granulopoiesis. *Nat Immunol* 2006;7:732-9.
- [82] Panopoulos AD, Watowich SS. Granulocyte colony-stimulating factor: molecular mechanisms of action during steady state and 'emergency' hematopoiesis. *Cytokine* 2008;42:277-88.
- [83] Krakauer T. Interleukin-8 production by human monocytic cells in response to staphylococcal exotoxins is direct and independent of interleukin-1 and tumor necrosis factor-alpha. *J Infect Dis* 1998;178:573-7.
- [84] Standiford TJ, Arenberg DA, Danforth JM, Kunkel SL, VanOtteren GM, Strieter RM. Lipoteichoic acid induces secretion of interleukin-8 from human blood monocytes: a cellular and molecular analysis. *Infect Immun* 1994;62:119-25.
- [85] Yao L, Lowy FD, Berman JW. Interleukin-8 gene expression in *Staphylococcus aureus*-infected endothelial cells. *Infect Immun* 1996;64:3407-9.
- [86] Schmeling DJ, Peterson PK, Hammerschmidt DE, Kim Y, Verhoef J, Wilkinson BJ et al. Chemotaxigenesis by cell surface components of *Staphylococcus aureus*. *Infect Immun* 1979;26:57-63.
- [87] Kobayashi SD, Voyich JM, Burlak C, DeLeo FR. Neutrophils in the innate immune response. *Arch Immunol Ther Exp (Warsz )* 2005;53:505-17.
- [88] Fujita T, Matsushita M, Endo Y. The lectin-complement pathway--its role in innate immunity and evolution. *Immunol Rev* 2004;198:185-202.
- [89] Hansen S, Holm D, Moeller V, Vitved L, Bendixen C, Reid KB et al. CL-46, a novel collectin highly expressed in bovine thymus and liver. *J Immunol* 2002;169:5726-34.
- [90] van de Wetering JK, van Golde LM, Batenburg JJ. Collectins: players of the innate immune system. *Eur J Biochem* 2004;271:1229-49.
- [91] Dziarski R, Platt KA, Gelius E, Steiner H, Gupta D. Defect in neutrophil killing and increased susceptibility to infection with nonpathogenic gram-positive bacteria in peptidoglycan recognition protein-S (PGRP-S)-deficient mice. *Blood* 2003;102:689-97.

- [92] Liu C, Xu Z, Gupta D, Dziarski R. Peptidoglycan recognition proteins: a novel family of four human innate immunity pattern recognition molecules. *J Biol Chem* 2001;276:34686-94.
- [93] Liu C, Gelius E, Liu G, Steiner H, Dziarski R. Mammalian peptidoglycan recognition protein binds peptidoglycan with high affinity, is expressed in neutrophils, and inhibits bacterial growth. *J Biol Chem* 2000;275:24490-9.
- [94] Brouwer N, Dolman KM, van HM, Sta M, Roos D, Kuijpers TW. Mannose-binding lectin (MBL) facilitates opsonophagocytosis of yeasts but not of bacteria despite MBL binding. *J Immunol* 2008;180:4124-32.
- [95] Fleit HB, Wright SD, Unkeless JC. Human neutrophil Fc gamma receptor distribution and structure. *Proc Natl Acad Sci U S A* 1982;79:3275-9.
- [96] Gounni AS, Lamkhioued B, Koussih L, Ra C, Renzi PM, Hamid Q. Human neutrophils express the high-affinity receptor for immunoglobulin E (Fc epsilon RI): role in asthma. *FASEB J* 2001;15:940-9.
- [97] Mansfield PJ, Hinkovska-Galcheva V, Carey SS, Shayman JA, Boxer LA. Regulation of polymorphonuclear leukocyte degranulation and oxidant production by ceramide through inhibition of phospholipase D. *Blood* 2002;99:1434-41.
- [98] Albrechtsen M, Yeaman GR, Kerr MA. Characterization of the IgA receptor from human polymorphonuclear leucocytes. *Immunology* 1988;64:201-5.
- [99] Eggleton P, Ghebrehiwet B, Sastry KN, Coburn JP, Zaner KS, Reid KB et al. Identification of a gC1q-binding protein (gC1q-R) on the surface of human neutrophils. Subcellular localization and binding properties in comparison with the cC1q-R. *J Clin Invest* 1995;95:1569-78.
- [100] Rabellino EM, Ross GD, Trang HT, Williams N, Metcalf D. Membrane receptors of mouse leukocytes. II. Sequential expression of membrane receptors and phagocytic capacity during leukocyte differentiation. *J Exp Med* 1978;147:434-45.
- [101] Ross GD, Jarowski CI, Rabellino EM, Winchester RJ. The sequential appearance of Ia-like antigens and two different complement receptors during the maturation of human neutrophils. *J Exp Med* 1978;147:730-44.
- [102] Dana N, Todd RF, III, Pitt J, Springer TA, Arnaout MA. Deficiency of a surface membrane glycoprotein (Mo1) in man. *J Clin Invest* 1984;73:153-9.

**[103]** Hickstein DD, Ozols J, Williams SA, Baenziger JU, Locksley RM, Roth GJ. Isolation and characterization of the receptor on human neutrophils that mediates cellular adherence. *J Biol Chem* 1987;262:5576-80.

**[104]** Myones BL, Dalzell JG, Hogg N, Ross GD. Neutrophil and monocyte cell surface p150,95 has iC3b-receptor (CR4) activity resembling CR3. *J Clin Invest* 1988;82:640-51.

## **2. MATERIALS AND METHODS**

### **2.1 Bacterial strain and culture condition**

*S. aureus* Newman strain was cultured in tryptic soy broth (TSB composition: casein peptone, 17 g/L, dipotassium hydrogen phosphate, 2.5 g/L, glucose, 2.5 g/L, sodium chloride, 5 g/L, soya peptone , 3 g/L) and 2% NaCl at 37°C. Bacteria were grown over night in 20 ml cultures volumes contained in 250 ml Erlenmeyer flasks, in a rotary shaker. Bacteria were collected by 10 minutes centrifugation at 4000 rpm.

### **2.2 Animal model**

CD1 out bred female mice (5 weeks old) from Charles River Italia were used to test the active immunization. The mice were immunized by intraperitoneal injection at days 0, 14 with 10 µg of recombinant proteins formulation in 0.20 ml in physiological solution in Alum adjuvant. A control group of mice were immunized with physiological solution and adjuvant alone. Nine days after the last immunization all groups were bled and the blood samples were collected to be used in serological analysis.

## **2.3 Cell cultures**

The HL-60 cell line was derived from peripheral blood leukocytes of a 36-year-old Caucasian female with acute promyelocytic leukemia. The wild type cell line (ATCC CCL-240) is available from ATCC, whose stock was obtained at passage 7. The main characteristic of this cell line is its multi-potentially to differentiate into various cell lineages. Environmental condition such as pH and multiple chemical inducers can greatly facilitate the differentiation of HL-60 cell lines into various myeloid lineages. Treatment with N, N-dimethylformamide (DMF) or other polar compounds induce granulocytic differentiation yielding polymorphonuclear-like cells (44% myelocytes and 53% PMNs).

## **2.4 Opsonophagocytosis Killing Assay**

The opsonophagocytosis killing assay (OPKA) is set up in a total reaction volume of 125  $\mu$ l and consists of 4 basic components in the reaction mixture.

The contents of this reaction typically consist of:

1. Differentiated HL60 cells
2. Bacteria
3. Complement

#### 4. Antibody source

##### Harvesting Differentiated HL60 cells

Differentiated HL-60 cells were centrifuged at 1100 rpm for 10 minutes at room temperature. The supernatant was removed completely and the cell pellet was washed by adding 20 ml of DPBS (Ca<sup>++</sup> and Mg<sup>++</sup>).

Before the last centrifugation, 50 µl of cell suspension were diluted 1/20 (950 µl) with DPBS Ca<sup>++</sup> and Mg<sup>++</sup> to be counted by hemocytometer, count cells using the following formula:

- #cells/ml= #cells counted x dilution factor x 10<sup>4</sup>
- Total# of cells= # cells counted x dilution factor x 10<sup>4</sup> X20

After the counting, the cells were centrifuged at 1100 rpm for 10 minutes at RT and the cell pellet was re-suspended in HBSS (without Ca<sup>++</sup> and Mg<sup>++</sup>).

##### Bacteria

*S. aureus* Newman wt strain was grown in TSB medium for 16-18 hours, and then was diluted in HBSS (with Ca<sup>++</sup> and Mg<sup>++</sup>) in order to obtain a final concentration of 75 000 CFU/well.

### Serum

All sera used in the assay (test or control sera) must be inactivated at 56°C for 30 minutes to remove the complement function, then mouse antisera was prediluted 1:50 in HBSS buffer (with Ca<sup>2+</sup>/Mg<sup>2+</sup>) and preincubated with bacteria for 20 minutes at 4°C.

### Complement

Rabbit complement provided by Calbiochem is kept at -20°C and drag up just few seconds before the use.

A set of negative controls is included in each experiment. They consisted of a sample without serum, one containing pre-immune serum, one without phagocytic cells and one with heat inactivated complement at 56°C for 30 min.

The plate, with the reaction mixture, was incubated at 37°C for 1h shaking at 600 rpm. After the addition of last components of reaction and after the incubation of 1h at 37°C, each sample was plated (at the appropriate dilution) on TSA agar plates.

The plates were incubated overnight at 37°C into CO<sub>2</sub> incubator and the day after the colonies were counted. The killing percentage was calculated respect to the sample without serum.

## 2.5 Invasion assay

A suspension of Newman *S. aureus* strain in PBS was added to HL-60 cells, cultured in RPMI and 10% heat inactivated human serum in 24-wells at multiplicity of infection 15:1 (the ratio bacteria: MOI=15:1). HL-60 cells and *Staphylococcus aureus* were co-cultured for 2h in a humidified atmosphere containing 5% CO<sub>2</sub>. Invasion was stopped by putting the plates on ice and washing the HL-60 twice with ice-cold PBS to remove non phagocytosed bacteria. Any remaining extracellular bacteria were killed by gentamycin treatment (50 µg -1). Then the medium was changed again to fresh media without antibiotic. At consecutive hours post-phagocytosis medium was aspirated and HL-60 cells were lysed. Both conditioned medium and cell lysates were plated onto TSA for CFU enumeration.

## 2.6 Bacterial labeling with PHrodo™

Bacteria were grown over night in TSB, washed twice with Phosphate Buffered Saline (PBS, pH 7.2–7.4, Gibco) and suspended in half volume of PBS-0.1% paraformaldehyde (PFA, Sigma). Cells were incubated at 37 °C for 30 min and kept at 2–8 °C in PBS-0.08%PFA. Immediately before labeling, cells were washed with PBS, suspended at 20 mg (wet weight)/ml using a freshly prepared 100 mM Sodium Hydrogen Carbonate solution pH 8.5 (Merck) and

split into aliquots of 750  $\mu$ l. A 10 mM stock solution of PHrodo™ Succinimidyl Ester (Invitrogen) in dimethyl sulfoxide (Sigma) was diluted in the bacterial suspension at a final concentration of 0.1 mM. Each sample was incubated for 45 min at room temperature in the dark and then added with 750  $\mu$ l of Hank's Balanced Salt Solution with Ca<sup>2+</sup> and Mg<sup>2+</sup> (HBSS, Gibco), then spin down with a bench top centrifuge for 60 s at 14,000  $\times$ g. The supernatant was aspirated and the pellet suspended in HBSS and stored in the dark at 4 °C for two months. Bacterial labeling was evaluated by FACS. The maximal fluorescence emission of pHrodo™ labeled GBS was 585 nm. The absolute concentration of labeled bacteria was determined by using TruCOUNT tubes (BD pharmingen). The beads contained in each tube were suspended in 100  $\mu$ l of PBS and added to 100  $\mu$ l of bacteria diluted 1/100 in PBS. The absolute cell count (N) was calculated using the following equation:  $N = (\text{number of events in region containing bacteria}) (\text{number of beads per test}) / (\text{no. of events in absolute count beads region})$ , where the number of beads per test was provided by BD Pharmingen together with TruCOUNT Absolute Count Tubes.

## 2.7 Fluorescence staining and confocal microscopy

Bacteria were grown in TSB at overnight condition, washed twice with Phosphate Buffered Saline (PBS, pH 7.2–7.4, Gibco) and suspended in half volume of PBS-0.08% paraformaldehyde (PFA, Sigma). Bacterial cells were incubated at 37 °C for 30min and kept at 2–8 °C in PBS-0.08%PFA. Immediately before labeling, cells were washed with PBS, suspended at 20mg (wet weight)/ml using a freshly prepared 100mM Sodium Hydrogen Carbonate solution pH 8.5 (Merck) and split into aliquots of 750 µl. A 10 mM stock solution of PHrodo™ Succinimidyl Ester (Invitrogen) in dimethyl sulfoxide (Sigma) was diluted in the bacterial suspension at a final concentration of 0.1 mM. Each sample was incubated for 45 min at room temperature in the dark and then added with 750 µl of Hank's Balanced Salt Solution with Ca<sup>2+</sup> and Mg<sup>2+</sup> (HBSS, Gibco), then spin down with a bench top centrifuge for 60 s at 14,000×g. The supernatant was aspirated and the pellet suspended in HBSS and stored in the dark at 4 °C for two months. After differentiation HL-60 cells were incubated for 10 minutes at 37°C with wheat germ agglutinin (WGA, 50 µg/ml Invitrogen), washed two times in PBS and resuspended in PBS. Differentiated HL-60 cells were dispensed in 6 microtiter plates and incubated with labeled bacteria for 2 hours in the presence of serum, under the same conditions and using the same concentration described for the invasion assay. After incubation, cells were washed twice with PBS (centrifuging the plate at 900 rpm for 5 min

at 2–8 °C) and fixed with 4% PFA in PBS for 5 min at 2–8 °C. After washing, bacteria and cells complex was mounted on a glass slide. Images were acquired on a Zeiss LSM 710 laser scanning confocal microscope.

## **2.8 Scanning electron microscopy**

Infected or non-infected cells were cultured on 6-wells plates in the presence of bacteria (MOI:15:1) at different time points. After the treatment, a drop of liquid cell suspension was placed on poly-L-lysine treated glass coverslip for five minutes. Then, the coverslip was fixed for immersion in a 2,5% glutaraldehyde solution in phosphate buffer 0,1 M pH 7,2 (PB) for 2 hours at 4°C, washed in PB, postfixed in 1% OsO<sub>4</sub> in PB for 30 min. at 4°C, dehydrated in ascending alcohol series, incubated for three time in tert-butanol and finally freeze dried. Afterwards the coverslip was mounted on aluminum stub, coated with 20nm gold in Balzers MED010 sputtering device and observed in Philips XL20 scanning electron microscope at 20kV.

## **2.9 Transmission electron microscopy**

Infected or non-infected cells were cultured on 6-wells plates in the presence of bacteria (MOI:15:1) at different time points. After the incubation,

cells/bacteria complex was fixed with Karnovsky solution (2% paraformaldehyde, 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.4, 30 min at room temperature) washed in the same buffer lacking the fixative and post-fixed in 2% osmium tetroxide for 1h. The pellets were dehydrated through serial incubation in graded series of ethanol solutions (30%, 60%, 90%, and 100%) and finally embedded in Epon. 60 nm sections, obtained at Reichert Ultracut E ultramicrotome, were counter-stained with uranyl acetate, lead citrate and examined with a Philips Tecnai g2 spirit electron microscope.

## **2.10 Tomography electron microscopy**

Tomography was performed with a transmission electron microscope CM200 field emission gun (Philips) operating at 200 kv, equipped with a 2,000 x 2,000 charge-coupled device camera TVIPS TemCam-F224HD and with the EMmenu4 and EM-Tools software packages (both from Tietz Video and Image Processing Systems GmbH). Stained 120-nm-thick sections were decorated on both faces with 10 nm colloidal gold particles before observation in the Philips transmission electron microscope. Gold particles were used as fiducial position markers for image alignment in the tomogram reconstruction procedure. For tomographic reconstruction, low-dose tilt series of images were recorded in

double tilt axis geometry, at 27,500x magnification (nominal pixel size 0.66 nm), with a maximum tilt range of about +60° -60° and tilt steps of 1°. Images were recorded with 1 µm of defocus.

### **2.11 Image analysis**

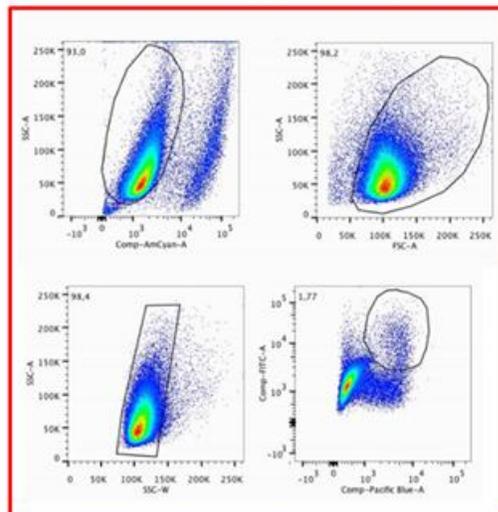
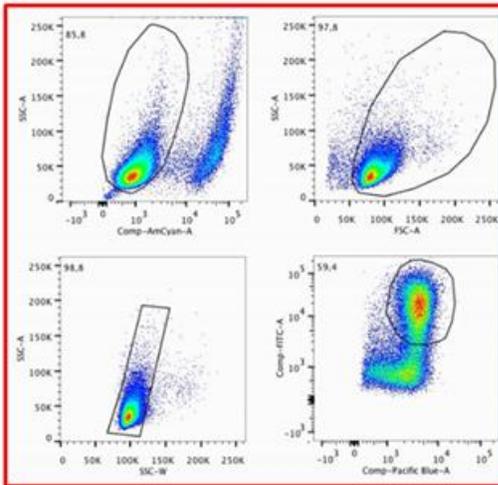
3D reconstruction was performed using R-weighted back-projection with IMOD software (Kremer et al., 1996; Mastronarde, 1997). We exported the 2D coordinates of the fiducial markers acquired with IMOD and pasted the two series of reference points together with simultaneous alignment. The reconstruction was then performed using a weighted back-projection algorithm for general geometry (Radermacher, 1992) and local refinement for distortions.

### **3. RESULTS**

#### **3.1 OPKA on Newman wt strain shows a killing percentage around 50 %**

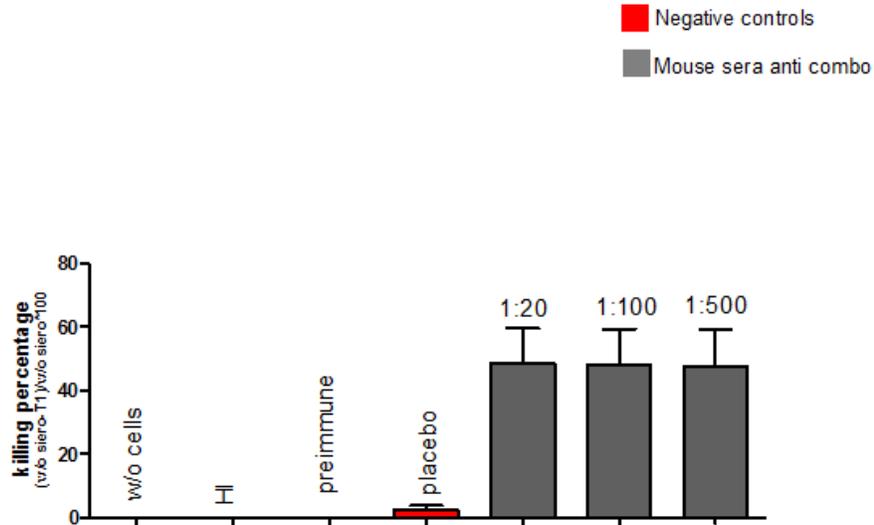
Opsonophagocytosis is the primary mechanism for clearance of pathogen from the host, and the measurement of opsonophagocytic antibodies appears to correlate with vaccine-induced protection. Opsonophagocytic assays are more attractive than other measures of in vitro protective immunity because they more closely resemble the mechanism of natural immunity, do not require the use of animal models, and appear to provide a closer correlation with serotype-specific vaccine efficacy than ELISAs. In order to assess if mouse sera immunized with the final vaccine formulation can elicit antibodies, we performed an opsonophagocytosis assay using HL-60 as effector cells. We randomly check the differentiation of cell line from primary cells into phagocytes by using flow cytometry analysis (FACS) that allow to test specific markers of HL-60. Live HL-60 cells were first gated based on LIVE/DEAD (figure 1) and then based on forward scatter versus side scatter cytogram. The percentage of live cells shown in figure 1 was 80% of total cells and this number varied from 72 to 85% in different experiments performed at different days [1]. Doublets were eliminated using SSC-W versus SSC-A plot in order to keep only singlets. Moreover HL-60 positive to CD-35 and CD11b receptors

were gated to identify the neutrophil effector cell population, which correspond in figure 1 to 80% of total live cells.



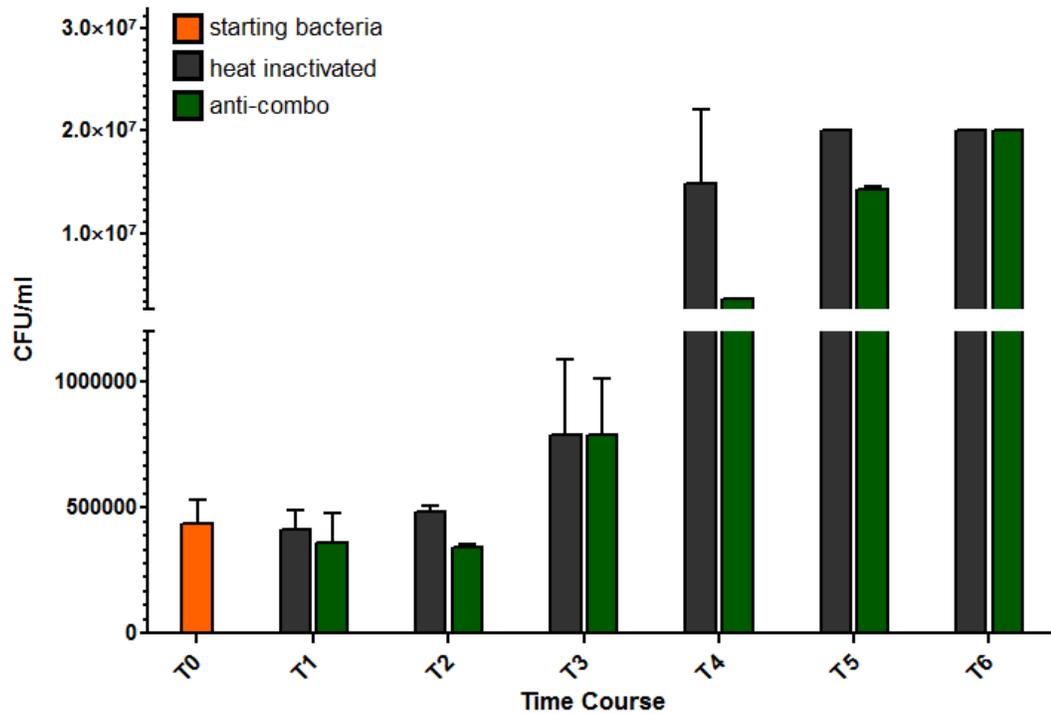
**Figure 1:** Gating strategy to check HL-60 differentiation into neutrophils by FACS analysis: discrimination between lived (gated region) and dead HL-60 cells. (B) Selection of HL-60 cells using forward scatter (FSC) and side scatter (SSC). (C) Discrimination of aggregates from singlets using side scatter–C versus side scatter (SSC-W). (D) Neutrophil identification with specific markers of HL-60 cells differentiation (anti CD-35 and anti CD-11b).

To further test if mouse sera immunized with the final vaccine formulation could elicit antibodies, we measured functional antibody activity against Newman strain in pre- and post-vaccination serum samples. Measurement of functional antibody activity was demonstrated by decreased number of colonies after 1 hours of incubation. Furthermore the opsonophagocytosis process was dependent upon the amount of functional antibody present in each sample. We tested four negative controls with all opsonophagocytosis components except neutrophils and complement. In addition mouse sera immunized only with alum formulation and pre-immune sera were tested. Moreover a mouse serum immunized with the final vaccine formulation was examined at three different dilutions starting from 1:20 to 1: 500. The differences in the percent uptake between pre- and a post-vaccination serum sample were shown in the figure 2. We observed that the obtained percentage of killing is around 50 % but did not decrease depending on the amount of functional antibody.



**FIGURE 2:** Opsonophagocytosis killing assay might be used as a correlate of protection for the assessment of functional activity in vitro of protein-induced antibodies in the presence of phagocytes and complement. The method was set up with Newman wt strain, HL-60 as effector cell, a rabbit complement source and mice antibodies sera immunized against combo formulation. The anti-combo mouse sera were tested at three different dilutions starting from 1:20 to 1:500, 5 fold dilution. The mix was incubated for 1 hour and was plated on TSA to measure bacterial survival as number of colonies (CFU). The red bars show the negative controls with all OPKA components except cells and rabbit complement respectively. Additionally a preimmune and a placebo sera were tested. The percent of killing was calculated respect to the sample without antibodies after 1h of incubation.

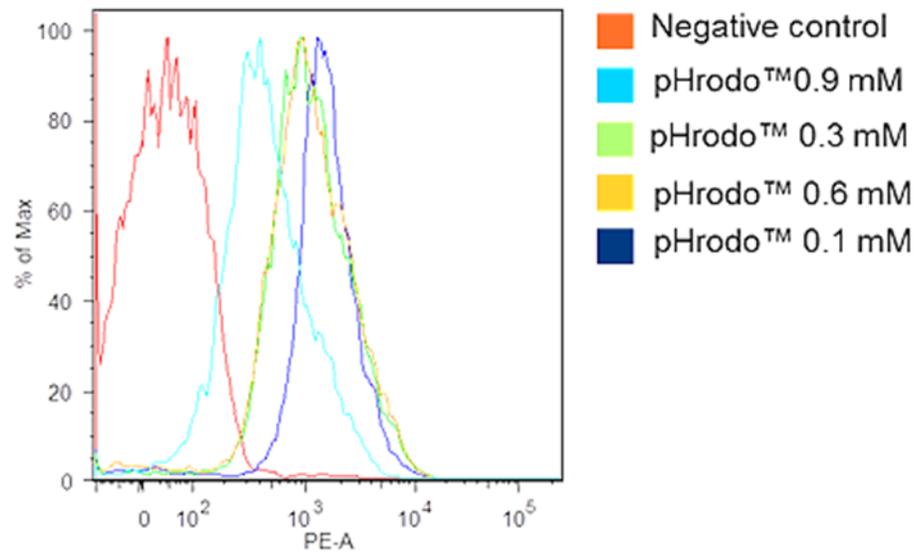
In the previous opsonophagocytosis experiment we observed that the phagocytic activity of neutrophils was activated already after 1 hour of incubation: by this time in fact a percentage of killing around 50% was detected. Then in order to verify if the cells maintained their phagocytic ability we performed opsonophagocytosis killing assay at different time points from 1 hour up to 6 hours. In this experiment we used the same condition applied for the previous one: infected HL-60 neutrophils were lysed at the indicated specific time intervals post phagocytosis and plated onto TSA plates. As expected the opsono assay showed that the majority of bacteria were internalized already at 1 hour and that the same trend was present at 2 hours of incubation. Unexpectedly an increase of bacterial growth occurred starting at 3 hours, with similar high number of *S. aureus* bacteria in both the sample without complement (negative control, grey bar) and in the post-vaccination serum sample (green bar) (figure 3).



**Figure 3:** OPKA was performed on Newman wt strain at six different time points. The assay was performed as described in the Material and Methods section. A positive (mouse sera immunized against final Novartis combination) and a negative control (sample without rabbit complement) were tested. We reported the number of colonies (CFU)/ml for each time intervals and as mean of two analytical section. The orange bar shows the starting number of bacteria before the incubation with the neutrophils cells, rabbit complement and antibodies. Starting form 3 hours of incubation *S. aureus* cells seem to duplicate in both negative and positive sample: the same trend was observed after 4, 5 and 6 hours of incubation.

### **3.2 Confocal microscopy analysis shows that *S. aureus* cell are engulfed by neutrophils after 1 hour of incubation**

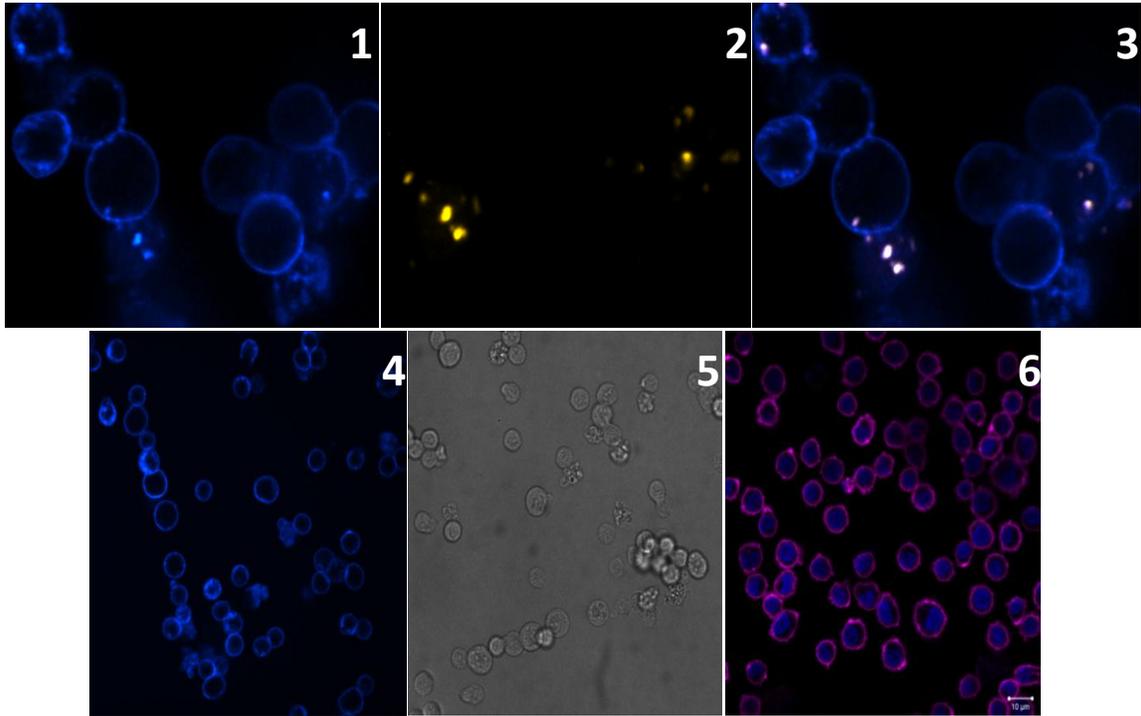
In order to investigate the type of interaction and internalization of *S. aureus* bacteria into the neutrophils we decided to perform a confocal microscopy analysis that could allow us to compare the opsono results with a morphological observation. Preparatory FACS analysis were set up to select the best condition of dye conjugation: differentiated HL-60 cells were incubated with pHrodo™ labeled bacteria in the presence of mouse sera immunized with the final vaccine formulation and complement for 1 hour at 37 °C. The pHrodo™ label is a pH-sensitive dye and attends as specific sensor of phagocytosis and endocytosis. In order to optimize the best conjugation between pHrodo™ and *S. aureus* cells, we tested different concentrations of pHrodo™ label and we analyzed them by FACS analysis. At the end of our analysis (figure 4), we choose the final concentration of 0.1 mM since the pick corresponding to this concentration (blue pick) resulted to be the most shifted one from the control pick(red pick).



**Figure 4:** FACS analysis was performed in order to check the best conjugation between pHrodo™ label and *S. aureus* cells. A 10 mM stock solution of pHrodo™ was diluted in the bacterial suspension at different concentration, from 0.9 mM to 0.1 mM. At the end of the analysis we identified pHrodo™0.1 mM as the best condition.

Once the dye condition was found we proceed with the confocal microscopy analysis: differentiated HL-60 cells were incubated for 1 hour at 37°C in the presence of rabbit complement with *S. aureus* Newman strain pHrodo™

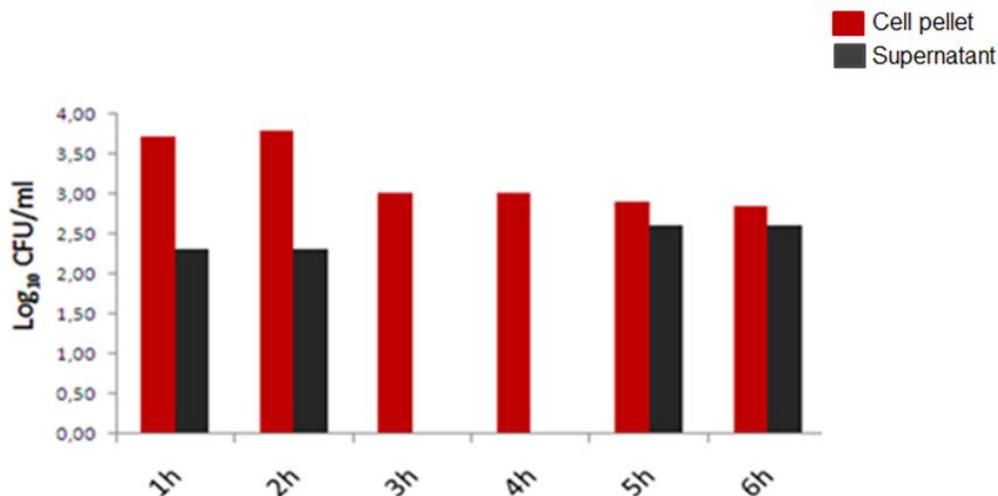
labeled bacteria. The plasma membrane of neutrophils was stained with wheat germ agglutinin (WGA): our observation indicated clearly that neutrophils (labeled in blue) maintain their shape, showing a round shape with a diameter around 5  $\mu$  and that *S. aureus* bacteria labeled with pHrodo™ are visible as a single yellow dot. Z stacks images of the sample were taken by confocal microscopy. Neutrophil plasma membrane (blue) and pHrodo™ labeled bacteria (yellow) are shown respectively in panel 1 and 2 of figure 5. The blue and the yellow images merged together (panel 3) clearly show that the internalized bacteria were brightly fluorescent after engulfment within neutrophils. The panel 5 shows the bright field of HL-60 cells whereas the panel 4 and 6 respectively show HL-60 dyed with WGA and phalloidin mixed with WGA before bacteria incubation.



**Figure 5:** confocal microscopy analysis shows that *S. aureus* cell are engulfed by neutrophils after 1 hour of incubation: differentiated HL-60 cells were incubated for 1 hour at 37°C in the presence of rabbit complement with *S. aureus* Newman strain pHrodo™ labeled bacteria. After washing, cells were fixed with 4% paraformaldehyde and plasma membrane of neutrophils was stained with wheat germ agglutinin (WGA). Panel 1 and panel 2 show respectively the plasma membrane of neutrophils in blue and internalized bacteria in yellow. The merged image (panel 3) clearly shows that the internalized bacteria were brightly fluorescent after engulfment within neutrophils. The panel 5 shows the bright field of HL-60 cells whereas the panel 4 and 6 respectively show HL-60 dyed with WGA and phalloidin mixed with WGA before bacteria incubation.

### ***3.3 S. aureus persists inside neutrophils until the cells lyse***

To determine the intracellular survival/killing rate of *S. aureus* cells we performed an invasion assay at different time points. Cultured HL-60 cells engulfed *S. aureus* strain Newman with the vast of bacteria being internalized by 1 hour. The infected HL-60 neutrophils were lysed at specific time intervals post-phagocytosis and plates on TSA plates. Based on literature data (ref) we selected a multiplicity of infection (MOI) of 15:1; the applied experimental condition, in term of ratio between bacteria and cells, was opposite to the condition optimized for the opsonophagocytosis killing assay (HL60:CFU=50:1): in fact while the aim of opsonophagocytosis killing assay is to assess the functional activity of antibodies, the aim of invasion assay is to quantify the ability of pathogenic bacteria to invade eukaryotic cells. At MOI 15:1 neutrophils could not completely eradicate the bacteria, since a burst of bacteria growth occurred after 5 hours of incubations. An explosion of live *S. aureus* in the conditioned media may indicate that the bacteria either proliferated intracellularly, with large number of bacterial cells being released into the media after neutrophils lysis, or that a small number of surviving intracellular bacteria escaped and multiplied vigorously in the media, killing the HL-60 cells in the process.

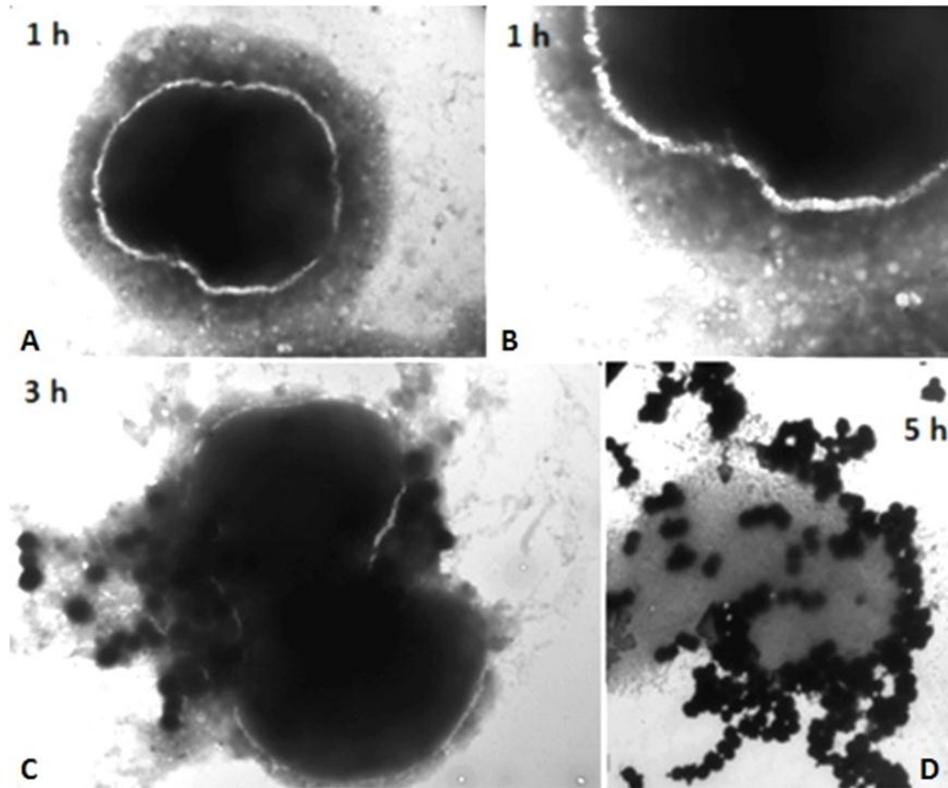


**Figure 6:** Invasion assay show that *S. aureus* persist intracellularly presenting a burst of growth after 5 hours. CFU of *S. aureus* were counted both in cell lysates and culture medium over six consecutive hours. Neutrophils were allowed to engulf *S. aureus* at MOI of 15:1 for 2 h and extracellular bacteria killed by gentamycin. Neutrophils were cultured in media without antibiotics. At consecutive hours post-phagocytosis medium was aspired and HL-60 were lysed. Both conditioned medium and cell lysates were plated onto TSA for CFU enumeration.

### ***3.4 Ultrastructural microscopy analysis shows that survival of bacteria in neutrophils affect the viability of host cells***

To determine whether bacteria engulfment affects the viability of host cells, we performed a negative staining analysis on HL-60 cells at different time points, starting from 1 hour to 6 hours. Negative staining is a sample preparation method in which biological samples are embedded in a thin layer of dried

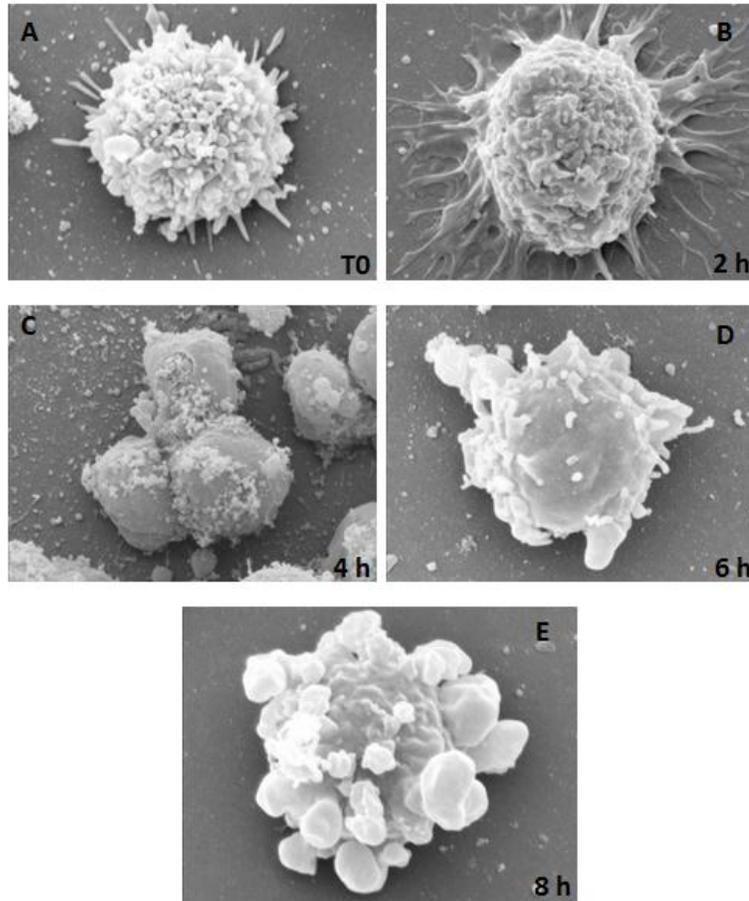
heavy metal salt to increase specimen contrast. For this reason negative staining electron microscopy allows a rapid examination of macromolecular complexes and offers us the possibility to obtain morphological and structural information of the whole bacterial cells and even of their superficial components as the cells membrane. Negative stained samples were than analyzed with a Transmission Electron Microscope (TEM) operating at 120kV. We firstly co-incubated bacteria and neutrophils in the presence of complement and mouse serum after 1, 3 and 5 hours of incubation respectively (figure 7) and after intense washing, we spotted 5 microliters of the samples on carbon coated grids. Negative stained samples were than observed at the TEM and images collected. After 1 hour (panel A) the cell membrane appears completely intact and undamaged even when observed at higher resolution (panel B). Starting from 3 hours of incubation (panel C), we observed many bacteria that invaded neutrophils causing a strong change in the cell membrane morphology that now appear totally disintegrated. By 5 hours the number of bacteria invading cells is considerably increased and the cell is completely dissolved.



**Figure 7:** Negative staining electron microscopy of HL-60 cells incubated with *S. aureus* bacteria. Bacteria and neutrophils were co-incubated in the presence of complement and mouse serum after 1, 3 and 5 hours respectively. After 1 hour (panel A) the cell membrane appears completely intact and undamaged even if it is observed at higher resolution (panel B). At 3 hours of incubation (panel C), bacteria invade neutrophils causing a strong change in the cell membrane morphology that is totally disintegrated. By 5 hours (panel D) the number of bacteria invading cells is increased and the cell is dissolved.

As a further step of our morphological analysis of the infected cells we decided to perform a scanning electron microscopy analysis (SEM) that gives high-resolution images and provides morphological and compositional information.

We used the same experimental condition described before for the negative staining analysis: we collected scanning electron microscopy images at different time points until 8 hours of incubation. In two independent experiments we found that engulfment of *S. aureus* clearly damage cell membrane by 4 hours of incubation (figure 8). Before the incubation (panel A) HL-60 cells are perfectly sticking on scanning microscopy slide, showing numerous protrusion/extension over the surface. Starting from 2 hours, there is a decrease of protrusion that suggests a decrease of adhesiveness (panel B). By 4 hours, we can appreciate a structural surface changes as well as loss of structural components. By 6 hours, as confirmed also by 8 hours, many blebs are present on HL-60 cell surface, demonstrating definitely the evolution of changes in cell membrane morphology. Collectively these data, together with invasion assay, argue that *S. aureus* persists inside HL-60 neutrophils in continuously decreasing number until the exhausted cells allow bacteria to escape into the conditioned medium, where they proliferate.

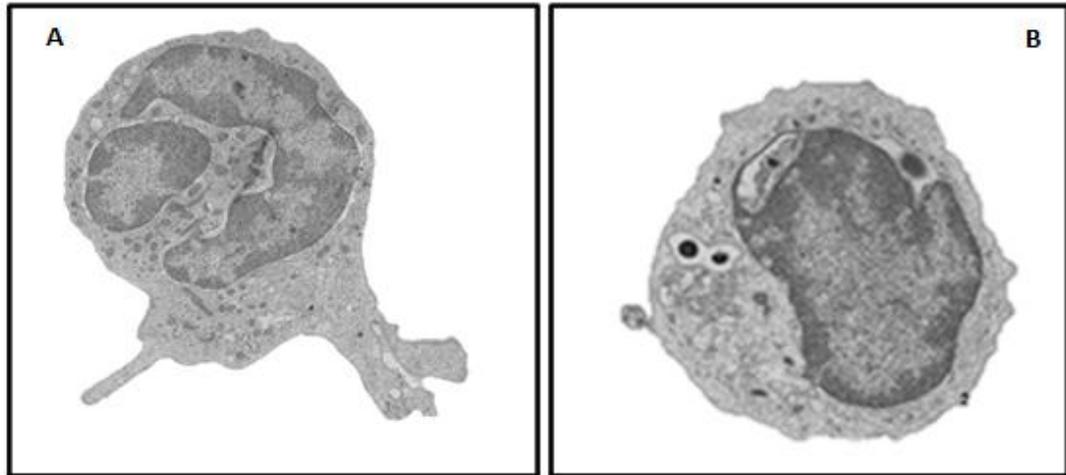


**Figure 8:** Scanning electron microscopy analysis (SEM) shows that survival of bacteria in neutrophils affect the viability of host cells. We collected scanning electron microscopy images at different time points until 8 hours of incubation. We found that engulfment of *S. aureus* clearly damage cell membrane by 4 hours of incubation. Before the incubation (panel A) HL-60 cells are perfectly sticking on scanning microscopy slide, showing numerous protrusion/extension over the surface. Starting from 2 hours, there is a decrease of protrusion that suggests a decrease of adhesiveness (panel B). By 4 hours, we can appreciate a structural surface changes as well as loss of structural components. By 6 hours, as confirmed also by 8 hours, many blebs are present on HL-60 cell surface,

demonstrating definitely the evolution of changes in cell membrane morphology.

### ***3.5 S. aureus perseveres in intracellular vacuoles of HL-60 cells***

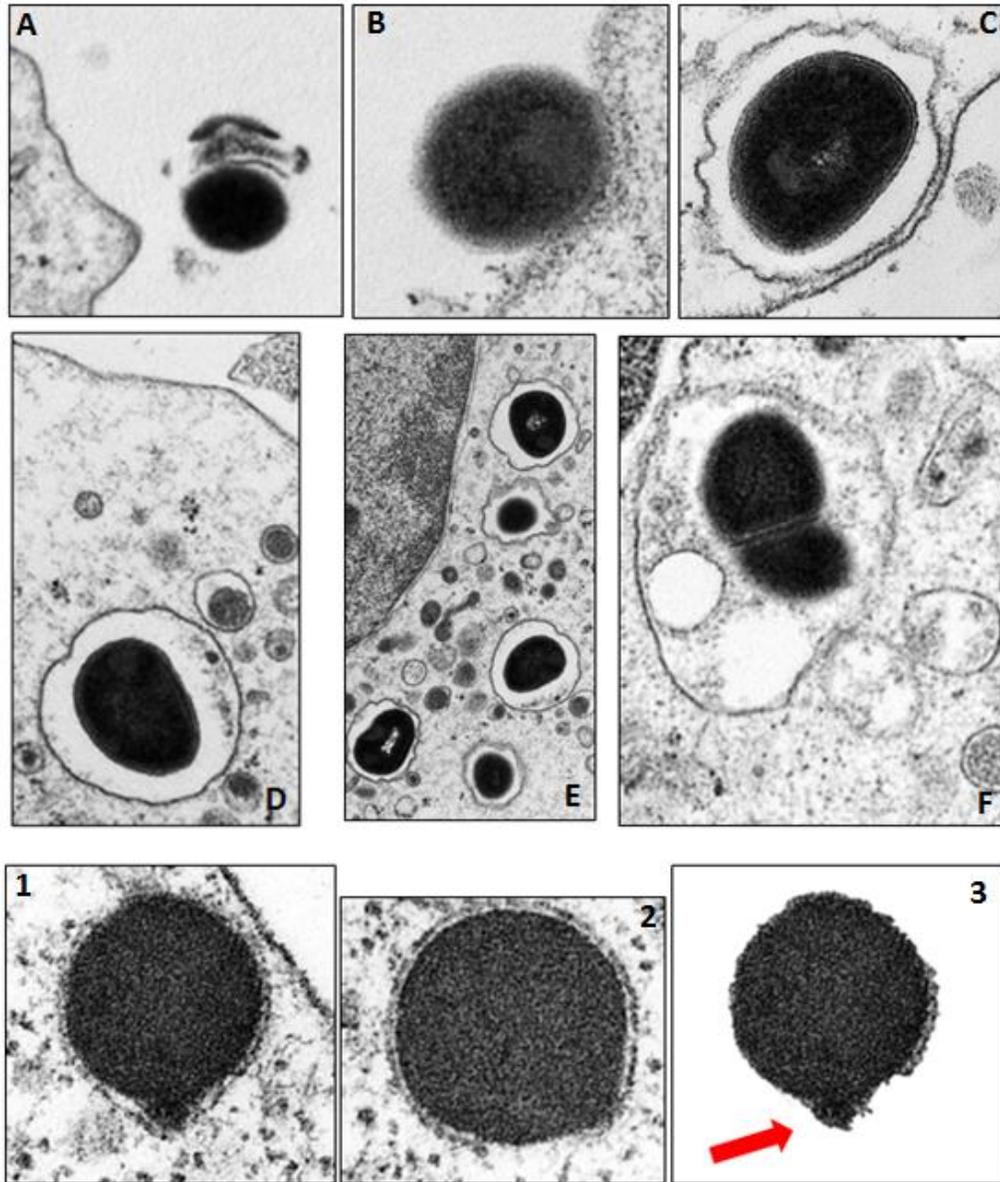
The strategies employed by obligate intracellular pathogen to avoid intracellular killing by professional phagocytes can be broadly categorized either as immediate escape from the phagosome into the cytoplasm either as modification of the phagosome preventing the fusion with lysosomes. Applying either scanning electron microscopy either negative staining techniques we appreciate the overall changes on HL-60 cells surface but we cannot obtain information on structural transformations within neutrophils. Furthermore in order to investigate specific localization of *S. aureus* we analyzed sections of HL-60 cells incubated with *S. aureus* for 2 hours, by transmission electron microscopy (TEM). We used epoxy resin to embed the specimens and we cut thin slices of material by an ultramicrotome. An overall image of the sectioned cell is shown in the Figure 9: the neutrophil HL-60 cells (panel A) have a nucleus that is segmented into two lobes and presents several mature granules; after the incubation with *S. aureus* bacteria HL-60 cells (panel B) assume an apoptotic morphology, including nuclear condensation and fragmentation as well as cytoplasmic vacuolization.



**Figure 9:** Transmission electron microscopy (TEM) micrographs show an overall picture of the whole HL-60 cells incubated with *S. aureus* for 2 hours. Neutrophil HL-60 cells (panel A) have a nucleus that is segmented into two lobes and presents several mature granules; after the incubation with *S. aureus* bacteria HL-60 cells (panel B) assume an apoptotic morphology, including nuclear condensation and fragmentation as well as cytoplasmic vacuolization.

In order to investigate the specific pathway adopted by *S. aureus* bacteria we performed a second transmission electron microscope analysis, using the same condition described in the previous experiment. Through transmission electron analysis (figure 9) we shown that phagocytosis following a sequence of events:

(a) recognition between epitopes on bacterial surface and receptors expressed on cell wall, (b) adherence of *S. aureus* to the HL-60 cell, (c) engulfment of the bacteria within a phagosome, and (d) transfer of the complex that appears distant to the cell membrane and. As expected, HL-60 cell is unable to kill the ingested pathogen: considerable numbers of bacteria were found in vacuoles whose membranes were partially or totally degraded. Finally, it is important to underline that dividing intact bacteria were frequently observed in vacuoles. Collectively these results strongly support our contention that *S. aureus* can survive intracellularly killing inside vesicular compartments.



**Figure 9:** Transmission electron micrographs on HL-60 and *S. aureus* cells were performed. Through this technique we demonstrated that phagocytosis event follows a sequence of specific events: (a) recognition between epitopes on bacterial surface and receptors expressed on cell wall, (b) adherence of *S. aureus* to the HL-60 cell, (c) formation of cup-like processes on the cell surface, and (d) elongation of the cup and engulfment of the bacteria within a

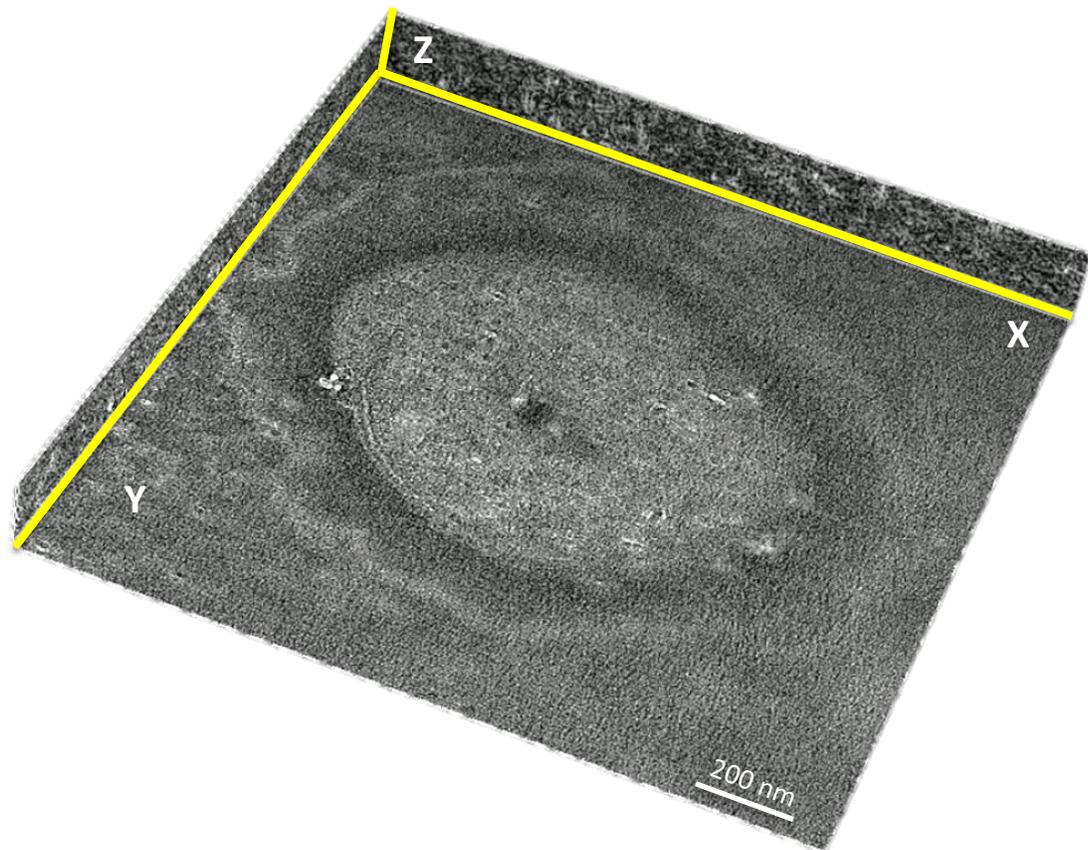
phagosome. As expected, HL-60 cell is unable to kill the ingested pathogen: considerable numbers of bacteria were found in vacuoles whose membranes were partially or totally degraded. Finally, it is important to underline that dividing intact bacteria were frequently observed in vacuoles. Collectively these results strongly support our contention that *S. aureus* can survive intracellularly killing inside vesicular compartments.

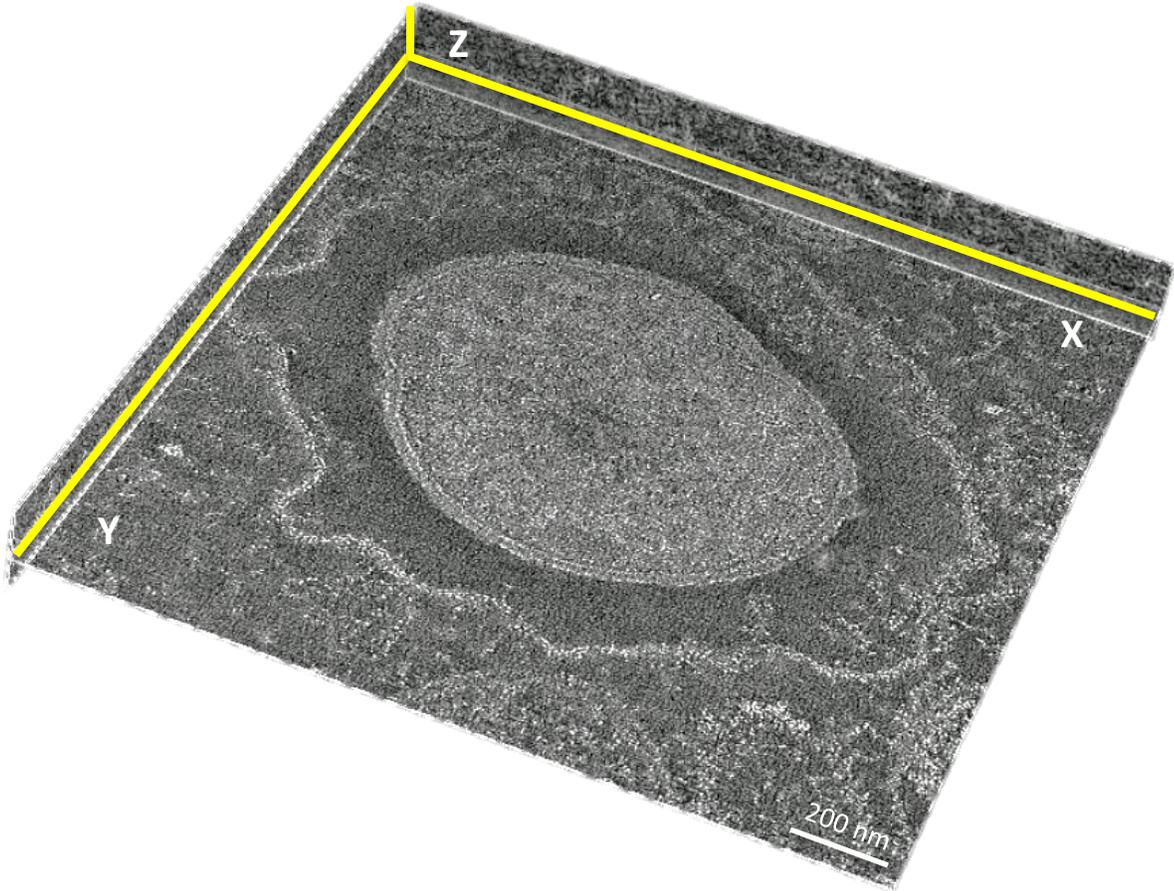
### ***3.6 Electron tomography analysis of S. aureus bacteria in intracellular vacuoles of HL-60 cells***

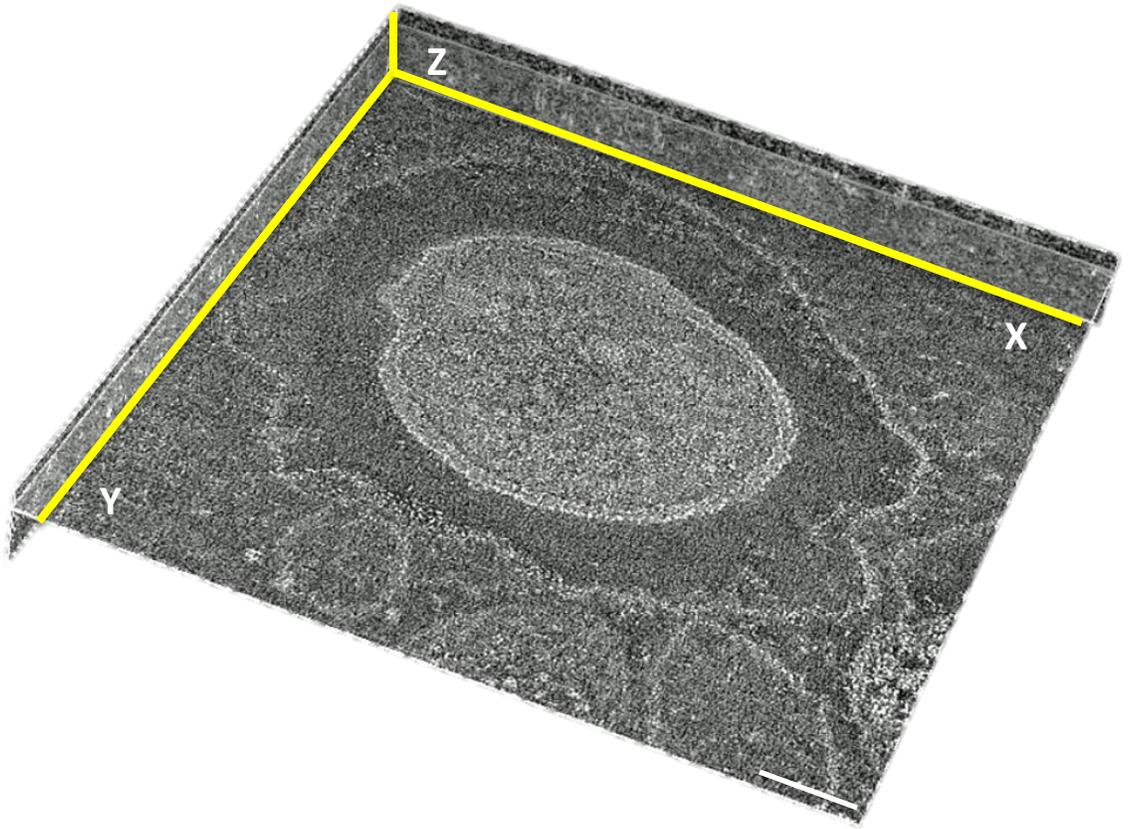
Visualizing the dynamic molecular architecture of cells is instrumental for answering fundamental questions in cellular and structural biology. Although modern microscopy techniques, including fluorescence and conventional electron microscopy, have allowed us to gain insights into the molecular organization of cells, they are limited in their ability to visualize multicomponent complexes in their native environment. Electron tomography (ET) allows cells, and the macromolecular assemblies contained within, to be reconstructed in situ, at a resolution of 2–6 nm. Electron tomography is a unique technique in structural biology research because it is the only tool that enables direct visualization of the cellular space at molecular resolution. In other words ET may bridge the resolution gap between cellular and structural biology.

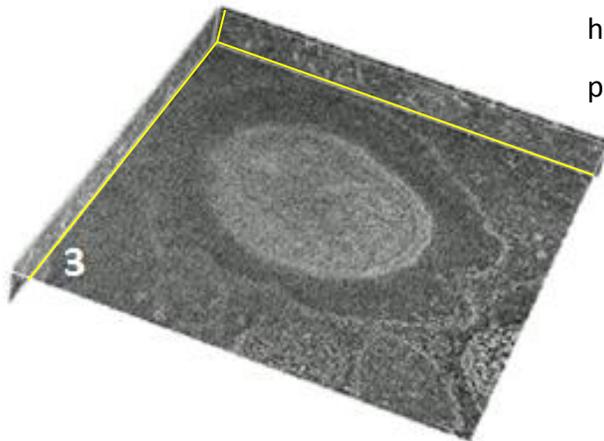
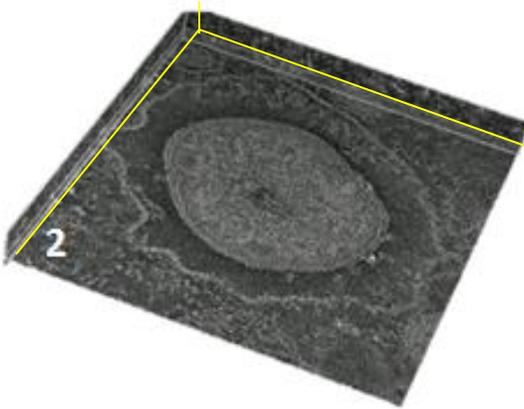
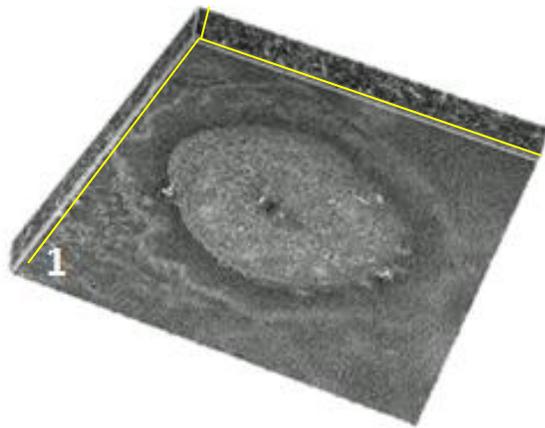
We collected 5 different tomograms and for each tomogram around 120 images were kept, one for each tilt angle. We reported a single whole picture (figure 10) corresponding respectively to the bottom (panel 1), center (panel 2) and top (panel 3) part of the tomogram. The detailed analysis of thin computational sections covering the whole volume for each of the tomograms included in this study was used to make important conclusions (Figure 10).

In electron tomography all useful 3D information is actually extracted from this analysis of planes. In addition the volumetric representation of reconstructed factories using programs such as Chimera helps us to summarize our findings and to highlight some important features of *S. aureus* cells. The extraordinary complexity of volumes generated by ET of highly-preserved cells is apparent in these representations. In order to facilitate their interpretation, noise reduction and segmentation have been applied to display the most important features. In our model the structure reveals the presence of intact bacterial membrane within neutrophil vacuoles. The *S. aureus* membrane appears completely undamaged and integral in contrast with the physiological process of phagocytosis through vacuoles progression. Moreover many vacuoles are present both inside and outside the vacuoles containing bacterium as usually happen during the phagocytosis process (Figure 11).

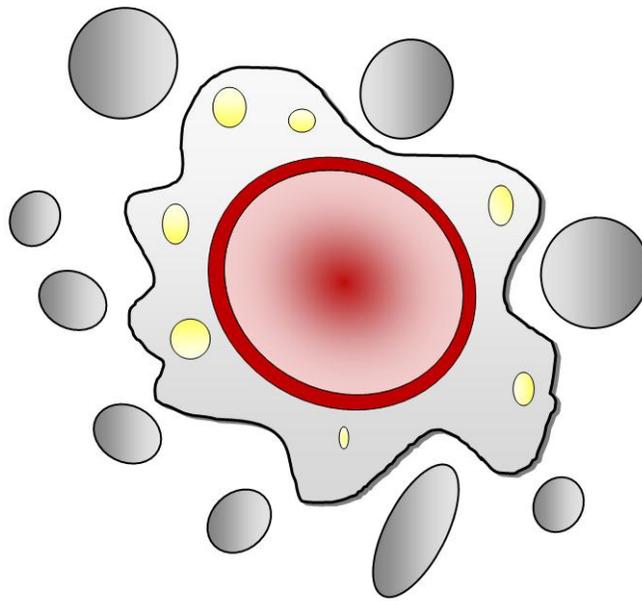








**Figure 10:** electron tomography on *S. aureus* bacteria trapped within HL-60 vacuoles: We reported a single whole picture (figure 10) corresponding respectively to the bottom (panel 1), center (panel 2) and top (panel 3) part of the tomogram. The *S. aureus* membrane appears completely undamaged and integral in contrast with the physiological process of phagocytosis through vacuoles progression. Moreover many vacuoles are present both inside and outside the vacuoles containing bacterium as usually happen during the phagocytosis process



**Figure 11:** A model of *S. aureus* survival within HL-60 neutrophils vacuoles. The *S. aureus* membrane appears completely undamaged and integral in contrast with the physiological process of phagocytosis through vacuoles progression. Moreover many vacuoles are present both inside and outside the vacuoles containing bacterium as usually happen during the phagocytosis process.

## 5. DISCUSSION

The principal immune effector mechanism by which humans are protected from Gram positive bacteria such as *S. aureus* is antigen specific antibody and complement dependent opsonophagocytosis [1]. This process can be measured in vitro using the opsonophagocytic killing assay (OPKA), which is a complex assay composed of live *S. aureus* bacteria, a complement source, phagocytic effector cells such as differentiated HL-60 cells, and test serum[2, 3].

During my PhD, we investigated the impact on the OPKA of *S. aureus* antigens vaccine formulation, measuring functional antibody activity against *S. aureus* Newman strain. We observed that the obtained percentage of killing is around 50 %, demonstrating that mouse antibodies immunized with *S. aureus* antigens vaccine formulation play a role during the clearance of pathogen (figure 2). Furthermore we have proven that the phagocytic activity of neutrophils was activated after 1 hour of incubation and that cells maintained this specific capability up to 2 hours. Unexpectedly we observed an increase of bacterial growth occurred starting from 3 hours, suggesting that HL-60 cells lost their phagocytic activity (figure 3)[4, 5]. These data suggest that *S. aureus* can survive inside neutrophils and supports current in vitro studies of *S. aureus* invasion and survival in epithelial cells, endothelial cells, and osteoblasts [6, 7]. This evidence of course is a significant problem because neutrophils are the most prominent

cellular defense against *S. aureus* infections and moreover suggest that the surviving of *S. aureus* inside neutrophils contributes to infections [8].

Subsequently, to determine the intracellular survival/killing rate of *S. aureus* cells we performed an invasion assay at different time points, demonstrating definitely that this bacterium can not only persist for several hours inside neutrophils, but ultimately is able to escape the intracellular confinement and proliferate rapidly in the conditioned media (figure 6)[9]. Furthermore by examining transmission electron microscopy micrographs of infected neutrophils at different time points, several important observations were made: after 1 hour of incubation the cell membrane appears completely intact and undamaged but starting from 3 hours many bacteria invaded neutrophils causing a strong change in cell membrane morphology that appear completely disintegrated.

As a further step of our morphological analysis we demonstrated by scanning electron microscopy technique that *S. aureus* compromises the viability of HL-60 neutrophils, confirming definitely the evolution of changes in cell membrane morphology. Taken together, these results strengthen the idea that *S. aureus* has evolved numerous mechanisms to evade host defense strategies employed by neutrophils, including the ability to modulate normal neutrophil turnover and that this critical process should contribute to dissemination of the pathogen to infections site.[10].

Finally in order to investigate the specific localization of *S. aureus* we performed an ultrastructural analysis by transmission electron microscopy: we observed that after the incubation with pathogen HL-60 assumed an apoptotic morphology, including nuclear condensation and fragmentation as well as cytoplasmic vacuolization (figure 9, panel B).

The development of electron microscopy and its application to biological studies allowed the morphology of many organelles to be revealed. Electron-microscopy can provide a high resolution view of the overall architecture of cells at high resolution of a few nanometers. More recently, the use of electron tomography has allowed this information to be extrapolated to three dimensions and to be used to build a molecular map of cells in physiologically relevant conditions at resolution of a few nanometers [11, 12]. To this purpose we recorded a series of two-dimensional (2D) electron micrograph projections at varying angles (tilt series) and then merging these projections to produce a 3D reconstruction. In our final model the *S. aureus* membrane appears completely undamaged and integral in contrast with the physiological process of phagocytosis through vacuoles progression. *S. aureus* bacteria show a homogenous distribution of the density in all the three dimensions (X, Y, Z). No preferential density distribution has been noticed and the cell turgor is maintained. All these evidences definitely explain the ability of the pathogen to survive inside the endosomal vacuoles and should be the cause of the low killing level.

## Bibliography

- [1] Nanra JS, Buitrago SM, Crawford S, Ng J, Fink PS, Hawkins J et al. Capsular polysaccharides are an important immune evasion mechanism for *Staphylococcus aureus*. *Hum Vaccin Immunother* 2012;9.
- [2] Romero-Steiner S, Frasch CE, Carlone G, Fleck RA, Goldblatt D, Nahm MH. Use of opsonophagocytosis for serological evaluation of pneumococcal vaccines. *Clin Vaccine Immunol* 2006;13:165-9.
- [3] Verbrugh HA, Peterson PK, Nguyen BY, Sisson SP, Kim Y. Opsonization of encapsulated *Staphylococcus aureus*: the role of specific antibody and complement. *J Immunol* 1982;129:1681-7.
- [4] Schaffer AC, Lee JC. Staphylococcal vaccines and immunotherapies. *Infect Dis Clin North Am* 2009;23:153-71.
- [5] DeLeo FR, Otto M. An antidote for *Staphylococcus aureus* pneumonia? *J Exp Med* 2008;205:271-4.
- [6] Vesga O, Groeschel MC, Otten MF, Brar DW, Vann JM, Proctor RA. *Staphylococcus aureus* small colony variants are induced by the endothelial cell intracellular milieu. *J Infect Dis* 1996;173:739-42.
- [7] Bayles KW, Wesson CA, Liou LE, Fox LK, Bohach GA, Trumble WR. Intracellular *Staphylococcus aureus* escapes the endosome and induces apoptosis in epithelial cells. *Infect Immun* 1998;66:336-42.
- [8] Gresham HD, Lowrance JH, Caver TE, Wilson BS, Cheung AL, Lindberg FP. Survival of *Staphylococcus aureus* inside neutrophils contributes to infection. *J Immunol* 2000;164:3713-22.
- [9] Kubica M, Guzik K, Koziel J, Zarebski M, Richter W, Gajkowska B 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:e1409.
- [10] Rigby KM, DeLeo FR. Neutrophils in innate host defense against *Staphylococcus aureus* infections. *Semin Immunopathol* 2012;34:237-59.
- [11] Chen S, McDowall A, Dobro MJ, Briegel A, Ladinsky M, Shi J et al. Electron cryotomography of bacterial cells. *J Vis Exp* 2010.

**[12]** Tocheva EI, Li Z, Jensen GJ. Electron cryotomography. Cold Spring Harb Perspect Biol 2010;2:a003442.

