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Carina da Silva Duarte

Dissertação de Mestrado apresentada à
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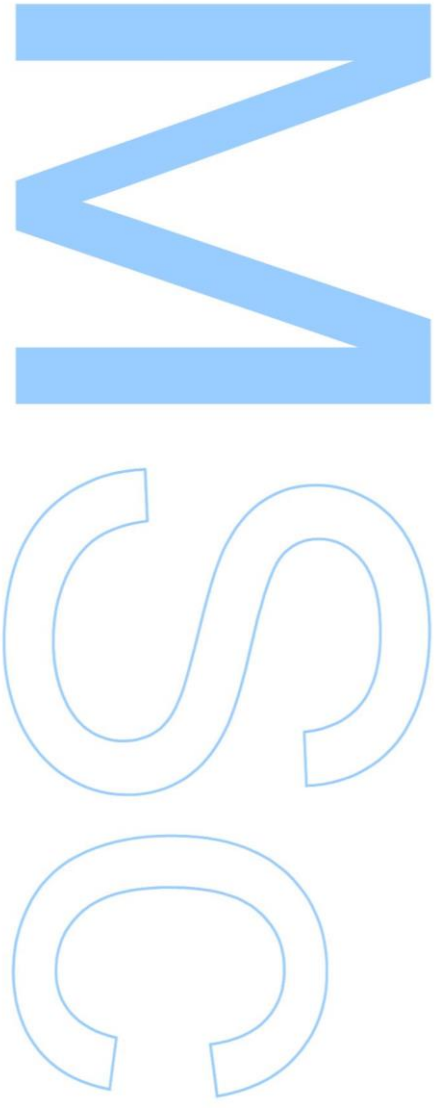

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Resumo

A bactéria *Staphylococcus epidermidis*, previamente encarada como um microrganismo comensal inofensivo da pele e membranas mucosas dos humanos, tem surgido como um importante agente patogénico oportunista. É a causa mais frequente de infeções associadas a dispositivos médicos implantados, o que está diretamente relacionado com a sua capacidade de formar estruturas em multicamadas protegidas por uma matriz, conhecidas por biofilmes. Em virtude da sua estrutura e fisiologia, o biofilme é intrinsecamente tolerante ao sistema imunitário do hospedeiro e à atuação de agentes antimicrobianos. Por consequência, a remoção do dispositivo médico passa, muitas das vezes, por ser a única solução plausível como tentativa de resolver a infeção, o que traz implicações ao nível da morbidade e acarreta custos inúmeros para o sistema de saúde público.

Muitos dos estudos conduzidos em infeções associadas a biofilmes são realizados usando sistemas *in vitro* que se focam na comparação entre a bactéria crescida de modo planctónico *vs* biofilme. No presente estudo foi avaliado o impacto dos ácidos teicóicos da parede (WTA, do inglês *wall teichoic acids*) na interação entre *S. epidermidis,* em modo planctónico, e o sistema imunitário usando uma estirpe na qual foi deletado o gene *tagO,* o primeiro gene da via biossintética dos WTA. Observou-se que, apesar da estirpe deficiente em WTA ter um fenótipo viável, apresenta um conjunto de defeitos que foram relacionados com a falta deste polímero. A perda da expressão de WTA culminou num aumento do tamanho da célula bacteriana, em alterações na divisão celular, e numa menor capacidade de formação de biofilme. A interação entre *S. epidermidis* com células dendríticas e macrófagos de murganho e humanos também indicou que os WTA terão um papel na resposta imune do hospedeiro a esta bactéria. Os WTA parecem estar implicados no reconhecimento da bactéria e na indução de uma resposta mais pro-inflamatória por parte dos macrófagos. Em células dendríticas, os resultados não foram concordantes entre as duas espécies. Enquanto em murganhos os WTA parecem estar envolvidos na evasão imune da bactéria a estas células, em humanos esses polímeros não parecem ter influência na resposta desencadeada. O papel dos WTA na evasão de *S. epidermidis* ao sistema imunitário do hospedeiro permanece, assim, por clarificar, embora este estudo tenha fornecido resultados preliminares nesse sentido. No seu conjunto, os resultados obtidos sugerem que a depleção dos WTA poderá induzir alterações estruturais da parede bacteriana, com uma possível alteração da expressão ou reorganização dos seus componentes, o que poderá por sua vez afetar o

reconhecimento da bactéria por parte das células do sistema imunitário. Uma futura caracterização da parede celular de *S. epidermidis* na ausência dos WTA será importante para confirmar esta hipótese.

Palavras-chave: *S. epidermidis*, ácidos teicóicos da parede, sistema imune inato, células dendríticas, macrófagos, biofilme.

Abstract

The bacterium *Staphylococcus epidermidis*, previously regarded as a harmless commensal microorganism of the skin and mucous membranes of humans, has emerged as an important opportunistic pathogen. It is the most frequent cause of infections associated with implanted medical devices, which is directly involved with its ability to form multi-layered structures protected by a matrix known as biofilms. Due to their structure and physiology, biofilms are intrinsically tolerant to the host immune system and to the action of antimicrobial agents. As a consequence, removal of the medical device is often the only plausible solution to eliminate the infection, which has implications for morbidity and poses a high economic burden for public health systems.

Many studies conducted on biofilm-associated infections have been done *in vitro* and were focused on comparing planktonic and biofilm growing bacteria. In the present study, the impact of wall teichoic acids (WTA) on the interaction between *S. epidermidis,* grown in the planktonic mode, and the immune system was evaluated using a strain defective in the *tagO* gene, the first gene of the WTA biosynthetic pathway. Although the WTA-deficient strain has a viable phenotype, it nevertheless presents a set of defects that were related to the lack of this polymer. Loss of WTA affected in an increase in the size of the bacterial cell, in defects on cell division, and a lesser capacity for biofilm formation. The interaction between *S. epidermidis* with mouse and human-derived dendritic cells and macrophages also appears to reveal a role for WTA in the host immune response to this bacterium. WTA seem to be involved in the recognition of the bacterium and the induction of a more pro-inflammatory response by macrophages. In dendritic cells, the results are not consistent between the two species. While in mice the WTA seem to play a role in the immune evasion of the bacteria to these cells, in humans these polymers do not seem to influence the triggered response. The role of WTA in the evasion of *S. epidermidis* from the host immune system remains to be clarified, although this study has provided preliminary results in this regard. Overall, the results presented here indicated that depletion of WTA could induce structural changes in the cell wall, with a possible alteration of the expression or reorganization of its components, which may influence the recognition of the bacteria by the cells of the immune system. Future characterization of the cell wall of *S. epidermidis* in the absence of WTA will be important to confirm this hypothesis.

Keywords: *S. epidermidis*, wall teichoic acids, innate immune system, dendritic cells, macrophages, biofilm.

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List of Abbreviations

AMP	Antimicrobial peptide
APC	Antigen-presenting cell
Bap	Biofilm-associated protein
Bhp	Bap homologue protein
BMDC	Bone Marrow-derived Dendritic Cell
BSA	Bovine Serum Albumin
CD	Cluster of differentiation
CFU	Colony Forming Unit
Cm	Chloramphenicol
CoNS	Coagulase-Negative Staphylococcus
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
Embp	Extracellular matrix-binding protein
FACS	Fluorescence-Activated Cell Sorting
FBS / FCS	Fetal Bovine Serum / Fetal Calf Serum
FVD	Fixable Viability Dye
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HBSS	Hank’s Balanced Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA	Human Leukocyte Antigen
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMD	Indwelling Medical Device
KO	Knockout

LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
M-CSF	Macrophage Colony-Stimulating Factor
MDM	Monocyte-Derived Macrophage
MFI	Mean Fluorescent Intensity
MHC	Major Histocompatibility Complex
MoDC	Monocyte-derived Dendritic Cell
MOI	Multiplicity of Infection
NF-kB	Factor nuclear kappa B
OD	Optical density
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate-Buffered Saline
PDG	Peptidoglycan
PGA	Poly-γ-Glutamic Acid
PI	Propidium iodide
PIA	Polysaccharide Intercellular Adhesin
PMN	Polymorphonuclear
PNAG	Poly-N-Acetylglucosamine
PRR	Pattern recognition receptor
PSM	Phenol-soluble modulin
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
S. aureus	*Staphylococcus aureus*
S. epidermidis	*Staphylococcus epidermidis*
SD	Standard deviation
Sdr	Short-chain dehydrogenase/reductase
TA	Teichoic acids
tag	*Teichoic acid glycerol*
Th	T helper
TLR	Toll-like receptor

TNF-α	Tumor necrosis factor-alpha
Treg	Regulatory T cell
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
WT	Wild-Type
WTA	Wall Teichoic Acid

CHAPTER 1: INTRODUCTION

1. *Staphylococcus epidermidis*

Firstly described in 1878, staphylococci are clustering gram-positive cocci facultatively anaerobic, nonmotile, non-spore forming that belong to the Micrococcaceae family (Kleinschmidt et al., 2015; Namvar et al., 2014). Staphylococci are classified in two main groups: coagulase positive, primarily identifying *Staphylococcus aureus* (*S. aureus*) and coagulase negative, which are distinguished by the ability or inability, respectively, to produce the blood-clotting enzyme coagulase (Nguyen et al., 2017; Otto, 2009). *Staphylococcus epidermidis* (*S. epidermidis*), the most frequently isolated member of the group of coagulase-negative staphylococci (CoNS) and one of the most abundant colonizers of human skin and mucous membranes, is a commensal bacterium of human epithelia. *S. epidermidis* predominantly colonizes moist areas such as the axillae, head, inguinal and perineal areas, anterior nares, conjunctiva, and toe webs (Becker et al., 2014; Otto, 2009; Vuong et al., 2002).

The skin, as a physical barrier and interface with the outside environment, in addition to protect against invasion by pathogens and foreign substances, is also colonized by large microbial communities, known as the microbiota (Grice and Segre, 2011). The skin microbiota has an extensive influence on the host and, under healthy conditions, is believed to be beneficial as it educates the immune system and outcompete with pathogens (Brown and Clarke, 2017). Thus, the colonization with *S. epidermidis* is thought to be important to the maintenance of a healthy skin microbiota as it competes with potentially harmful microorganisms, in particular, *S. aureus* (Otto, 2012). On the other hand, once the epithelial protective barrier is compromised, by any type of trauma or insertion of a medical device, *S. epidermidis*, a microorganism usually innocuous, or even beneficial, can penetrate the host and become pathogenic (Nguyen et al., 2017). As such, this bacterium, once regarded as relatively innocuous, has emerged as an important opportunistic pathogen, becoming the most frequent cause of device-related infections (Otto, 2012), especially among immunocompromised and critically ill patients (McCann et al., 2008). This is directly related to its capability to adhere and form multi-layered and highly structured biofilms on native host tissues and on the surface of indwelling medical devices (IMDs) (McCann et al., 2008; Otto, 2009; Vuong et al., 2002).

2. Importance of *S. epidermidis* in Health Care

Medical devices, such as pacemakers, heart valves, intravenous and dialysis catheters, vascular prostheses, cerebrospinal fluid shunts and endotracheal tubes, save millions of lives worldwide. However, the use of implanted foreign bodies presents an inherent risk of developing biofilm-associated infections due to the colonization of their surfaces by microorganisms (França and Cerca, 2012). Over the years, with the advance of medicine, there have been drastic changes in patient demographics. The growing number of premature newborns and multimorbid, chronically ill, elderly and immunocompromised patients, along with the increasing use of IMDs, have contributed to the rising number of medical device-related infections (Becker et al., 2014). Infecting microorganisms can originate from different sources, such as skin at the insertion site, colonization of the medical device before implantation and due to shedding from healthcare workers (McCann et al., 2008). CoNS species were not always considered pathogens, however, a singular increase in the use of medical devices and invasive techniques, combined with the greater immunobiological weakness of patients, provided an opportunity for normal commensal organisms, such as *S. epidermidis*, to be considered pathogens of relevance in the context of infections associated with biofilm formation in medical devices (França and Cerca, 2012).

By adhering to a tissue surface, *S. epidermidis* can establish a lifelong commensal relationship with the host. In general, the commensal lifestyle of this bacterium prevents the colonization of pathogenic bacteria such as *S. aureus* (Kleinschmidt et al., 2015). However, *S. epidermidis* has also been associated with the onset of invasive infections. Unlike *S. aureus*, which can produce a large number of extracellular enzymes and toxins, *S. epidermidis*, with a distinctly reduced arsenal of toxins, normally is unable to cause infections in healthy hosts (Sabaté Brescó et al., 2017), with the only exception being native valve endocarditis (Caputo et al., 1987). To change from a commensal to a pathogenic lifestyle, *S. epidermidis* requires a predisposed host (Vuong et al., 2002), which includes preterm newborns, immunocompromised patients and patients with IMDs (Sabaté Brescó et al., 2017). For example, the immature immune system and the impaired skin and mucosal barriers of preterm newborns, as well as the systematic exposure to invasive procedures, such as the insertion of IMDs, predispose these hosts to develop *S. epidermidis* infections (Becker et al., 2014). The use of the IMDs can actually predispose and facilitate infection, contributing to the emergence of *S. epidermidis* as an opportunistic pathogen, since any bacterium accidentally introduced

into the surgical site is capable to adhere and accumulate on the surface of the device (Sabaté Brescó et al., 2017).

S. epidermidis is known as the most frequent causative agent of infections on IMDs. The infection usually begins with the introduction of bacteria from the skin of the patient, or that of health care personnel, and the fact that this bacterium is a permanent and ubiquitous colonizer of human skin and mucous membranes results in a high probability of contamination during device implantation (Otto, 2009). Moreover, the use of intravenous and prosthetic devices allows not only access to the bloodstream but also provide a surface to where the bacterium can attach and, subsequently, form a biofilm (Kleinschmidt et al., 2015). The ability to establish biofilms on IMDs is a major virulence mechanism of *S. epidermidis* and make these infections difficult to eradicate (Vuong et al., 2002). Cells within biofilms, when compared to their planktonic (free-floating) counterparts, are more tolerant to several classes of antibiotics (Cerca et al., 2005), as well as to the attack from the host immune system (Cerca et al., 2006; Vuong et al., 2002). This can be explained by the restricted penetration in the biofilm structure, the decreased growth rate and a distinct genetic phenotype of the cells, and the presence of biofilm persister cells (McCann et al., 2008). Furthermore, the bacterium can disperse from the original site of colonization and can cause infections in other suitable sites (McCann et al., 2008). Therefore, the colonization of such medical devices results in infections characterized as predominantly subacute or chronic, which are often recurrent in nature (Kleinschmidt et al., 2015).

Although severe complications or life-threatening diseases only rarely develop among *S. epidermidis* caused infections, their frequency and the fact that they are notoriously difficult to treat, constitute a serious clinical problem (Otto, 2009). Since the antimicrobial treatment has little or no effect in biofilm-associated infections, treatment can be exceptionally challenging, and the complete removal and replacement of the device, combined with the administration of prolonged antibiotic therapy, is often required as an attempt to eradicate the infection (Rogers et al., 2009). In situations where this is not a viable option, patients are subject to intermittent antibiotic therapy throughout their lives (Costerton et al., 2003). These approaches involve risks to patients, leading to increased morbidity and, occasionally, mortality rates among affected patients (Otto, 2009). Furthermore, these infections have a significant economic impact on the public health care system (Rogers et al., 2009).

The strains that are found in the hospital setting are subjected to the selective pressure of antibiotics and, in case of Staphylococcus spp., the resistance to antibiotics is widespread across the genus, such as methicillin, the antibiotic of choice for the treatment of staphylococcal infections (França and Cerca, 2012). This, together with the factors listed above, emphasize the clinical problem of this bacterium.

3. Biofilm formation

S. epidermidis mostly lacks components recognized as "virulence factors", such as toxins or tissue-damaging exoenzymes, that are produced by the more virulent *S. aureus* (Vuong et al., 2002). Most of the factors that facilitate the establishment of infection and persistence of this bacterium in the host are the same that contribute to its commensal life in the human skin and mucosa (Otto, 2012). The most important mechanism of virulence, which is responsible for the success of *S. epidermidis* as a pathogen, is the ability to adhere to surfaces and form biofilms, a highly structured bacterial community, embedded in a protecting extracellular matrix (Otto, 2013; Vuong et al., 2002). Biofilm protects bacteria from exogenous environmental factors and endogenous host factors, in both the colonizing and infectious states (Otto, 2013). This mode of bacterial growth has characteristic physiology and architecture that facilitate evasion from the host defence and confers marked tolerance to several antimicrobial agents (Otto, 2009). First, the extracellular matrix can act as a mechanical barrier making difficult the penetration of immune cells and the diffusion of some antibiotics. Second, biofilm cells exhibit downregulation of basic cellular processes such as cell division, protein synthesis and DNA replication, which may explain the significantly limited activity of many antibiotics that target actively growing cells (Mah and O'Toole, 2001). Third, the existence of "persisters", cells that are highly recalcitrant to antimicrobial agents, represent the major concern in biofilm treatment (Lewis, 2010; Otto, 2012).

The development of a bacterial biofilm is a multistep process that can be divided into three phases: 1) initial adhesion, 2) intercellular aggregation and maturation, and 3) detachment (O'Toole et al., 2000) (Fig.1). Initial adhesion may occur to abiotic surfaces, such as the uncovered plastic surface of IMDs, or to biotic surfaces, such as host tissues or human matrix protein-covered devices (Otto, 2012). During the second stage, bacteria multiply and accumulate as multi-layered cell clusters, occurring the formation of fluid-filled channels to ensure the nutrient and oxygen distribution to deeper layers of the

biofilm, give the mature biofilm its typical three-dimensional structure. The last stage is characterized by the detachment of single or clusters of cells from the biofilm and subsequent dissemination, via the bloodstream, to start the colonization at distal sites, leading to the secondary focus of infection (Becker et al., 2014; O'Toole et al., 2000).

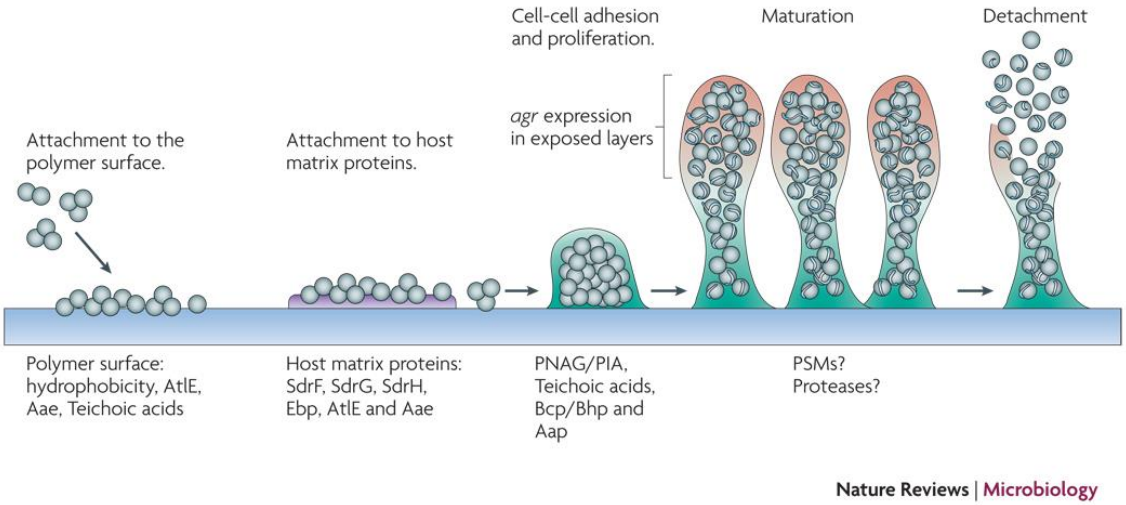


Fig. 1 - Phases of biofilm development: initial adhesion, maturation and final detachment. Attachment may occur directly to a surface, such as the polymeric surface of a medical device, or to host matrix proteins of the “conditioning film” that forms on the device after implantation. Maturation stage is characterized by intercellular aggregation through adhesive molecules and the formation of channels that require disruptive factors. These disruptive factors possibly correspond to detergent-like peptides and proteases expressed in exposed layers of biofilm under control of the agr system, which is also involved in the last phase of biofilm development, detachment. Molecules involved in biofilm formation are shown. Aae, autolysin/adhesin; Aap, Accumulation-associated protein; AtlE, autolysin E; Bcp/Bhp, Bap homologue protein; Ebp, Elastin-binding protein; PNAG/PIA, poly-N-acetyl-glucosamine/polysaccharide intercellular adhesin; PSMs, phenol-soluble modulins; SdrF/SdrG/SdrH, Serine-aspartate repeat-containing protein F/G/H (Otto, 2009).

3.1 Initial Bacterial Adhesion

The first stage of *S. epidermidis* colonization may either proceed as the direct attachment of the bacteria to the polymer surface or through binding to host-derived matrix proteins present in the conditioning film that forms on the medical device surface within seconds following implantation (McCann et al., 2008; Vuong et al., 2002). Bacterial adhesion is a critical step in the development of medical device-associated infections.

3.1.1 Adhesion to abiotic surfaces

The adhesion to an abiotic surface may occur immediately after implantation of a medical device or in *in vitro* systems, although it is not clear whether this mechanism has a high significance in the *in vivo* situation, once the device rapidly becomes coated by host matrix proteins (Vuong et al., 2002). This mechanism is dependent on the physicochemical properties of the device and bacterial surface and is mainly governed

by hydrophobic or electrostatic interactions (Otto, 2013). As the plastic surface of a device is hydrophobic, the main parameter that determines bacterial adhesion is the hydrophobicity of the bacterial cell surface (Vacheethasanee et al., 1998). Specific bacterial surfaces molecules such as the abundant surface protein AtlE, a bifunctional adhesin and autolysin, the staphylococcal surface protein 1 and 2 (SSP-1 and SSP-2), which are organised in fimbria-like structures, and the Bhp (homologue of the Biofilm-Associated Protein, Bap, from *S. aureus*) are also involved in this process (McCann et al., 2008; Otto, 2009). Extracellular DNA, generated through an AtlE mediated lysis of a subpopulation of bacteria, also seems to play a role in the adherence (Qin et al., 2007). It is believed that those molecules contribute to the hydrophobic character of the bacterial cell surface rather than mediate attachment via specific, receptor-mediated interactions (Otto, 2013).

3.1.2 Adhesion to biotic surfaces

The adhesion to biotic surfaces is governed by different and much more specific interactions than the ones mentioned above. After implantation, the surface of the medical device becomes coated with a conditioning film consisting of host human proteins and glycoproteins such as fibronectin, vitronectin, fibrinogen, elastin and collagen as well as coagulation products (platelets and thrombin) (Kleinschmidt et al., 2015; Sabaté Brescó et al., 2017). Some of these host proteins may serve as important receptors for the adhesins expressed on the bacterial cell surface. The staphylococcal surface-anchored proteins, collectively called MSCRAMMs (“microbial surface components recognizing adhesive matrix molecules”), with specificities for a series of different human matrix proteins, play a role in tethering bacteria to host tissues or devices surfaces coated by host matrix proteins (Bowden et al., 2005; Otto, 2012). In *S. epidermidis* it has been identified adhesins for fibrinogen (SdrG, also called “fibrinogen-binding protein” (Fbe)), fibronectin (Embp – “extracellular matrix-binding protein”), vitronectin (AtlE and “autolysin/adhesin” (Aae)), collagen (SdrF/GehD) and elastin (EbpS – “elastin-binding protein”) (Otto, 2012; Sabaté Brescó et al., 2017). Embp is a bifunctional protein that besides the adhesin function it is also involved in the accumulation stage of biofilm formation (Christner et al., 2010), GehD is an extracellular lipase (Bowden et al., 2005) and, AtlE, it is also involved in primary cell wall turnover through the ability to hydrolyse peptidoglycan (Otto, 2012). Additionally, wall teichoic acids (WTAs), that can be found in the cell wall linked to peptidoglycan, enhance the initial adhesion by binding to the adsorbed fibronectin on the medical devices (Hussain et al., 2001).

3.2 Intercellular Aggregation and Maturation

After primary attachment to abiotic or biotic surfaces, the formation of multilayered cell aggregates on top of cells attached to the surface constitutes the second stage of biofilm formation (Vuong et al., 2002). This stage comprises intercellular adhesion, that links bacteria together during proliferation, and formation of mushroom-like structures and channels that are thought to deliver nutrients to all cells in the biofilm and facilitate the removal of metabolic waste (Le et al., 2018; McCann et al., 2008; O'Toole et al., 2000). Cell-cell adhesion is mediated by many different surface macromolecules, such as exopolysaccharide, proteins, teichoic acids and extracellular DNA, that form the extracellular biofilm matrix (Le et al., 2018; Otto, 2009).

The most common molecule associated with biofilm formation in *S. epidermidis* is the polysaccharide intercellular adhesin (PIA), constituted by poly-N-acetyl glucosamine (PNAG), which surrounds and connects bacterial cells (Mack et al., 1996). This polysaccharide, synthesized by proteins encoded in the *icaADBC* operon, was long considered indispensable for biofilm formation, but further studies identified some *S. epidermidis* strains that are capable to form biofilms without harbouring the *ica* locus (Kogan et al., 2006; Rohde et al., 2007). In some strains, the biofilm formation may be partially, or exclusively, mediated by specific surface proteins such as the Bap homologue protein (Bhp) and the Accumulation associated protein (Aap) (Kleinschmidt et al., 2015; Otto, 2009). However, evidence suggested that PIA-dependent biofilms appear to be more structured and robust than those dependent on proteinaceous factors (Rohde et al., 2007). Moreover, teichoic acids and extracellular DNA originating from lysed bacteria, due to its polyanionic character, can interact with other staphylococcal surface polymers, thereby increasing intercellular aggregation and contributing to biofilm maturation (Otto, 2009; Otto, 2013).

3.3 Detachment

Disruptive processes are required not only for the formation of channels but also for the detachment of cells clusters from the biofilm which are essential to control biofilm thickness (O'Toole et al., 2000; Otto, 2012; Otto, 2013). This last stage of biofilm development has important clinical implications, since the detached cells spread to other sites, via the bloodstream, establishing additional infections that can be more severe,

such as endocarditis, but also may cause acute infections like bacteraemia (McCann et al., 2008; Otto, 2013).

In *S. epidermidis*, the quorum sensing system agr (accessory gene regulator), a regulatory system of gene expression that responds to cell density through cell-cell communication, have been implicated in biofilm structuring and in the detachment process. The evidence comes from the observations that agr expression in biofilms is limited to the most exposed layers, where the regulation of the detachment occur, and strains that are dysfunctional in the agr system, or agr-negative, exhibit thicker biofilms and a reduction in dispersal (Vuong et al., 2004a). The mechanisms underlying the disintegration of biofilms in *S. epidermidis* are still poorly understood, however, enzymatic degradation of biofilm exopolymers and disruption of non-covalent interactions by detergent-like molecules seem to be involved (Otto, 2009). Phenol-soluble modulins (PSMs), which are secreted in a quorum-sensing controlled fashion, are peptides with surfactant properties (Otto, 2013) that are shown to lower the surface tension at the biofilm interface and lead to the separation of bacterial cells from the biofilm (McCann et al., 2008). Moreover, this reduction in cell-to-cell bonding facilitates the formation of channels in a mature biofilm (Kleinschmidt et al., 2015). The fact that *S. epidermidis* exoproteases and PSMs are strictly regulated by agr system support the idea about their involvement in these processes (Otto, 2009).

4. Interaction between *S. epidermidis* and the immune system

Research efforts have been mainly focused in understanding *S. epidermidis* in-host evolution, however, how the host responds to the bacterium remains elusive. Although a limited set of defence mechanisms, such as the production of antimicrobial peptides (AMPs), are present on the skin, bacteria that penetrate the epithelial barrier encounter more and stronger host defence mechanisms (Otto, 2009; Otto, 2013). Thus, bacteria must evade the host immune system in order to survive in the human body and the *S. epidermidis* approach consist of a series of mechanisms that are in general of a more “passive” nature (Otto, 2012). *S. epidermidis* is well equipped with factors that promote its persistence rather than those that actively attack the host, such as the production of aggressive toxins, demonstrating the low virulence potential of this bacterium (Otto, 2009).

4.1 Immune Response to *S. epidermidis* as a commensal

In healthy individuals, the interaction between *S. epidermidis* as a commensal microorganism with the host immune system is believed to play a role in the induction of immunological tolerance (Sabaté Brescó et al., 2017), as demonstrated recently in murine models with the topical application of this bacterium (Naik et al., 2015; Scharschmidt et al., 2015). Also, the role of this bacterium in the prevention of pathogen overgrowth on the skin has been attributed to the stimulation of the immune system (Nguyen et al., 2017). However, it remains to be proven whether these findings are applicable to humans.

Scharschmidt et al. reported that the skin colonization with *S. epidermidis,* during a defined period of neonatal life, established antigen-specific tolerance to the bacterium by triggering a local, as well as systemic, specific $CD4^+$T cell response, as evidenced by the enrichment of specific $CD4^+$ T regulatory (Treg) cells in both the skin-draining lymph nodes and the spleen (Scharschmidt et al., 2015). On the other hand, Naik et al. demonstrated that the application of *S. epidermidis* on the skin induces $CD8^+$T cells expressing interleukin (IL)-17A, which resulted from the action of skin-resident dendritic cells subsets and was not associated with inflammation (Naik et al., 2015). In an epicutaneous model of *Candida albicans* infection, it was shown that IL-$17A^+CD8^+$T cells provided immunity against cutaneous fungal infection (Naik et al., 2015). This study suggested that the immune system can be modulated by resident bacteria in the skin, since the generated adaptive immune response may help to promote protective innate immune responses and can contribute to controlling inflammation (Sabaté Brescó et al., 2017). In another study, *S. epidermidis* lipoteichoic acids (LTA) have been shown to reduce skin inflammation through a mechanism involving Toll-like receptor (TLR) 2 (Lai et al., 2009). However, this observation must be clarified, since the purity of LTA is extremely difficult to achieve and LTA preparations, even those commercially available, usually have TLR2-stimulating lipopeptide contaminants (Nguyen et al., 2017). Overall, these findings reveal the capacity of the commensal *S. epidermidis* to specifically shape cutaneous immunity and, although the experimental data of Scharschmidt et al. and Naik et al. studies were quite different, showed that the colonization with this bacterium induces an adaptive T cell response in mice, with consequent decrease infection burden (Nguyen et al., 2017; Sabaté Brescó et al., 2017). However, such immune signature remains to be proven in humans.

4.2 Immune Response to *S. epidermidis* during infection

Staphylococcus can cause many types of infections, ranging from acute diseases, such as skin infections and sepsis, to chronic diseases, such as implant-associated infections. An important immune-evasion mechanism employed by *S. epidermidis* is its ability to form biofilms on host tissues and implanted medical devices, that protect bacteria from the host immune system. The most serious complication of biofilm-originated infections is the dispersion of biofilm cells that can culminate in sepsis (de Vor et al., 2020).

Many clinical studies conducted on biofilm-associated infections are *in vitro* studies that have focused on the comparison between planktonic and biofilm growing bacteria. In general, *S. epidermidis* seems to induce and maintain a low inflammatory infection profile, which may contribute to the persistence of the infection and prevent the eradication by the host immune system (Le et al., 2018). Since the host immune response is not protective or enough to clear the infection, most *S. epidermidis* biofilm-associated infections have a chronic and indolent course (Nguyen et al., 2017). Furthermore, this low inflammatory infection profile makes diagnosis difficult in the early stages of infection and, thus, when *S. epidermidis* biofilm infections are diagnosed, often when there is a mature biofilm, the sequelae are more severe and complicated to treat (Le et al., 2018). An overview of the host immune response to *S. epidermidis* is illustrated in Fig. 2.

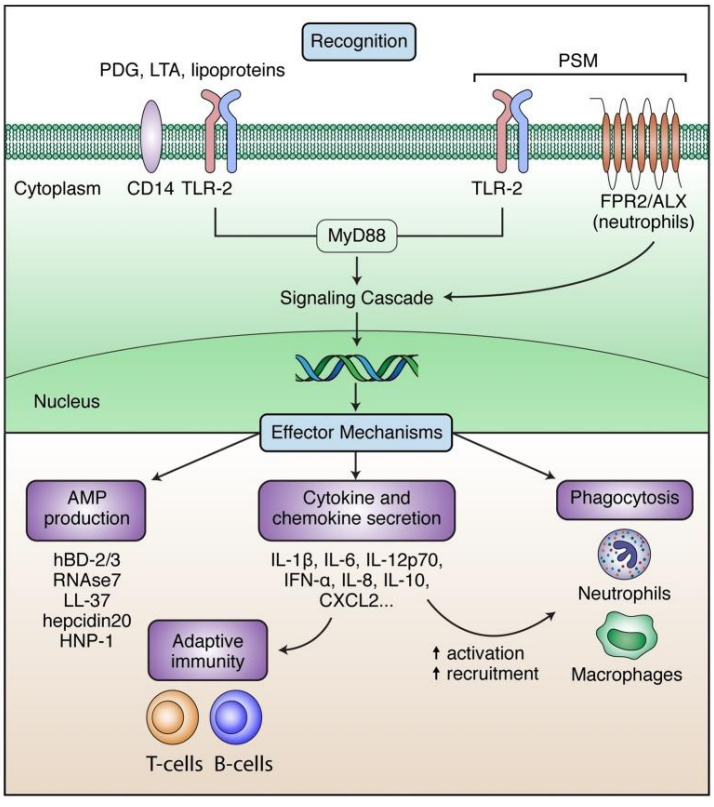


Fig. 2 – The host immune response against *S. epidermidis* infection. Recognition of *S. epidermidis* via TLR-2, which heterodimerize with TLR-1 and TLR-6, or by other receptors such as FPR2/ALX activate the signalling cascade that triggers various effectors mechanisms: production of AMPs, phagocytosis by neutrophils and macrophages and secretion of cytokine and chemokine play a role in various processes of the innate and adaptive immune response. AMP, antimicrobial peptide; LTA, lipoteichoic acid; hBD, human-beta defensin; HNP, human neutrophil peptide; PDG, peptidoglycan; PSM, phenol-soluble modulin; TLR, toll-like receptor. (Sabaté Brescó et al., 2017)

Recognition is the first step in the development of the innate immune response and results in the activation of several signalling pathways that ultimately culminate in the expression of many molecules required for the response. Mediators of innate immunity can also stimulate the adaptive immune response and influence the type of adaptive immune response that develops (Abbas et al., 2014).

4.2.1 Innate Immune Response

Early recognition of bacteria by the innate immune system is fundamental for the success of bacterial clearance. Immune and tissue cells express pattern-recognition receptors (PRRs) that recognize evolutionary conserved microbial structures termed “pathogen-associated molecular patterns” (PAMPs). The toll-like receptors (TLRs) are one of the most largely studied classes of PRRs that recognize products of a wide variety of microorganisms (Abbas et al., 2014). There is evidence suggesting that TLR2 mediates host defence against *S. epidermidis*. TLR2 is found on the cell membrane, where it can recognize various PAMPs in the extracellular environment. TLR2 can heterodimerize with TLR1 and TLR6, extending the repertoire of specificities of the TLR system (Abbas et al., 2014). TLR2 can recognize ligands of the bacterial cell wall, such as lipoproteins, peptidoglycan (PDG) and LTA, although some of its putative ligands are still a matter of controversy (Fournier, 2013). Secreted components, such as *S. epidermidis* PSM, can also activate the immune system, via recognition by TLR2/TLR6 heterodimers (Hajjar et al., 2001). In *S. epidermidis*, the recognition via TLR2 has been demonstrated in endothelial cells, keratinocytes or human fibroblast (Sabaté Brescó et al., 2017), and human embryonic kidney (HEK) cells transfected with TLR2 (Strunk et al., 2010). Furthermore, an up-regulation in the transcription of TLR2 and the TLR adaptor molecule, MyD88, was observed in response to *S. epidermidis* bacteraemia, using a neonatal model of intravenous infections in mice (Kronforst et al., 2012). In addition, the use of TLR2-deficient (*TLR2*$^{-/-}$) mice showed that TLR2 signalling accelerates the clearance of bacteraemia (Cole et al., 2016; Strunk et al., 2010), although *TLR2*$^{-/-}$ mice could still resolve a bloodstream infection, suggesting that the response to *S. epidermidis* can occur via TLR2-independent pathways (Cole et al., 2016). Accordingly, additional receptors pathways may be engaged, such as NOD-like receptors that recognize *S. epidermidis*-derived PDG and the formyl peptide receptor 2 (FPR2/ALX) expressed in neutrophils which can sense PSMs, even if their contribution has not yet been proved *in vivo* (Sabaté Brescó et al., 2017).

Antimicrobial peptides (AMPs), more recently known as host defence peptides, are a heterogeneous group of amphipathic peptides, generally positively charged, that have broad-spectrum activity. AMPs are expressed by many innate immune cells, such as neutrophils and macrophages and, in this way, AMPs are one of the first molecules that invading microorganisms encounter. Their mechanisms of action include the direct killing of microbial pathogens and immune modulation. The antibacterial activity of AMPs can be divided into membrane targeting, through pore formation on the bacterial membrane that can occur following the interaction between the cationic AMP and the negatively charged bacterial cell wall components such as teichoic acids, or by interfering with essential cellular processes such as DNA, protein and cell wall synthesis, after translocation into the cytoplasm. AMPs can also act by modulating the host defences, through the recruitment and activation of immune cells, indirectly promoting pathogen clearance and/or control of inflammation (Kumar et al., 2018). The production of AMPs is crucial to innate defence on the human skin. *In vitro* studies have shown that *S. epidermidis,* or its culture supernatants, increased the expression of β-defensin 2 (hBD-2) and hBD-3 but not hBD-1 in human keratinocytes (Lai et al., 2010; Ommori et al., 2013). This could function in a beneficial way enhancing antimicrobial defence against invading pathogens, but can also contribute to the defence against *S. epidermidis* superficial infections since it has been shown that β-defensin 2 and 3, *in vitro*, have activity against *S. epidermidis* (Huang et al., 2007). In the context of IMD-associated infections, many immune cells, such as neutrophils and macrophages, produce AMPs that are commonly located in the phagolysosomes, where they contribute to the killing of the invading bacteria (Sabaté Brescó et al., 2017). Although the mechanism of action is still unknown, some AMPs, namely hBD-3 (Zhu et al., 2013) and human liver-derived AMP hepcidin 20 (Hep20) (Brancatisano et al., 2014) have been shown to interfere with *S. epidermidis* attachment and/or biofilm formation *in vitro*.

Cells that have specialized phagocytic functions are the first line of defence against microorganisms when the epithelial barrier is breached. Neutrophils, also known as polymorphonuclear (PMN) leukocytes, and macrophages are the primary effector cells in the innate immune system. These cells are recruited into sites of infections, following host signals (chemokines, AMPs) or by sensing bacterial components, and have receptors that directly bind to bacteria (PRRs) or high-affinity receptors for certain opsonins (antibodies, complement proteins) that are coating the bacterial cell wall. Bacteria are internalized into phagocytic vacuoles (phagosomes), which fuse with lysosomes to form phagolysosomes and where most of the microbicidal mechanisms are

concentrated. Bacteria are destroyed intracellularly by the action of reactive oxygen species (ROS), as well as non-oxygen-dependent processes such as proteolytic enzymes and AMPs (Abbas et al., 2014). It is generally accepted that the biofilm mode of growth protects *S. epidermidis* from phagocytosis by effector cells, although conflicting findings have been reported in the literature. A study carried out by Spiliopoulou *et al.* reported a significant increase of phagocytosis by monocyte-derived macrophages when stimulated with biofilm-grown bacteria compared with planktonic cells (Spiliopoulou et al., 2012), while studies by others have shown an attenuation in the phagocytosis by human PMNs (Vuong et al., 2004b) and murine macrophages (Schommer et al., 2011), which was linked to the immune evasion effect of biofilm exopolysaccharide (PNAG) and biofilm formation *per se*. On the other hand, there is a consensus about the effects of *S. epidermidis* biofilm on phagocytic killing. Phagocytic killing is strongly attenuated in human macrophages (Spiliopoulou et al., 2012) and PMNs (Kristian et al., 2008; Vuong et al., 2004b) since the biofilm-derived *S. epidermidis* cells are capable to survive more efficiently within these effector cells than planktonic bacteria or *ica*-negative mutant strain. In addition, when a rabbit anti-PNAG antibody was used as an opsonin, the cells within biofilms were more resistant than their planktonic counterparts. This observation was associated with a large amount of antigen within the biofilm matrix that prevent antibody binding close to the bacterial cell surface, where it is necessary to mediate efficient opsonic killing (Cerca et al., 2006). When comparing the phagocytosis of *S. epidermidis* and *S. aureus* biofilms, it appears that the latter were more infiltrated and phagocytosed, although this not always correlated with higher phagocytic killing, since the bacteria have several mechanisms to prevent destruction by phagocytes and to persist in the intracellular environment (Moldovan and Fraunholz, 2019). In *S. epidermidis*, although it apparently does not have such mechanisms, some strains are killed less efficiently. This may be related to the low ability to induce an oxidative response of neutrophils, or simply by their biofilm mode of growth (Sabaté Brescó et al., 2017). Together, these reports can, in part, explain the chronic character often associated with *S. epidermidis* infections.

The activation of phagocytes might involve the action of the complement system. The complement system consists of several plasma proteins that can opsonize microbes, promote the recruitment of phagocytes to the site of infection, and directly kill microbes by the assemble of membrane attack complex (MAC). The activation of the complement system occurs in three ways: 1) the classical pathway, that is dependent on antibody-recognition, 2) the alternative pathway, that is triggered by direct recognition of microbes,

and 3) the lectin pathway that is initiated by the interaction between mannose-binding lectin or ficolins and microbial glycoproteins and glycolipids (Reis et al., 2019). There is evidence that biofilm-producing *S. epidermidis* strains elicit a more robust activation and release of complement components than planktonic bacteria or PIA-negative isogenic mutants, as measured by C3a formation (Aarag Fredheim et al., 2011; Kristian et al., 2008). However, such complement release did not translate to the higher killing of *S. epidermidis* biofilm cells by PMNs. In fact, the biofilm formation protects *S. epidermidis* from IgG and C3b opsonization since the deposition of these molecules was paradoxically diminished on *S. epidermidis* biofilm surfaces, which might contribute to the evasion of PMN mediated killing (Kristian et al., 2008). This discrepancy between C3a induction and C3b deposition substantiates the early observation that *S. epidermidis* cells embedded in a biofilm are protected from PMN killing when rabbit anti-PNAG-specific antibody was used as an opsonin (Cerca et al., 2006; Kristian et al., 2008). These findings show that biofilm formation and/or extracellular material, namely PIA, may be important for *S. epidermidis* immune evasion (Kristian et al., 2008) and implicate the opsonic deposition as one aspect of immune response that can be modulated in *S. epidermidis* biofilm-associated infections (Le et al., 2018).

The cells of the innate immune system interact with one another and with other host cells during innate and adaptive immune responses. Many of these interactions are mediated by cytokines. Cytokines are a heterogeneous group of secreted proteins produced by different cell populations that mediate and regulate all aspects of immunity (Abbas et al., 2014). *In vitro* studies have reported the release of the pro-inflammatory cytokines IL-6 and tumor necrosis factor (TNF)-α by monocyte-derived dendritic cells stimulated with commensal strains of *S. epidermidis* and *S. aureus*, although the stimulation with commensal *S. epidermidis* strains elicited a more anti-inflammatory profile, with high levels of IL-10 (Laborel-Préneron et al., 2015). Accordingly, these observations were verified using *in vivo* models, where high levels of the cytokines IL-6 and IL-10 were detected in the serum and peritoneal exudates at the first hours pos-challenge with *S. epidermidis* (Ferreirinha et al., 2016). The importance of the regulatory cytokine IL-10 was evaluated in a study using a mouse model of catheter-associated *S. epidermidis* biofilm infection. The results obtained suggested that IL-10 may be important to regulate the inflammatory response in this model but do not have an impact in bacterial clearance, since the IL-10 KO mice showed an increased level of pro-inflammatory cytokines as well as an increase in weight loss, although the bacterial accumulation in the catheter was similar in both IL-10 KO and WT mice (Gutierrez-Murgas et al., 2016). When

compared with planktonic bacteria, biofilm bacteria induced less amounts of the pro-inflammatory cytokines TNF-α, IL-1β, IL-6 as well as IL-12 by monocyte-derived macrophages (Spiliopoulou et al., 2012). In the same study, peripheral blood mononuclear cells (PBMCs) produced lower amounts of TNF-α, IL-12, IFN-γ, IL-6 and IL-1β and elevated levels of the cytokine IL-13 when stimulated with biofilm bacteria than when stimulated with planktonically grown bacteria (Spiliopoulou et al., 2012). Furthermore, *S. epidermidis* biofilm-positive strains, when compared with isogenic biofilm negative strains, induced a reduced inflammatory response in macrophages with significantly diminished NF-kB activation and IL-1β production (Schommer et al., 2011). Therefore, it can be hypothesized that the low levels of pro-inflammatory cytokines secretion triggered by *S. epidermidis*, together with high levels of IL-10, may contribute to the sub-acute nature and persistence of *S. epidermidis* biofilm-related infections (Sabaté Brescó et al., 2017).

Other important messengers for the recruitment and migration of circulating leukocytes from the blood to extravascular sites are chemokines, a large family of structurally homologous cytokines (Abbas et al., 2014). The CXCL8 (IL-8) is the major chemokine supporting neutrophil migration into tissues (Abbas et al., 2014) and their secretion has been described post-challenge with *S. epidermidis* (Ivarsson et al., 2013; Spiliopoulou et al., 2012) or their surface components (Stevens et al., 2009). Neutrophil-chemokine attractants CXCL-1 and CXCL-2 were also found increased in animals infected with *S. epidermidis* (Ferreirinha et al., 2016; Gutierrez-Murgas et al., 2016).

4.2.2 Adaptive Immune Response

Protective immunity against microbes is also mediated by later responses of adaptive immunity. The adaptive immune response can be divided into T cell-mediated cellular immunity and humoral immunity, which is mediated by antibodies produced by B lymphocytes. The knowledge about the adaptive immune response in biofilm-associated infections is limited, in part, because of the difficulty to develop models of long-term *S. epidermidis* biofilm infections (Nguyen et al., 2017). Vuong et al., using an *in vivo* model, founds that immunocompromised mouse (T cell-deficient or lacking both T and B lymphocytes) were more susceptible to *S. epidermidis* biofilm-associated infection than immuno-competent mice, indicating an important role for adaptive immunity in this type of infection and supporting the clinical evidence that patients with a dysfunctional immunological system are more susceptible to device-related *S. epidermidis* infection (Vuong et al., 2008).

The antigen-presenting cells (APCs), primarily dendritic cells, are specialized in display microbial antigens for recognition by lymphocytes and provide signals that promote the proliferation and differentiation of lymphocytes (Abbas et al., 2014). The lifelong interactions of *S. epidermidis* as a commensal with humans is expected to elicit adaptive immune responses. As mentioned previously, the action of skin resident dendritic cells subsets was responsible for orchestrating T cell responses in a mouse model that enhanced protection against pathogen invasion (Naik et al., 2015). Upon infection, it can be anticipated that the tissue-resident DCs or that migrate there, once activated by the encounter with a microbe, will shape adaptive immune responses (Sabaté Brescó et al., 2017). The activation of DCs stimulated with *S. epidermidis* has been reported in *in vitro* studies, as evidenced by the upregulation of maturation markers CD83, HLA-DR and the most pronounced CD86, on their surface (Balraadjsing et al., 2020). The cytokine secretion by DCs stimulated with *S. epidermidis* is poorly described and studies have generated inconsistent results, possibly due to different sources of DCs that activated distinct pathways. Thus, further investigations are required to clarify the role of different DCs subsets on T cell polarization (Sabaté Brescó et al., 2017).

Humoral immunity is mediated by antibodies, that can recognize microbial antigens and target microbes for elimination by effector mechanisms. The identification of immunogenic proteins is an important step for advancing in the development of therapeutic and prophylactic measures. As an example, the immunization of mice with proposed immunogenic proteins AtlE, GehD lipase and staphylococcal conserved antigen B (ScaB) elicited the production of specific antibodies that are capable to opsonized *S. epidermidis in vitro* (Pourmand et al., 2006). In another study, immunized animals with staphylococcal major amidase (Atl-AM) showed higher antibody titers and, when challenged with a lethal dose of *S. epidermidis*, have a better rate of surviving and lower bacterial load compared to mock-immunized animals (Sabaté Brescó et al., 2017). Furthermore, an *in vitro* study performed using antibodies against PNAG, since this molecule is the principal component that mediated biofilm accumulation, showed the efficacy of antibodies in interfering with biofilm formation, although this observation is dependent on the *S. epidermidis* strain used and has not yet been tested *in vivo* (França et al., 2013).

Cell-mediated immunity promotes the destruction of microbes within phagocytes or the killing of cells infected by microbes, where they are inaccessible to circulating antibodies and phagocytic destruction, in order to eliminate reservoirs of infection (Abbas et al.,

2014). Although T cell-mediated immune responses to *S. epidermidis* are poorly characterized, considering the cytokine profile induced by *S. epidermidis*, it may be expected a Th1/Th17 polarization in their infections (Sabaté Brescó et al., 2017). An *in vitro* study showed that *S. epidermidis* is recognized by human serum IgG and the activation of human monocyte-derived dendritic cells by bacteria opsonized enhanced Th17 responses (Den Dunnen et al., 2012). Interferon (IFN)-γ, which is a Th1-type cytokine, reduced the susceptibility to *S. epidermidis* infection when subcutaneous injections of this cytokine were administrated to an *in vivo* model of biomaterial-associated infection (Boelens et al., 2000). In concordance, mice challenged with PNAG-positive *S. epidermidis* strain presented higher proportions of splenic IFN-γ-producing $CD4^+$ T cells and IL-$17A^+$ $CD4^+$ T cells (Ferreirinha et al., 2016).

5. *S. epidermidis* Cell Wall

The cell wall plays an important role in bacterial physiology but is also involved in pathogenesis and host immune response. A schematic representation of the *S. epidermidis* cell surface is illustrated in Fig. 3. Gram-positive bacteria are surrounded by a complex cell envelope with thick layers of peptidoglycan, which consists of linear glycan chains cross-linked by covalent bonds between short attached peptides (Brown et al., 2013). The peptidoglycan polymer provides structure to the cell, which stabilizes the cell membrane, and serves as a scaffold to which proteins and other glycopolymers can be anchored (Swoboda et al., 2010).

Teichoic acids (TA) are a major class of cell surface glycopolymers found in a wide range of Gram-positive bacteria (Swoboda et al., 2010). These anionic polymers are structurally diverse and contain phosphodiester-linked polyol repeat units (Brown et al., 2013). Due to their abundance, they contribute to the high density of negative charge in the cell wall (Swoboda et al., 2010). There are two types of teichoic acids, the lipoteichoic acids (LTAs) and the wall teichoic acids (WTAs). The first is anchored to the bacterial membrane via a glycolipid intercalated in the membrane and extend into the peptidoglycan layer, while the WTAs are covalently attached to peptidoglycan via a linkage unit and extend outwardly (Swoboda et al., 2010). They are implicated in cation homeostasis, regulation of autolytic enzymes, biofilm development, anchoring of cell surface proteins, cell division and protection against antimicrobial peptides (Brown et al., 2013).

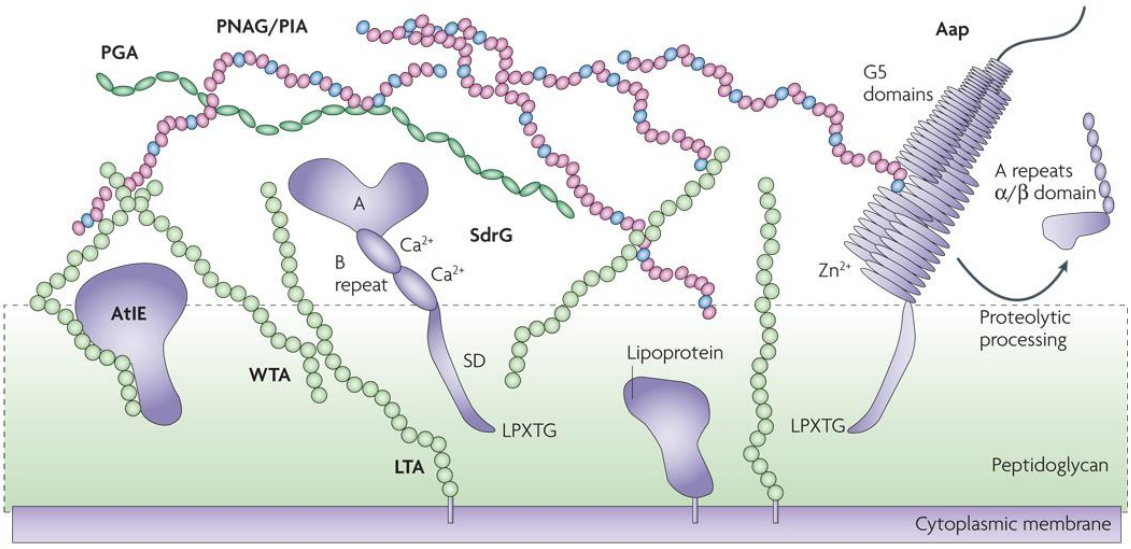


Fig. 3 – The cell surface of *S. epidermidis*. *S. epidermidis* contain a cell wall consisting of a thick peptidoglycan layer with anchored proteins and polysaccharides. Teichoic acids are classified in WTA, covalently attached to peptidoglycan, and LTA, anchored to the cytoplasmic membrane. Surface proteins can be covalently attached to peptidoglycan, such as SdrG and Aap, or through non-covalent interactions with surface polymers, such as AtlE. The PNAG and PGA, protective exopolymers produced by *S. epidermidis*, surround the bacterial surface and can interact with others cell surface components. All these constituents of the cell wall are involved in biofilm formation. (Otto, 2009)

Surface proteins can be covalently bound to the *S. epidermidis* surface through sortase-catalysed anchoring. These proteins, such as SdrG and Aap, harbour a characteristic LPXTG amino acid motif at the C-terminal, whose threonine residue is attached to the peptidoglycan (Otto, 2009). SdrG, an example of the Sdr protein family, is composed by a serine/aspartate (SD) repeat region, B repeats that harbour a Ca^{2+} binding EF-hand domain and A region which binds fibrinogen (Otto, 2009). The Aap protein aggregates through ZN^{2+}-dependent G5 domains, forming fibril-like structures on the bacterial surface, and require proteolytic processing for its activation (Yarawsky et al., 2020). The same domains are known to bind with N-acetylglucosamine and can, therefore, potentially interact with PNAG (Otto, 2009). Many autolysins can be anchored to the bacterial surface through non-covalent interactions, probably with polymers such as teichoic acids (Otto, 2009). AtlE, a bifunctional adhesin and autolysin, contribute to the hydrophobic character of the bacterial surface and can bind matrix proteins (vitronectin) (Otto, 2009, 2012).

All enumerated proteins are implicated in biofilm formation. AtlE promotes bacterial adhesion to abiotic surfaces since this process is mainly governed by cell surface hydrophobicity (Otto, 2013). Furthermore, AtlE, as well as serine/aspartate repeat family proteins such as SdrG and SdrF (a collagen-binding protein), due to their potential to interact with matrix proteins, promote adhesion to biotic surfaces (Otto, 2012). The Aap

protein form fibrils that are thought to connect cells in the biofilm matrix, promoting intercellular aggregation, and can also bind to PNAG, forming a biofilm network consisting of protein and polysaccharide (Otto, 2009).

S. epidermidis produces protective exopolymers, such as PGA and PNAG. The polymer PGA is thought to contribute to osmotolerance, promoting the bacterial growth at high salt concentrations, and is involved in *S. epidermidis* resistance to neutrophil phagocytosis and AMPs (Otto, 2009). PNAG, also named PIA, is a cationic β1-6 linked N-acetylglucosamine homopolymer, that surrounds and connects bacterial cells in a biofilm and has been found to protect *S. epidermidis* from immunoglobulins and complement deposition, AMPs and phagocytosis (Kristian et al., 2008; Vuong et al., 2004b). This polymer probably interacts with PGA and teichoic acids, which are negatively charged (Otto, 2009).

6. Wall Teichoic Acids

Wall teichoic acids (WTAs) are the most abundant glycopolymers attached to the peptidoglycan in many gram-positive bacteria, constituting up to 60% of the total cell wall mass (Brown et al., 2013; Swoboda et al., 2010). The chemical structure of WTA is composed by a disaccharide linkage unit, which is highly conserved across bacterial species, and the main chain of phosphodiester-linked polyol repeat units that confer structural diversity to the polymer (Brown et al., 2013). Although their composition varies among organisms, all WTAs are characterized by a negatively charged anionic backbone that is covalently attached to peptidoglycan via a phosphodiester bond to the C6 hydroxyl of the *N*-acetyl muramic acid (Brown et al., 2013; Swoboda et al., 2010). The most usual structures are composed by a long chain of either glycerol phosphate (Gro-P) or ribitol phosphate (Rbo-P) repeating units (Fig. 4) (Swoboda et al., 2010). These units can be tailored with monosaccharides, commonly glucose or N-acetylglucosamine, or by D-alanylation, a tailoring modification that introduces cationic D-alanine residues that may alter the net charge of the polymer (Brown et al., 2013). In *S. epidermidis*, the structure of the WTA was found to be a poly(glycerol phosphate) (Archibald et al., 1968; Sadovskaya et al., 2004).

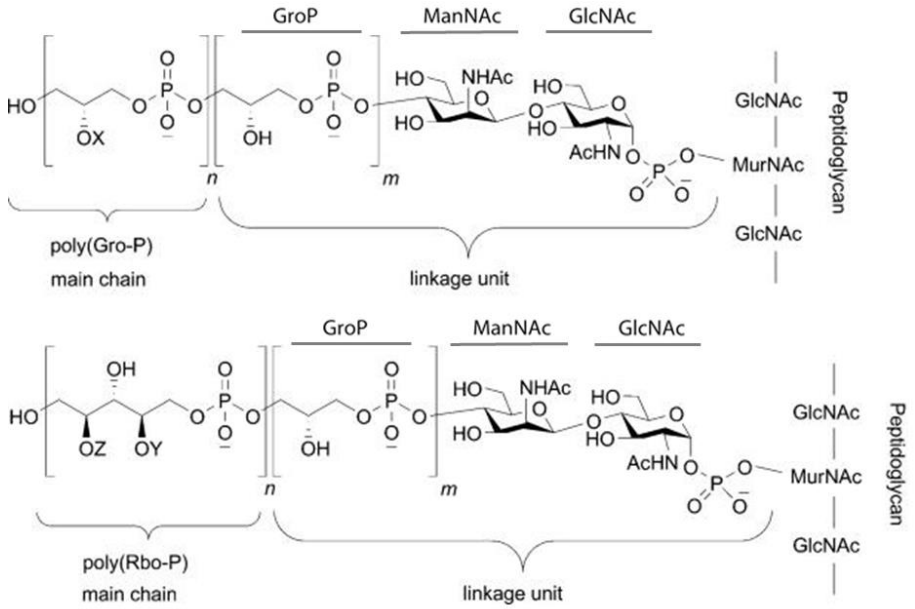


Fig. 4 – Representative chemical structures of the WTA polymer. The polymer consists of a common linkage unit and the main chain of phosphodiester-linked polyol repeats, and it is covalently attached to peptidoglycan via a phosphodiester linkage between the phosphate of the linkage unit and the MurNAc. The most common structures are composed of a ManNAc(β1→4)GlcNAc-1P with glycerol phosphates attached to the ManNAc followed by repeat units of Gro-P or Rbo-P that extended from the Gro-P end of the linkage unit. GlcNAc, *N*-acetylglucosamine; Gro-P, glycerol-phosphate; ManNAc, *N*-acetylmannosamine; MurNAc, *N*-acetylmuramic acid; Rbo-P, ribitol-phosphate. (Swoboda et al., 2010))

The WTA biosynthesis was first characterized in *Bacillus subtilis* 168 which contain WTA composed of Gro-P repeating units (Swoboda et al., 2010). The genes encoding proteins involved in the synthesis of these WTAs are annotated as *tag* genes (**t**eichoic **a**cid **g**lycerol) (Brown et al., 2013; Swoboda et al., 2010). The synthesis starts in the cytoplasm and TagO catalyses the first synthetic step with the transfer of GlcNAc-1-P to an undecaprenyl phosphate carrier lipid anchored in the bacterial membrane (Brown et al., 2013; Swoboda et al., 2010). The transfer of ManNAc from UDP-ManNAc to the C4 hydroxyl of the GlcNAc catalyzed by TagA follows to form a β-linked disaccharide (ManNAc(β1→4)GlcNAc) (Brown et al., 2013). TagB, a glycerophosphate transferase, transfers a single GroP unit from CDP-glycerol to the C4 hydroxyl of ManNAc, to complete the synthesis of the WTA linkage unit (Swoboda et al., 2010). In the case of the biosynthesis of WTA composed of Rbo-P polymers, the enzymes involved are designated as TarO, TarA and TarB for **t**eichoic **a**cid **r**ibitol and have the same biochemical functions of the Tag enzymes (Brown et al., 2013). These first steps in the pathway for WTAs biosynthesis (Fig. 5) are highly conserved across all strains characterized so far (*B. subtilis* 168, *B. subtilis* W23 and *S. aureus*), but after these steps, the pathways diverge (Brown et al., 2013).

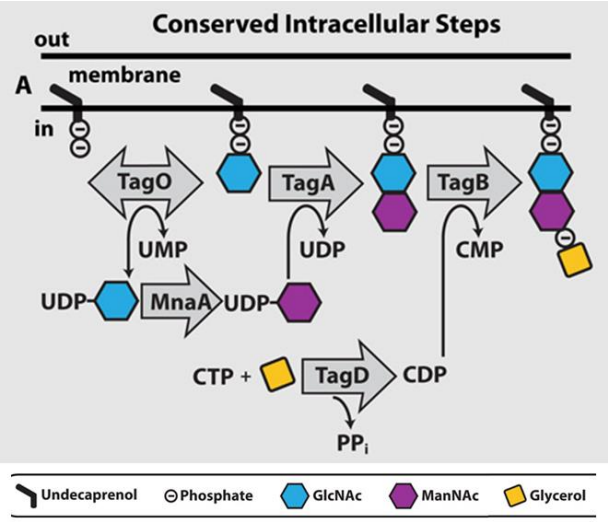


Fig. 5 – The initial steps of WTA biosynthesis. The synthesis of WTA linkage unit is catalysed in three steps by TagO, TagA and TagB enzymes, and these steps have been found in all pathways characterized so far. TagD, a cytidylyltransferase, catalyses the transfer of GroP to CTP, originating CDP-glycerol. CDP/CTP/CMP, cytidine di/tri/monophosphate; GlcNAc, *N*-acetylglucosamine; ManNAc, *N*-acetylmannosamine; PPi, pyrophosphate. (Brown et al., 2013)

Succinctly, once the synthesis of the linkage unit is complete, GroP or RboP units are added to assemble the main chain. In *B. subtilis* 168 this step is carried out by TagF, a polymerizing cytidylyl transferase, that attaches GroP units to the linkage unit (Swoboda et al., 2010). The assembly of the main chain in *B. subtilis* W23 requires two enzymes that are suggested to function as a primase/polymerase pair: TarK, which transfers a single RboP to the linkage unit, and TarL which is responsible for the polymerization of the poly(ribitol phosphate) chain (Swoboda et al., 2010). In *S. aureus* TarF mediates the transfer of a single unit of GroP to the linkage unit and the synthesis of poly(ribitol phosphate) continues by a single enzyme, TarL, which is a bifunctional primase/polymerase (Brown et al., 2013). After assembly, the lipid-linked WTA polymer is modified inside the cell by a glycosyltransferase and then translocated through the bacterial membrane by a two-component ABC transporter (TagGH or TarGH) (Brown et al., 2013; Swoboda et al., 2010). Outside the cell, the WTA polymer is coupled to peptidoglycan by an unidentified transferase and is modified by the attachment of the D-alanine esters (Swoboda et al., 2010).

Considering their location, abundance and polyanionic nature, the WTAs polymers play varied functions in bacterial physiology. Due to the dense network of negative charges that WTAs form on the gram-positive cell surface, they are implicated in the regulation of cation homeostasis (Swoboda et al., 2010). These polymers can bind cationic groups, including extracellular metal cations, that are thought to minimize the repulsive interactions between nearby phosphate groups, which in turn influence the cell wall

integrity (Swoboda et al., 2010). Furthermore, WTAs provide a reservoir of ions close to the cell surface that can indirectly modulate the activity of some enzymes (pH-sensitive cell-wall associated enzymes such as autolysins) (Biswas et al., 2012) and could help to mitigate the fluctuations in osmotic pressure between the inside and outside of the cell (Swoboda et al., 2010). D-alanylation can modulate the capacity of WTAs for cations by masking the negative charges of the polymer (Brown et al., 2013). In addition to providing binding sites for cations, WTAs are necessary for proper localization and/or activity of cell wall hydrolases (autolysins), since the strains lacking WTAs exhibit defects in septal positioning and number, failure to effectively separate during cell division and have increased autolysis rates (Biswas et al., 2012; Brown et al., 2013; Schlag et al., 2010). These strains also present morphological abnormalities, such as increased cell size and a nonuniform thickening of the peptidoglycan cell wall (Brown et al., 2013).

WTAs and their tailoring modifications affect the cell surface characteristics (charge and hydrophobicity) playing a role in protecting bacterial from adverse conditions. These polymers are involved in temperature tolerance, osmotic stress (Brown et al., 2013; Vergara-Irigaray et al., 2008), and protection against human antibacterial fatty acids (Kohler et al., 2009). The modification of WTAs by D-alanylation also modulates interactions between the cell surface and the environment. The absence of D-alanyl esters or the reduction in their content on the teichoic acids increases susceptibility to cationic antimicrobial peptides. Presumably, the absence of D-alanyl esters increases the negative charge density on the cell surface attracting these positively charged molecules (Kristian et al., 2003; Peschel et al., 1999). A reduction in the alanine residues on the cell wall also results in increased sensitivity of bacteria to glycopeptides antibiotics and to lytic activity of enzymes that are produced by neutrophils in the context of infection (Swoboda et al., 2010).

As major components of the cell surface, WTAs influence bacterial interactions with various surfaces, having effects on adhesion and colonization. WTA-deficient cells show a reduced initial adherence to artificial surfaces and impaired capability to form biofilms (Gross et al., 2001; Holland et al., 2011; Vergara-Irigaray et al., 2008). Removal of D-alanine esters has the same effects, potentially due to the increased repulsive forces between bacteria and the surface (Brown et al., 2013). The lack of WTAs or D-alanine esters also impaired the adhesion to host tissue, which attenuates the bacteria' ability to colonize and infect the host (Brown et al., 2013). Since the WTAs polymers have important roles in physiology and are required for host colonization and infection, they

have been categorized as virulence factors (Brown et al., 2013; Swoboda et al., 2010). Therefore, the WTA pathway, or in other words, the enzymes involved in the biosynthesis of these polymers, is a possible target for novel antimicrobial drugs and vaccines (Swoboda et al., 2010).

7. Objectives of the present work

The overall aim of the present work is to study the role of WTA in *S. epidermidis* virulence and capacity to survive and to establish infection in the host, using a WTA-deficient strain. The following specific objectives were outlined:

i) to analyze the phenotype and bacterial growth of *S. epidermidis* strains;

ii) to characterize the *in vitro* interaction of *S. epidermidis* strains with host innate immune cells, such as macrophages and dendritic cells

.

CHAPTER 2: MATERIALS AND METHODS

1. Bacterial strains

S. epidermidis strains used in this study are listed in Table 1. All strains were maintained in 30% glycerol at -80ºC. Bacterial strains were propagated in either Tryptic Soy Broth (TSB) or Tryptic Soy Agar (TSA) (BD Difco™, USA), prepared according to the manufacturer's instructions, and in the presence of chloramphenicol (10 µg/mL) when appropriated (complemented strain).

Table 1 – *S. epidermidis* strains used in this study.

Strain	Description[a]	Reference
1457	Wild-type (WT), isolated from a central venous catheter infection	Mack et al., 1992
1457Δ*tagO*	Mutant strain carrying a deletion of *tagO* gene	França et al., unpublished
1457ΔtagO +pRB473*tagO*	Mutant strain complemented with gene *tagO*, Cm^R	França et al., unpublished

[a] Abbreviations: Cm^R, resistance to chloramphenicol.

2. Optimization of bacterial growth conditions

2.1 Bacterial growth curve

A single colony of *S. epidermidis* strains grown on TSA plates was inoculated in TSB and incubated overnight at 37 ºC and 180 rpm in a shaking incubator (3031-GFL, Germany). Cells were recovered by centrifugation (10 min, 3000 ×*g*, 4 ºC) and resuspended in sterile and apyrogenic phosphate-buffered saline (PBS). Upon optical density (OD) measurement of overnight planktonic cultures, a fresh culture was prepared with a starting OD_{640nm} of 0.05 (±0.05). *S. epidermidis* cultures were allowed to grow for 24h at 37 ºC and 180 rpm in a shaking incubator (3031-GFL, Germany). Aliquots of the culture were collected at each time point (every hour until 8h and a final time-point at 24h) and the OD values were measured. The *S. epidermidis* growth curve for each strain was obtained by the representation of the optical density at 640 nm as a function of time.

2.2 Calibration curve for each strain relating OD and CFU counts in different conditions

A single colony of each *S. epidermidis* strains, grown on TSA plates, was inoculated in different conditions: 1) TSB, 2) TSB supplemented with 10% human AB serum (Capricorn Scientific) and 3) TSB supplemented with 10% bovine serum albumin (BSA). The cultures were incubated overnight (to late exponential growth: 14h-16h) at 37 ºC and 180 rpm in a shaking incubator (3031-GFL, Germany). Cells were recovered by centrifugation (10 min, 3000 ×*g*, 4 ºC) and resuspended in the respective conditions: 1) PBS, 2) PBS supplemented with 2% human AB serum and 3) PBS supplemented with 2% BSA. Upon OD measurements of overnight planktonic cultures, the OD_{640nm} was adjusted to 1.00 (±0.05) and several dilutions were prepared in order to obtain OD values from 0.06 to 1. The OD values for each dilution were measured and the samples serially diluted and plated onto TSA for culturable bacteria counting. The plates were incubated for up to 24h at 37 ºC before counting the CFU. Calibration curves for each strain, at each condition, were created by plotting OD_{640nm} values versus culturable bacteria counts (log_{10} CFU/mL). OD-CFU calibration curves of the supplementation condition with human AB serum were selected and utilized for further studies.

2.3 Bacterial cell counts using flow cytometry

The value of 0.25 (±0.05) for WT strain and 0.50 (±0.05) for the mutant (Δ*tagO* or ΔT*)* and complemented (Δ*tagO::tagO* or ΔT::T*)* strains were selected as corresponding to the same number of culturable bacteria, approximately 2×10^8 colony forming units (CFU)/mL. To obtain the correspondence between the culturable and viable bacteria, cell concentration and viability were evaluated by flow cytometry. Briefly, overnight planktonic cultures were adjusted to the same concentration of culturable bacteria (2×10^8 CFU/ml) as described above and diluted 1:200 considering the flow cytometer instrument sensitivity previously determined by beads calibration curve ($\approx1\times10^5$ – 2.5×10^7 beads/ml). Diluted bacterial suspensions were co-stained with propidium iodide (PI, 5 µg/ml) and SYTO (1:5000, SYTO™ BC dye, Molecular Probes Inc.) and flow cytometric quantification was carried out using a BD Accuri C6 Plus flow cytometer (BD Bioscience, CA, USA). SYTO fluorescence was detected on the FL1 channel while PI fluorescence was detected on the FL3 channel. Gating was done in FL1 vs FL3 dot plot to obtain the percentage of viable ($SYTO^+PI^-$) and non-viable/dead bacteria ($SYTO^+PI^+$ and $SYTO^-PI^+$). Data were analysed using the FlowJo v.10.6.1 software.

3. Bacterial growth conditions

3.1 Planktonic mode of growth

A single colony of *S. epidermidis* strains grown on TSA plates was inoculated in TSB supplemented with 10% human AB serum (Capricorn Scientific) and incubated overnight (to late exponential growth: 14h-16h) at 37 ºC and 180 rpm in a shaking incubator (3031-GFL, Germany). Cells were recovered by centrifugation (10 min, 3000 ×*g*, 4 ºC) and resuspended in sterile and apyrogenic phosphate-buffered saline (PBS) supplemented with 2% human AB serum. The optical density (OD) of overnight cultures was read at 640 nm and adjusted to 0.25 (±0.05) for WT and 0.50 (±0.05) for mutant (Δ*tagO* or ΔT*)* and complemented (Δ*tagO::tagO* or ΔT::T*)* strains. The inoculum was confirmed by CFU counts on TSA plates after incubation at 37 ºC for up to 24h.

3.2 Biofilm mode of growth

To start biofilm cultures, 1.5 µL of a 2×10^8 CFU/mL bacterial suspension was inoculated into 150 µL of TSB supplemented with 0.4% of glucose (Merck, Germany) and 10% human AB serum (Capricorn Scientific) ($TSB_{0.4\%Glucose+10\%human\ serum}$) in a 96-well polystyrene plate (Orange Scientific, Braine-l' Alleud, Belgium) and incubated at 37 ºC with shaking, at 80 rpm, for 24h. After 24h, the culture medium was removed and 150 µL of fresh $TSB_{0.4\%Glucose+10\%human\ serum}$ was added, followed by additional 24h of growth, under the same temperature and agitation conditions. Biofilms were washed twice with apyrogenic PBS and disrupted using a sterile embolus from 1 mL syringe. Then, biofilms were sonicated for 10 s at 18W (Branson model W 185 D, Heat Systems Ultrasonics, CT, USA), with the sonicator tip placed at the air-liquid interface, in order to dissociate cell clusters. Biofilm production by different strains was evaluated by measuring OD_{640nm} and determining CFU. These parameters were measured for each individual biofilm in order to compare the biofilm formation between strains. The assays were performed in duplicate (2 biofilms/strain).

4. Transmission electron microscopy (TEM) imaging

Exponential-phase bacterial cells were obtained by diluting down overnight cultures (12h in the conditions mentioned above for the planktonic mode of growth) to 3×10^7 CFU/mL and then grown for 6h at 37 ºC with shaking at 250 rpm. The cells were collected by centrifugation (5000 x*g*, 10 min, 4 ºC) and washed thrice with metal-free ultrapure water to remove salts. To fix the cells, 2.5% (w/v) glutaraldehyde / 2% (w/v) paraformaldehyde

in cacodylate buffer 0.1 M (pH 7.4) was added and the samples were incubated overnight. Upon washed in 0.1M sodium cacodylate buffer, the samples were post-fixed with 2% (w/v) osmium tetroxide in the 0.1M sodium cacodylate buffer overnight, followed by additional fixation overnight with 1% (w/v) uranyl acetate. The dehydration of samples was performed with graded series of ethanol and propylene oxide and included in EPONTM resin by immersing the samples in increasing series of propylene oxide to EPON^{TM} (till 0:1 ratio) for 60 min each. The inclusion of samples in EPONTM resin was performed in a silicon mold. Sections with approximately 60 nm thickness were prepared using a diamond knife on an RMC Ultramicrotome (PowertTome, USA) and recovered to 200 mesh Formvar Ni-grids, followed by staining on the grids with 2% (w/v) uranyl acetate and saturated lead citrate solution. Each of these sections was viewed at 80 kV in a JEM 1400 microscope (JEOL, Japan) and digital images were taken using a CCD digital camera Orious 1100 W (Tokyo, Japan).

5. Phagocytic activity of RAW 264.7 macrophages against *S. epidermidis*

5.1 Macrophage Culture

The murine macrophage-like cell line RAW 264.7 was cultivated in complete Roswell Park Memorial Institute (cRPMI, RPMI-1640 supplemented with 10% FBS, 2% L-Glutamine, 1% HEPES buffer, 1% Penicillin-Streptomycin and 50 µM β-mercaptoethanol (all from Sigma Aldrich) in a humidified atmosphere with 5% CO_2 at 37 ºC. Macrophages were kept by serial passages in T-75 flasks (Brand). After confluent growth, cells were recovered by detachment with a cell scraper. Cell concentration and viability were determined by Trypan blue (Sigma Aldrich) staining in a hemocytometer.

5.2 Phagocytosis quantification assay

Suspensions of *S. epidermidis* in the planktonic mode of growth were prepared as described above, resuspended in antibiotic-free cRPMI medium and used to infect previously plated RAW 264.7 at a multiplicity of infection (MOI) of 1 Macrophage to 10 Bacteria (MOI 1:10). Macrophage cell suspensions were transferred to 96-well plates (flat bottom, Sarstedt, Nümbrecht, Germany) (1×10^5 cells/well) and incubated for 1h, at 37 ºC and 5% CO_2, to allow macrophage adherence, before the addition of bacteria. Thereafter, bacterial suspensions were added (1×10^6 CFU/well) and plates were centrifuged, at 300 ×*g* for 2 min, to synchronize phagocytosis, followed by incubation at

37 ºC in the presence of 5% CO_2. Macrophages were allowed to internalize bacteria for up to 3h. At 30min, 1h and 3h post-challenge, culture supernatants were discarded and serum-free pre-warmed cRPMI containing gentamicin (50 µg/mL) (PanReac AppliChem) was added for 60 min to eliminate extracellular bacteria. After gentamicin treatment, cells were washed with PBS and the release of the gentamicin-protected bacteria (i.e. intracellular bacteria) was performed by lysing macrophages with 0.1% (w/v) saponin in PBS for 15 min. Lastly, macrophage lysates were serially diluted in PBS and plated on TSA plates. After 24h of incubation at 37 ºC, the number of culturable bacteria present at each time point was assessed by CFU counts allowing to monitor the uptake of bacteria by macrophages.

6. Interaction of *S. epidermidis* with Mouse Innate Immune Cells

6.1 Mice

BALB/c mice were kept at the Animal Facility of the Instituto de Investigação e Inovação em Saúde (i3S), Porto, Portugal. All procedures involving mice were performed according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123), the directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes, and Portuguese rules (DL 113/2013). Experiments were approved by the institutional board responsible for animal welfare (ORBEA) at i3S and authorization to perform the experiments was issued by the competent national authority (Direcção Geral de Alimentação e Veterinária) reference number 014036/2019-07-24.

6.2 Differentiation of Mouse Bone Marrow-Derived Dendritic Cells (BMDC)

BALB/c mice were anaesthetized using CO_2 and then euthanized by cervical dislocation. Upon brief immersion in 70% ethanol, femurs and tibias were removed under aseptic conditions. Both ends of the bone were cut off and, using a needle of a syringe that was inserted into the bone cavity (25-G for femurs and 27-G for tibias), the bones were flushed with cold Hank's Balanced Salt Solution (HBSS) (Sigma Aldrich), in order to obtain undifferentiated bone marrow cells. The cell suspension was passed through a 70 µm pore filter, centrifugated at 300 ×*g* and the supernatant was discarded. Cells were resuspended in cRPMI medium and counted in a hemocytometer. Cell concentration

was adjusted to 1×10^6 cell/mL in cRPMI supplemented with murine granulocyte-macrophage colony-stimulating factor (GM-CSF, 20 ng/mL) in order to differentiate cells into BMDC and distributed into 6-well plates (5mL/well) (Brand). Finally, plates were incubated at 37 ºC with 5% CO_2 atmosphere for 7 days. The medium was renewed every two days. On day 8, the cells were recovered by carefully pipetting the medium against the plate, washed with antibiotic-free cRPMI, counted, and seeded at a desire concentration in a 96-wells plate (round bottom) for incubation with *S. epidermidis* strains.

6.3 Stimulation of mouse BMDC with *S. epidermidis* strains

BMDC prepared as described above were seeded in 96-well round-bottom plates (Sarstedt, Nümbrecht, Germany) at 1×10^5 cells/well. BMDC were mixed with culturable bacterial cells at a MOI of 1:4 (4×10^5 CFU/mL) and 1:10 (1×10^6 CFU/mL) and the plates were incubated at 37 ºC and 5% CO_2 for 2h. After 2h, gentamicin (PanReac AppliChem) was added to the medium at a final concentration of 50 µg/mL and cells were incubated for a further 22h. At the end of the incubation period, the plates were centrifugated at 300 ×*g* during 10 min and the culture supernatants collected and stored at -80 ºC until cytokine quantification by ELISA. Unstimulated and LPS stimulated cells (2 µg/mL, Sigma Aldrich) were used as negative and positive controls, respectively.

6.4 Evaluation of mouse BMDC activation

BMDC activation was evaluated through the detection of cell surface activation markers using flow cytometry. After the period of infection with bacteria and incubation (24h), BMDC were washed in PBS solution and stained with fixable viability dye (FVD) for 30 min on ice, protected from light, to distinguish between live (FVD^-) and dead (FVD^+) cells. Next, cells were washed and incubated with a mixture of fluorochrome-conjugated antibodies: anti-mouse CD11c-FITC conjugated (clone N418), anti-mouse F4/80-APC/Cy7 conjugated (clone BM8), anti-mouse Ly6G-AF647 conjugated (clone 1A8), anti-mouse CD80-PE conjugated (clone 16-10A1), anti-mouse CD86-PECy7 conjugated (clone GL1), anti-mouse MHC-PerCP conjugated (clone M5/114.15.2) (all from eBioscience), and were kept on ice for 20 min in the dark. Cells were then washed again and fixed by incubating for 20 min in 2% paraformaldehyde at room temperature. After fixation, a washing step was followed, cells were resuspended in FACS buffer (PBS with 2%FCS and 10mM Sodium Azide) and analysed by flow cytometry. All washing steps consisted in the addition of PBS solution followed by centrifugation at 300 ×*g* for 5 min

at 4 ºC to discard the supernatant. Data analyses were performed using FlowJo v.10.6.2 software.

6.5 Cytokine quantification by ELISA

The levels of the cytokines IL-1β, IL-6, IL-10, IL-12p70 and TNF-α secreted by BMDC were quantified by sandwich ELISA according to the manufacturer's instructions (Mouse IL-1β Uncoated ELISA Kit; Mouse IL-6 Uncoated ELISA Kit; Mouse IL-12p70 Uncoated ELISA Kit; Mouse TNF-α Uncoated ELISA Kit, all from Invitrogen by Thermo Fisher Scientific, Vienna, Austria and Mouse IL-10 DuoSet® ELISA Development System, R&D Systems, Minneapolis, MN).

7. Interaction of *S. epidermidis* with Human Innate Immune Cells

7.1 Ethics statement

Buffy coats of healthy blood donors were obtained through a collaboration protocol with Centro Hospitalar de São João (CHSJ), Porto, Portugal, and used for investigation purposes. All studies were approved by CHSJ Ethics Committee for Health (References 259 and 260/11), in agreement with the Helsinki declaration. Informed consent was obtained from all subject before blood donation.

7.2 Differentiation of human monocytes into macrophages and dendritic cells

Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of healthy donors by density gradient centrifugation using Histopaque-1077 (Sigma Aldrich), according to manufacturer's instructions. $CD14^+$ monocytes were then purified from PBMCs by magnetic-activated cell sorting using CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Human $CD14^+$ monocytes were resuspended in cRPMI medium, at a cell density of 1×10^6 cells/mL and cultured in 6-well plates (Brand). To differentiate cells into monocyte-derived macrophages (MDMs), GM-CSF or macrophage colony-stimulating factor (M-CSF), at a final concentration of 20 ng/mL, was used. Differentiation to monocyte-derived dendritic cells (MoDCs) was triggered by the addition of GM-CSF (100 ng/mL) plus IL-4 (20 ng/mL). Cells were incubated at 37 ºC in a humidified 5% CO_2 incubator for 7 days, and the medium was renewed every three days. After 7 days, dendritic cells were harvested by gently pipetting the medium against the plate and adherent macrophages were detached from the culture plates with ice-cold

5 mM EDTA in PBS solution. Cells were then washed, counted and resuspended at the desired concentration in antibiotic-free cRPMI medium before incubation with *S. epidermidis* strains in 96-wells plates (round and flat bottom, respectively, for dendritic cells and macrophages).

7.3 Stimulation of human MoDCs and MDMs with *S. epidermidis* strains

After the 7-day-incubation period, MoDCs and MDMs were prepared as described above and seeded in 96 well-plates (round and flat bottom, respectively) (Sarstedt, Nümbrecht, Germany) at 1×10^5 cells/well. The indicated cells were infected with all *S. epidermidis* strains at a MOI of 1:10 (1×10^6 CFU/mL) and the plates were incubated for 2h at 37 ºC with 5% CO_2. After the challenge, 50 µg/mL of gentamicin was added to medium and cells were incubated for an additional 22h in the same conditions. Then, the plates were centrifugated at 300 ×*g* during 10 min and the culture supernatants were collected and stored at -80 ºC until cytokine quantification by ELISA. Unstimulated and LPS stimulated cells (2 µg/mL, Sigma Aldrich) were used as negative and positive controls, respectively.

7.4 Evaluation of human MoDC activation

The expression of surface markers associated with MoDC activation was evaluated by flow cytometry. After the period of infection with bacteria and incubation (24h), cells were washed in PBS solution and incubated with FVD for 30 min on ice, in the dark. The MoDCs were subsequently incubated for 30 min on ice, protected from the light, with the appropriate conjugated antibody cocktail at previously optimized concentrations. Cells were then fixed with 2% paraformaldehyde, for 20 min at room temperature, washed with PBS solution and resuspended in FACS buffer (PBS with 2%FCS and 10mM Sodium Azide) before flow cytometric analysis. All steps in this protocol were preceded by washing the cells, which consisted of adding PBS solution, centrifuging (300 ×*g*, 5 min, 4 ºC) and removing the supernatant. Flow cytometry data analysis was performed using FlowJo v.10.6.2 software.

The following specific cell surface antibodies were used to stain MoDCs: anti-human CD14-PE conjugated (clone 61D3), anti-human CD11c-APC conjugated (clone BU15), anti-human CD83-FITC conjugated (clone HB15e), anti-human CD80-BV510 conjugated (clone 2D10), anti-human CD86-PECy5 conjugated (clone IT2.2) and anti-human HLA-PECy7 conjugated (clone L243).

7.5 Cytokine quantification by ELISA

Cytokine production, by MoDCs and macrophages, was assessed in the human cell culture supernatants using ELISA. ELISA kits for IL-1β, IL-6, IL-8, IL-10, IL-12p70 and TNF-α were purchased from R&D Systems (Minneapolis, MN) and the procedure was performed following instructions provided by the manufacturer.

8. Statistical Analysis

Statistical analysis was carried out with GraphPad Prism 8 Software (CA, USA) and data were analysed by one-way ANOVA. Data were reported as means ± SD. P values of <0.05 were considered statistically significant.

CHAPTER 3: RESULTS AND DISCUSSION

1. Characterization of *S. epidermidis* strains

In the present study, a mutant *S. epidermidis* strain lacking WTAs was used. Firstly, a comparative analysis between the Δ*tagO* (or ΔT) and WT strains was performed to explore the effect of *tagO* deletion on bacterial growth and phenotype. The mutant strain complemented with the *tagO* gene in a plasmid (*ΔtagO::tagO* or ΔT::T*)* was used as a control to ensure that the observed mutant phenotype was actually due to the loss of the *tagO* gene.

1.1 Characterization of bacterial growth

Growth characteristics of the different strains were assessed through the bacterial growth curves by hourly measuring the OD_{640nm} for 8 hours plus an additional time point at 24 hours (Fig. 6A). To estimate and to compare the growth rates of the strains, an exponential regression in the log phase was obtained for each strain (Fig. 6B) that provided the parameters gathered in Table 2.

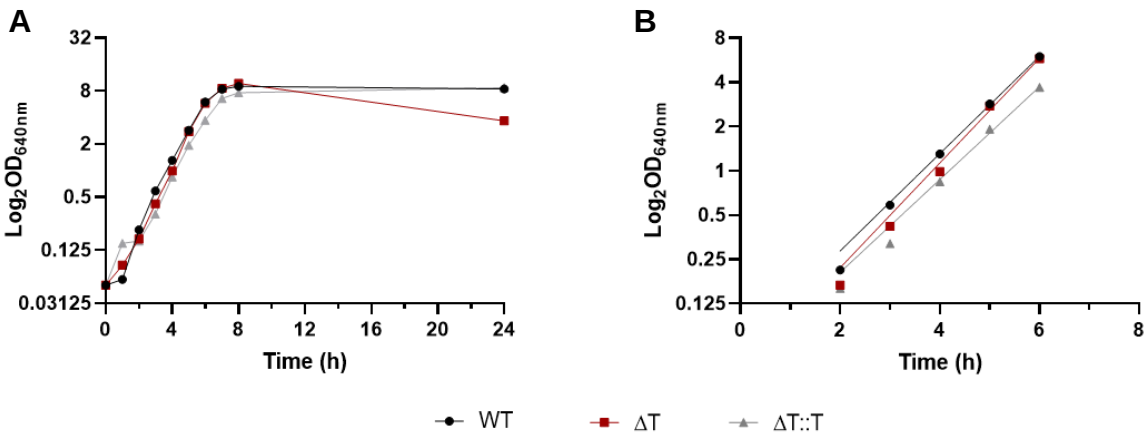


Fig. 6 – Characterization of bacterial growth of *S. epidermidis* strains. (A) Bacterial growth curves of *S. epidermidis* strains by measurement of optical density at 640 nm every hour until 8h and a final point at 24h. (B) Exponential regression fits in the log phase of the growth curves. Representative result of two independent experiments (n=2). OD, optical density. WT, Wild Type. ΔT, mutant strain. ΔT::T, complemented strain.

The evolution of the number of bacteria over time follows the typical growth curve in a closed system with four phases: lag phase (0h-2h), exponential phase (2h-6h), stationary phase (from 6h) and death phase, this last one only evident in the mutant strain (ΔT) at the 24hours time-point (Fig. 6A). The absorbance of the culture began to decrease in

tagO mutant during prolonged incubation in the stationary phase, which can be explained by the increased susceptibility of that strain to autolysis. This is consistent with earlier studies of *S. aureus* WTA mutants, in which it has been reported that the absence of WTAs could induce spontaneous autolysis (Schlag et al., 2010; Vergara-Irigaray et al., 2008). Schlag et al., 2010 showed that WTAs prevent the binding of autolysins to the cell wall and that these are more concentrated in the septum region, where it is speculated that there is less or premature WTA (Schlag et al., 2010). The possible uniform distribution of these proteins on the cell wall of the mutant strain justifies the greater susceptibility to autolysis.

In the exponential phase of growth, the cells divide at a constant rate that is expressed as the doubling time of the bacterial population, which corresponds to the time interval required for the bacteria to divide. The WT and complemented strains exhibited similar growth rates, while the mutant strain presents the lowest doubling time (Table 2), as can also be seen in Fig. 6B by the higher slope relatively to other strains.

Table 2 - Parameters obtained by exponential regression in the log phase of bacterial growth for each strain. Doubling time is calculated by the formula ln (2)/μ. y0, nº of cells at the time (t) zero (0h); μ, specific growth rate (number of divisions per cell per unit time). WT, wild type. ΔT, mutant strain. ΔT::T, complemented strain.

Strain	Equation: y= $yo \times e^{\mu t}$	Doubling Time (h)
WT	$y = 0{,}0449 \times e^{0.8269\,t}$	0.838248
ΔT	$y = 0{,}0282 \times e^{0.8973\,t}$	0.772481
ΔT::T	$y = 0{,}0311 \times e^{0.8086\,t}$	0.857219

1.2 Optimization of growth conditions for *S. epidermidis*

1.2.1 Influence of the culture medium for bacterial growth

Staphylococci are known to grow in clusters since the daughter cells remain attached to each other after division, which may lead to an erroneous enumeration of CFU. Besides, it has been reported that the *tagO* deletion leads to an increase in intercellular aggregation, which is possibly related to the increased surface hydrophobicity in these mutants (Holland et al., 2011). To overcome this issue, different growth conditions were tested. The bacterium was grown overnight and prepared in the next day with: 1) only medium, 2) supplementation with human AB serum and 3) supplementation with BSA. These conditions were chosen in order to test whether opsonization with serum-derived

proteins could reduce the aggregation of the bacteria. The OD_{640nm} versus CFU counting graphs for each condition are shown in Fig. 7.

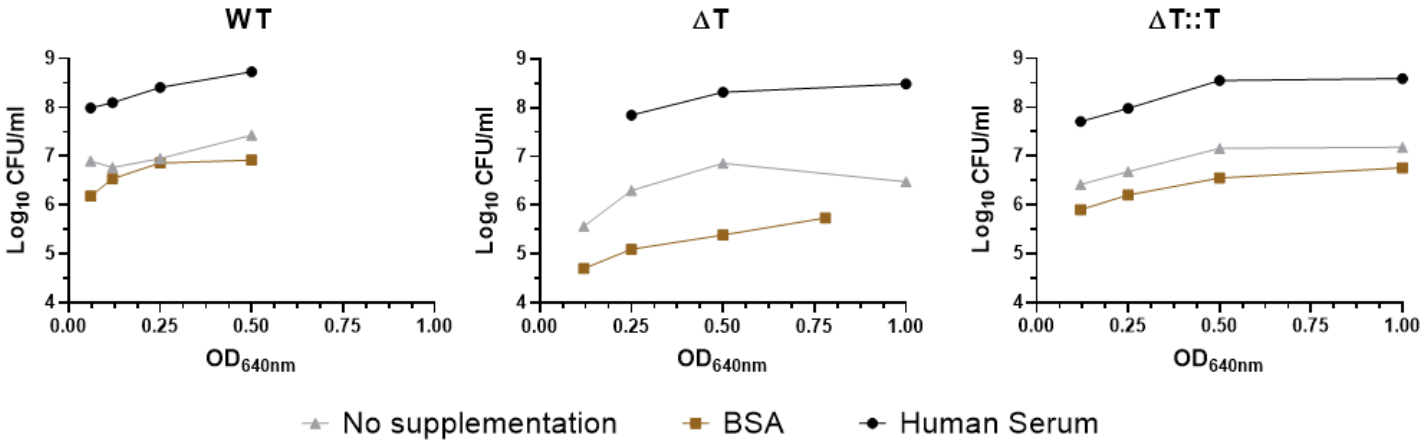


Fig. 7 – Evaluation of bacterial growth in different conditions. All *S. epidermidis* strains were grown overnight with only TSB medium (*No Supplementation*), supplementation with BSA (*BSA*) or supplementation with human AB serum (*Human Serum*). For each condition, the OD_{640nm} of bacterial suspension was adjusted to a range of values between 0.06 and 1.00, and the respective CFU counts were determined. Each point represents the result of a single measurement. OD, optical density. WT, Wild Type. ΔT, mutant strain. ΔT::T, complemented strain.

From the results shown in Fig. 7, it can be seen that the type of culture medium supplementation influences the relationship between OD and CFU counts. Under growing conditions with BSA supplementation or medium alone, the increase in OD does not always translate into a linear increase in CFU. This is most evident in the mutant strain for higher OD values. The aggregation of the bacterium, which is more notorious at higher concentrations, may lead to an underestimation of the CFU counts.

On the other hand, the bacterium grew better in medium supplemented with human AB serum, with culturable cell counts mostly above 10^8 CFU/mL. In this condition, a linearity relationship between OD and CFU was observed in the tested range. It is “assumed” that the presence of serum may be contributing to the reduction of bacterial aggregation, providing a more accurate CFU value. The use of human serum in cultures, being a factor of the host, also helps to mimic the *in vivo* situation. In this way, the bacterium used in all experiments was grown in the presence of 10% human AB serum.

1.2.2 Normalization of bacterial concentration by OD and CFU

Considering the culture medium optimization, calibration curves for all *S. epidermidis* strains in the condition of human AB serum supplementation were elaborated (Fig. 8). The purpose of this was to normalize the concentration of bacteria (CFU/mL) by

measurement of optical density to facilitate the adjustments of inoculum in all subsequent experiments.

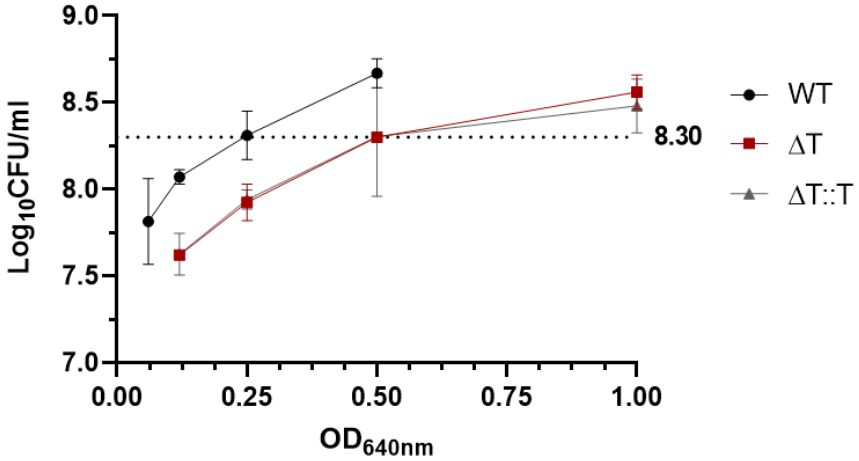


Fig. 8 – Calibration curves for all *S. epidermidis* strains. The calibration curves were obtained for each strain by plotting optical density at 640 nm versus culturable bacteria counts (log_{10}CFU/mL). The line identifies the point of log_{10}CFU/mL equal at 8.30, corresponding to approximately 2×10^8 CFU/mL. Each point represents the mean of two independent experiments (n=2). Bars correspond to mean plus SD. CFU, count forming unit. OD, optical density. WT, Wild Type. ΔT, mutant strain. ΔT::T, complemented strain.

The OD values of 0.25 for the WT and 0.50 for the mutant and the complemented strains were established as corresponding to approximately 2×10^8 CFU/mL, as shown by the line that intersects these points on Fig. 8. As can be seen from the SD in the figure, this relationship may change slightly and may not be exactly linear. However, there is a clear correspondence to the range of log values equal to 8. In microbiology, it is common to use a logarithmic scale that transforms a large number of bacteria into a smaller one that is easier to work and interpret. Therefore, the relation between OD and CFU/ml determined here was used to adjust the bacterial concentration when necessary.

1.2.3 Relationship between culturability and viability

The CFU value corresponds to bacterial cells that are culturable on medium plates and form colonies. This cultivation method is used to estimate the viable bacteria, however, in many bacterial species, there are “viable but non-culturable” (VBNC) cells that are not detected by the plate count technique. To obtain the correspondence between the viability and culturability for *S. epidermidis* cells, the number of viable bacteria was determined by flow cytometry using a live/dead analysis (SYTO and PI staining). This counting protocol was subject to numerous optimizations to overcome constraints caused by the aggregating nature of this bacterium (sonication was a discarded option for counting planktonic bacteria and supplementation with human AB serum allowed to

resolve this issue) and by the cytometry itself (threshold, range of linearity, dyes concentration).

The number of viable bacteria was determined for the OD values previously established as corresponding to the same number of "culturable bacteria" in all strains (Fig.8). The applied gating strategy is shown in Fig. 9. The number of viable bacteria and the correspondence with culturability for each strain is represented by the graphs in Fig. 10.

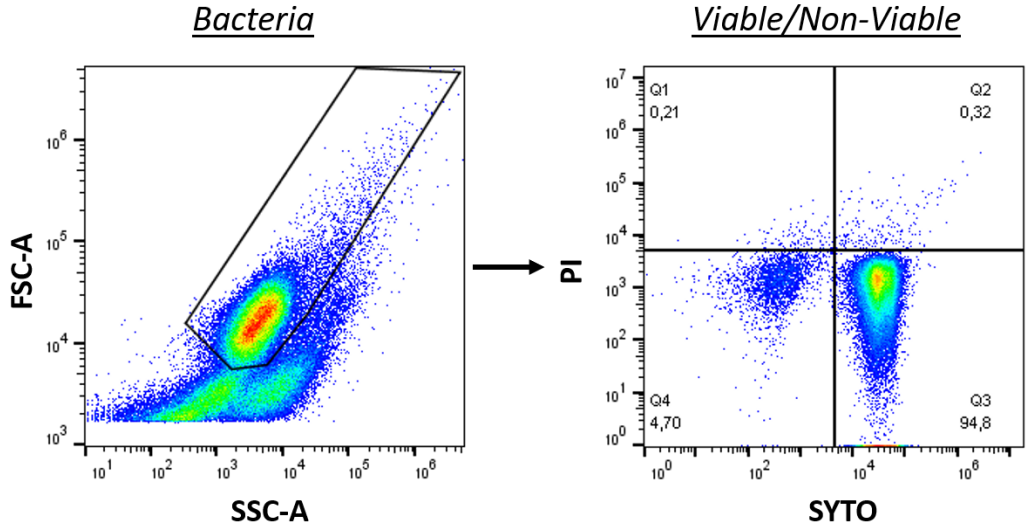


Fig. 9 – Representative analysis of flow cytometry for quantification of viable *S. epidermidis* cells. After overnight growth, the OD_{640nm} of the bacterial suspensions was adjusted to 0.25 for the WT strain and 0.50 for the mutant and complemented strains and the bacterium was stained with SYTO and PI for live cell/dead cell discrimination. Briefly, the gating strategy consisted in the selection of the bacterial population (FSC-A vs SSC-A) and the quantification of viable cells ($SYTO^+PI^-$ in Q3) and non-viable or dead cells ($SYTO^+PI^+$ and $SYTO^-PI^+$ in Q2 and Q1, respectively). This representative scheme corresponds to the analysis for the WT strain.

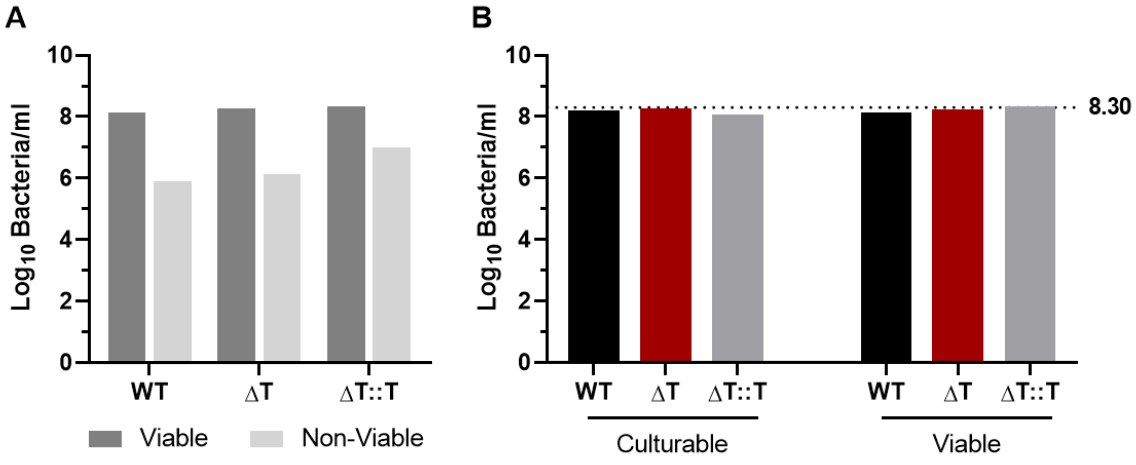


Fig. 10 – Relationship between viability and culturability in *S. epidermidis*. The OD_{640nm} of bacterial suspension was adjusted to 0.25 for the WT strain and 0.50 for the mutant and complemented strains and the bacteria were quantified. **(A)** Viable and non-viable bacteria in the suspension were determined by flow cytometry with SYTO and PI staining. **(B)** Comparison between the numbers of culturable bacteria in the suspension as determined by CFU counts and the number of viable bacteria quantified by flow cytometry, for the OD values referred previously. The line in the graph B identifies the point of log_{10}bacteria/mL equal at 8.30, which corresponds to approximately to 2×10^8 CFU/ml. Each bar represents the result of a single measurement. WT, Wild Type. ΔT, mutant strain. ΔT::T, complemented strain.

Of note, these data refer to a single experiment that was not yet repeated. In general, the number of viable bacteria in suspension appears to correspond to bacteria capable of being cultivable (Fig. 10B). Thus, the plate count method should provide a good estimate of the bacterial concentration in terms of viability. Taking into account this first indication, the OD values selected previously seems to correspond not only to the same number of culturable bacteria but also to viable bacteria in suspension.

The cytometric analysis allows us to verify the existence of a very few aggregates in the bacterial suspensions (Fig. 11).

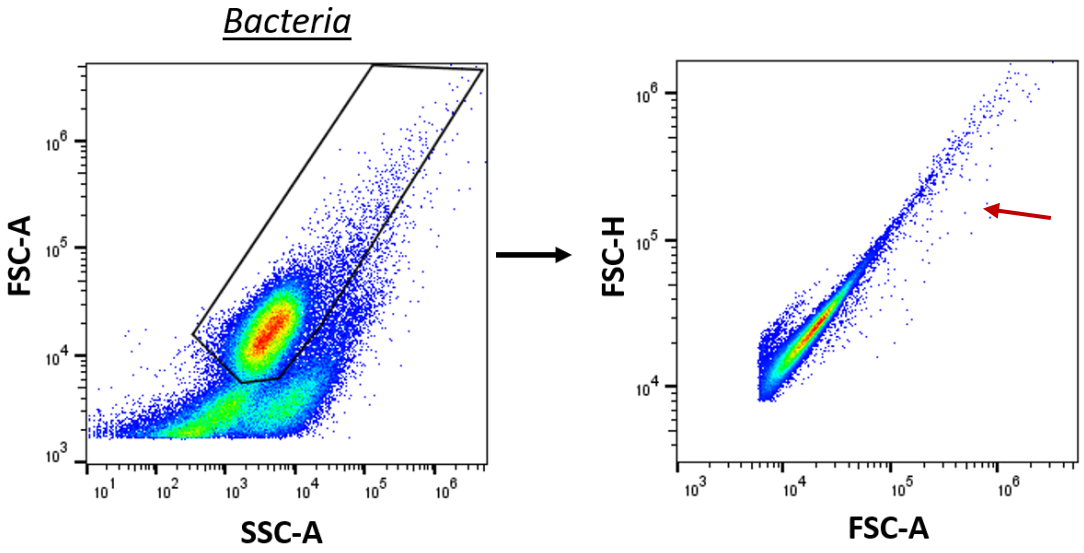


Fig. 11 - Representative analysis of flow cytometry illustrating the bacterial population with singlets and aggregates. The population of interest, corresponding to the total cells of *S. epidermidis*, was selected (FSC-A vs SSC-A) and the presence of aggregates was analysed (FSC-H vs FSC-A). The vast majority of the bacterial population was found to correspond to singlets, with a reduced amount of aggregates that are shown in increased FSC-A/FSC-H (red arrow).

This reinforces the choice of supplementing the growth medium with human serum, as a strategy to decrease the aggregation naturally present in this bacterium.

1.3 Phenotypic characterization of *S. epidermidis* strains

To address if the WTA-deficient strain has a similar phenotype compared to the WT, we used transmission electron microscopy (TEM). Representative images of each strain are illustrated in Fig. 12 and correspond to bacteria in the exponential phase of growth.

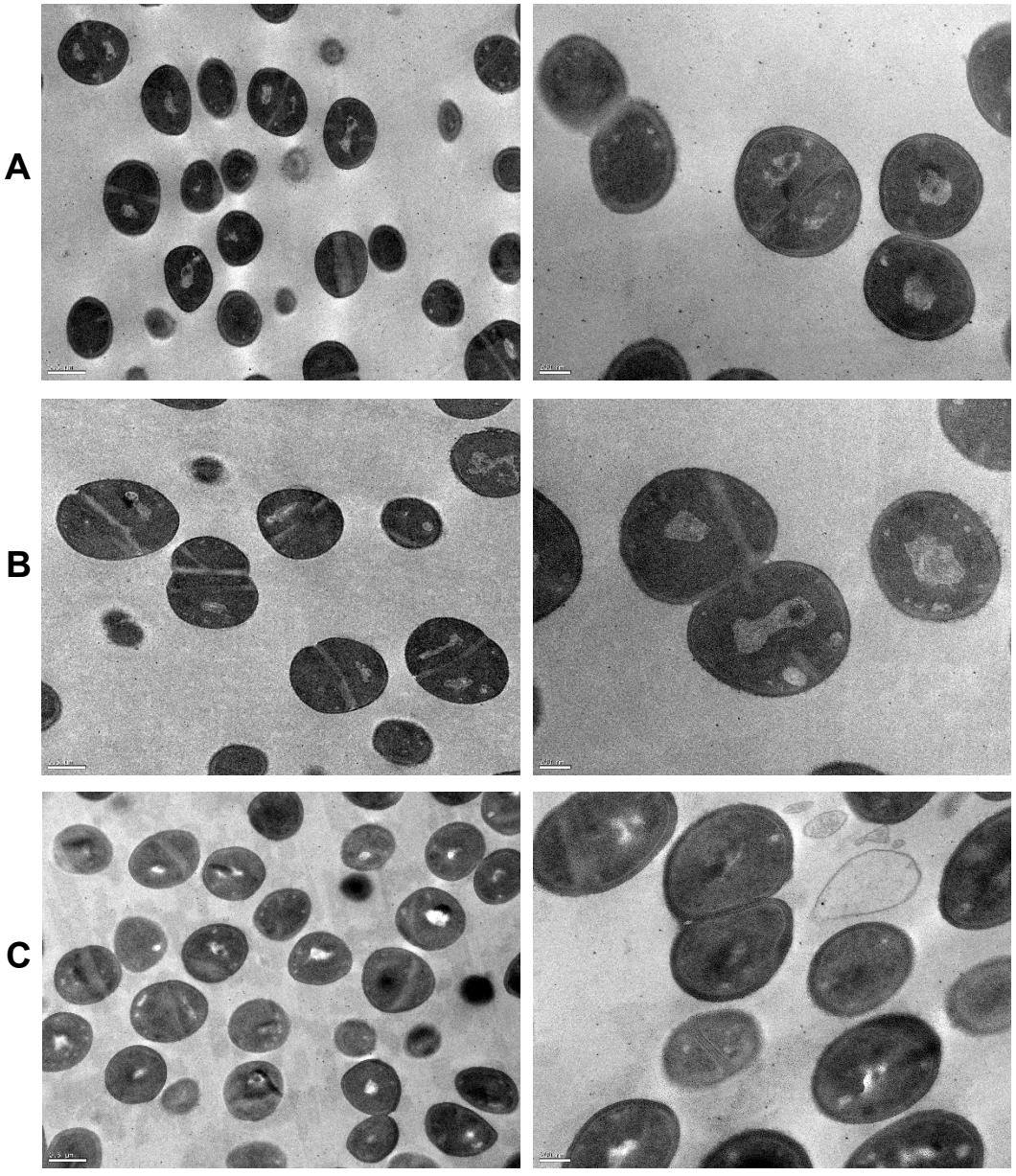


Fig. 12 - Representative TEM images of *S. epidermidis* strains in the exponential phase of growth showing dividing cells. **(A)** Wild-Type cells. **(B)** The mutant strain that lacking wall teichoic acids (WTA). **(C)** Complemented strain. Images on the left with 25 000x magnification (scale bar: 0.5µm) and images on the right with 50 000x magnification (scale bar: 0.2µm).

Compared to WT *S. epidermidis*, the *tagO* mutant appears to have a higher size. Since the dimension of bacterial cells (size and shape) interfere with OD reading through the amount of light that reaches the detector, these discrepancies in cell size between strains

goes in line with the differences of OD encountered previously. As the bacteria were in the exponential growth phase, it was observed that the vast majority of cells were actively dividing. The division planes in the mutant were frequently placed at nonorthogonal angles, leading to asymmetric divisions, and it was common to find more than one division septum (Fig. 12B). This phenotype has already been described in *S. aureus* after treatment with tunicamycin (Santa Maria et al., 2014).

Morphological analysis of the mutant strain in division (Fig. 12B), together with the decrease in absorbance over the stationary phase which can be related to an increase in the rate of autolysis (Fig. 6A), highlights the role of WTA in bacterial cell division, as already described in *S. aureus* (Biswas et al., 2012; Schlag et al., 2010).

2. Quantification of *S. epidermidis* phagocytosis by macrophages

The crucial function of the macrophages as cells of the innate immune system is their capacity of phagocytosis. To evaluate the susceptibility of the WTA-depleted strain to phagocytosis, RAW 264.7 macrophages were incubated with *S. epidermidis* for different periods of time (30 min, 1h and 3h) that allowed to follow the internalization of the bacteria by these cells. A multiplicity of infection (MOI) of 1 macrophage cell for 10 bacteria (1:10) was used. The concentration of bacteria inside the macrophages over time after the infection is shown in the graph in Fig. 13. The quantification of the phagocytic process was expressed as the percentage of bacteria that were phagocytosed relatively to the number of bacteria incubated initially (Fig. 14).

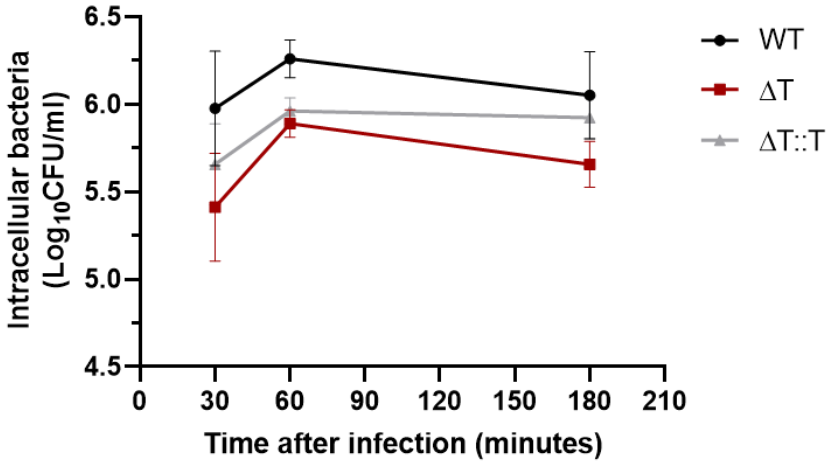


Fig. 13 – Quantification of bacteria phagocytosed by RAW 264.7 macrophages over time. The macrophage cell line was infected with *S. epidermidis* strains at a MOI of 1:10 for up to 3 hours and the bacteria engulfed by these cells was quantified by CFU count at different time points post-infection: 30 min, 1h and 3h. Each point represents the mean of three independent experiments, each performed in duplicate. Error bars are presented and correspond to mean plus SD. WT, Wild Type. ΔT, mutant strain. ΔT::T, complemented strain.

The results show a trend in the amount of the bacteria that was internalized by RAW 267.4 cells over time after the infection (Fig. 13). All *S. epidermidis* strains were rapidly phagocytosed by macrophages with phagocytosis rates greater than 70% at 30 minutes post-infection (Fig. 14A). The highest percentage of phagocytosis was registered after 1hour contact, with the WT strain was significantly more phagocytosed than the others (Fig. 14B). After 3 hours, there was a slight decrease in the percentage of bacteria inside macrophages (Fig. 14C). This observation may be explained by the occurrence of intracellular bacteria killing and shows that infection was being controlled, even in the absence of WTA.

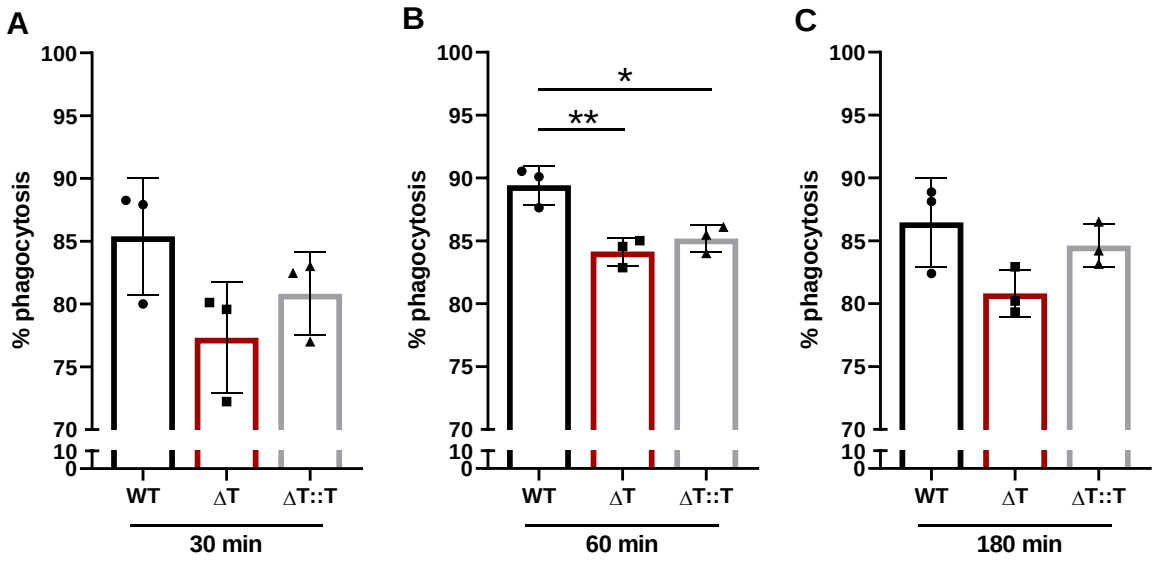


Fig. 14 – Quantitative comparison of the percentage of bacteria phagocytosed by RAW 264.7 macrophages at each time point. The macrophage cell line was infected with *S. epidermidis* strains at a MOI of 1:10 for up to 3 hours and the bacteria engulfed by these cells was quantified by CFU count at different time points post-infection: **(A)** 30 min, **(B)** 1h and **(C)** 3h. The phagocytosis rate was calculated by dividing the CFU of the intracellular bacteria by the CFU of the initial inoculum used to infect the cells. Each point on the graph represents an independent experiment, each performed with duplicates. Bars correspond to mean plus SD. Data were analysed by one-way ANOVA with Tukey post hoc test and the asterisks indicate a statistically significant difference. * $P<0.05$; ** $P<0.01$. WT, Wild Type. ΔT, mutant strain. ΔT::T, complemented strain.

In general, the mutant strain is less phagocytosed by the macrophage cell line than the *S. epidermidis* WT strain. Since the absence of WTAs leads to a reduction in the uptake of bacteria, it can be suggested that: 1) WTA molecules are involved in the recognition of *S. epidermidis* by macrophages; and/or 2) their absence can generate changes in the cell wall that make molecules that compromise the phagocytic process more exposed.

3. *S. epidermidis* stimulation of mouse bone marrow-derived dendritic cells

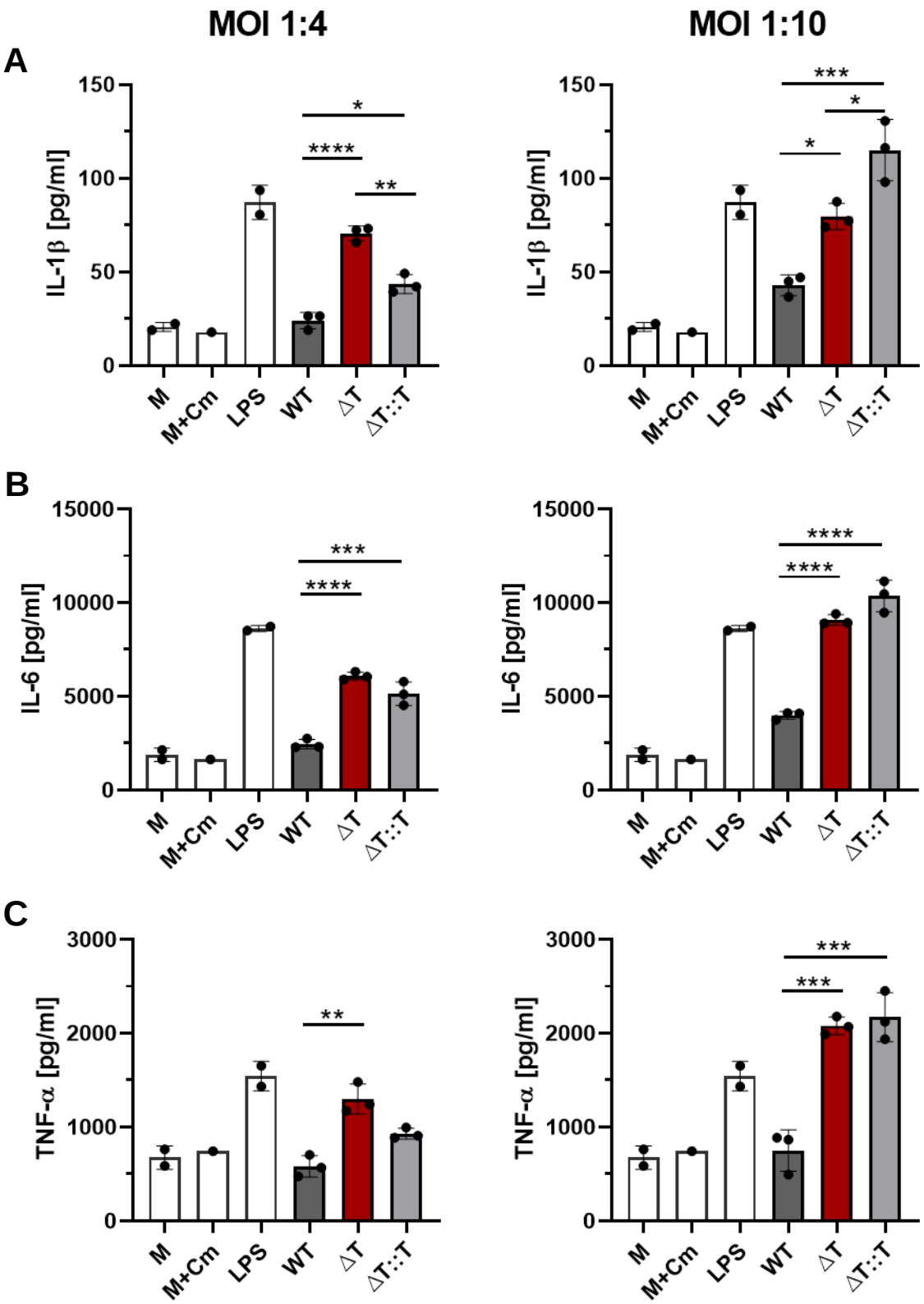
Dendritic cells are a highly specialized type of APCs. Upon recognition of pathogens via PRRs, such as TLRs, DCs are strongly activated. This event is characterized by the upregulation of co-stimulatory molecules, necessary for naïve T cell priming, and secretion of cytokines, which lead to the recruitment of other immune cells and contribute to T helper differentiation. In the mouse model, the bone marrow-derived dendritic cells (BMDCs) have been widely used for *in vitro* DC research (Fuertes Marraco et al., 2012).

3.1 Quantification of cytokines

To assess the cytokine profile of BMDCs in response to *S. epidermidis* strains after 24h incubation, the levels of IL-1β, IL-6, TNF-α, IL-12p70 and the anti-inflammatory cytokine IL-10 were quantified by ELISA in culture supernatants of BMDCs stimulated with *S. epidermidis* at two different multiplicities of infection (1:4 and 1:10, which correspond to 1 DC to 4 and 10 bacteria, respectively) (Fig.15). Culture supernatants of BMDCs stimulated with LPS were used as positive controls, while supernatants of unstimulated BMDCs were used as negative controls (incubation with medium alone as a control for the WT and mutant strains or medium with chloramphenicol as a control for the complemented strain).

The production of the pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α, was shown to be significantly higher in BMDCs stimulated with the mutant strain compared to stimulation with the WT. The same was observed for IL-12p70, a key cytokine driving Th1-type responses, although at a much lower production than the previous ones. Contrary to this more pro-inflammatory profile, the levels of the anti-inflammatory cytokine IL-10 were also found increased in cells infected with the mutant strain, with statistical significance relative to WT.

Globally, the mutant strain depleted of WTA (ΔT) stimulated greater production of pro-inflammatory cytokines and anti-inflammatory IL-10 by mouse BMDCs, comparatively to the WT strain, regardless of the multiplicity of infection used. These results demonstrate that the absence of WTA led to a more exacerbated response by dendritic cells in terms of cytokine production.


MOI 1:4
MOI 1:10
A
IL-1β [pg/ml]
150
100
50
0
*
****
**
***
*
*
M
M+Cm
LPS
WT
ΔT
ΔT::T
B
IL-6 [pg/ml]
15000
10000
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C
TNF-α [pg/ml]
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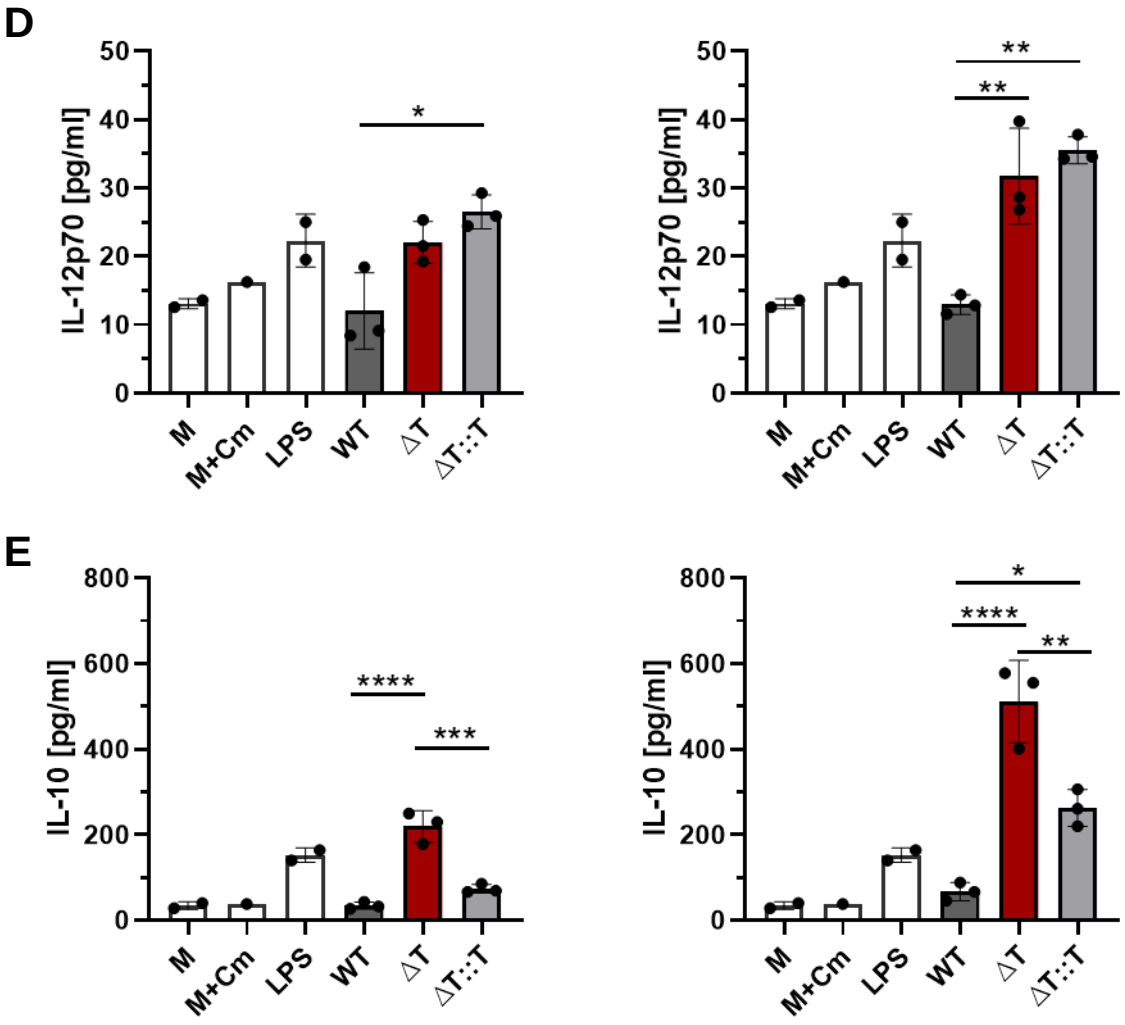


Fig. 15 - Quantification of cytokine levels in supernatants of BMDCs stimulated with *S. epidermidis*. BMDCs from BALB/c mice were stimulated with *S. epidermidis* at two different multiplicities of infection: at a MOI of 1:4 (1 DC: 4 Bacteria) and 1:10 (1 DC: 10 Bacteria). After 24h incubation, the levels of the cytokines **(A)** IL-1β, **(B)** IL-6, **(C)** TNF-α, **(D)** IL-12p70 and **(E)** IL-10 produced by BMDCs were quantified in culture supernatants by ELISA. The LPS group represents the positive control which corresponds to culture supernatants of BMDCs stimulated with LPS. As negative controls, culture supernatants from unstimulated BMDCs were used: incubation with only medium (M group) as a control for the WT and ΔT or medium with chloramphenicol (M+Cm group) as a control for the ΔT::T. Representative result of three independent experiments, each performed with triplicates. Bars correspond to mean plus SD. Data were analysed by one-way ANOVA with Tukey post hoc test and the asterisks indicate a statistically significant difference. * P<0.05; ** P<0.01; *** P<0.001; **** P<0.0001. Cm, chloramphenicol. M, medium. WT, Wild Type. ΔT, mutant strain. ΔT::T, complemented strain.

It can be hypothesized: 1) a role for WTA in immune evasion, given the lower production of cytokines by BMDC in infection with the WT; and/or 2) the depletion of WTA can lead to a restructuring of the cell wall, allowing greater exposed or expression of PAMPs capable of triggering more inflammatory responses. The recognition of these PAMPs by BMDCs may be responsible for the increased production of cytokines in infection with the mutant strain, compared to the WT.

In *S. aureus*, the WTA molecules have already been shown to be involved to some extent in immune evasion, protecting bacteria against human antimicrobial fatty acids on the skin (Kohler et al., 2009). It remains to be seen whether these polymers are involved in evasion by other mechanisms.

3.2 Evaluation of mouse BMDC activation

To examine the effect of the infection with *S. epidermidis* strains on the maturation of BMDCs after 24h incubation, the surface expression of activation markers was assessed by flow cytometric analysis. The gating strategy applied to analyse the population of live dendritic cells is shown in Fig. 16A. The expression of MHC Class II, CD80 and CD86 by dendritic cells is shown in the overlapping histograms and graphs of Fig. 16B, C and D, respectively. The expression of these markers on cells unstimulated and stimulated with LPS was evaluated as controls.

The infection with *S. epidermidis* led to the activation of dendritic cells, as can be seen by the increased expression of surface markers MHC Class II, CD80 and CD86, compared to controls corresponding to unstimulated cells (M and M+Cm groups) (Fig. 16B to 16D). This is verified regardless of the MOI used. However, analysing the histograms in Fig. 16 it is possible to verify that a large amount of the BMDCs did not upregulate the assessed markers. A possible reason for this may be due to the 24-hours infection time being too late to assess the activation of the BMDCs stimulated with *S. epidermidis*. It would be interesting to analyse in different infection times to evaluate activation kinetics.

The stimulation of dendritic cells by the different strains does not lead to differences in the expression of the analysed markers, except for the expression of the co-stimulatory molecule CD80, which is significantly increased on cells infected with the mutant strain, compared to infection condition with the WT, at a MOI of 1:10 (Fig.16C).

In general, the results show that all strains of *S. epidermidis* activate dendritic cells. The increased expression of these markers after infection also indicate that all strains induced an increase in the ability to antigen presentation by these cells, a process that requires the MHC molecule loaded with the antigen and co-stimulatory molecules.

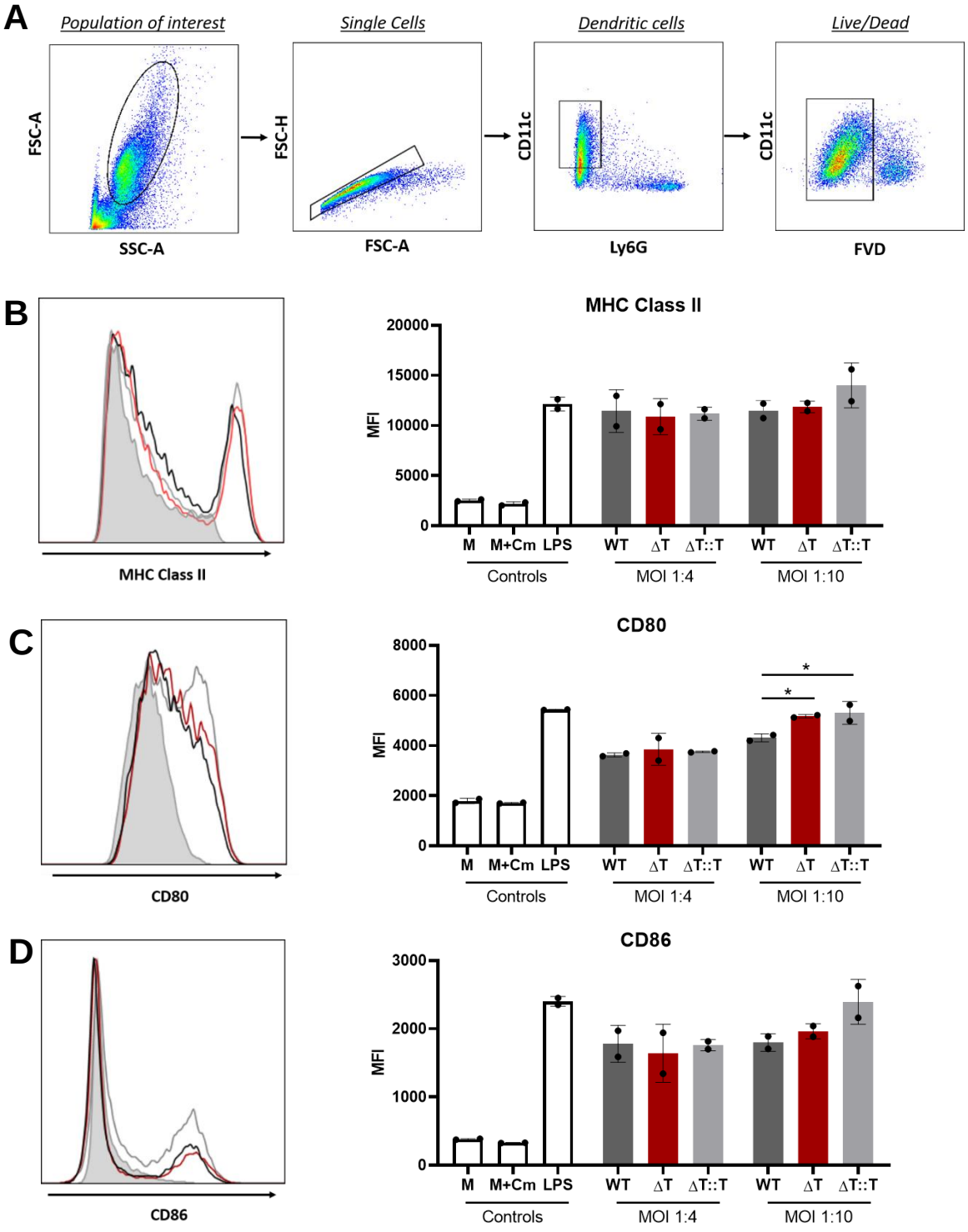


Fig. 16 – Maturation analysis of BMDCs stimulated with *S. epidermidis*. BMDCs from BALB/c mice were stimulated with *S. epidermidis* at two different multiplicities of infection: at a MOI of 1:4 (1 DC: 4 Bacteria) and 1:10 (1 DC: 10 Bacteria). After 24h incubation, the expression of surface activation markers was evaluated. **(A)** The gating strategy to analyse the population of viable BMDCs consisted in the selection of the population of single dendritic cells, without neutrophils ($Ly6G^+$), and with viability (FVD^-). The expression of the following activation markers was evaluated: **(B)** MHC Class II, **(C)** CD80 and **(D)** CD86. An overlay of histograms is shown for each activation marker that represents unstimulated BMDCs (shaded in grey) and BMDCs stimulated with the WT (black line), mutant (red line) and complemented (grey line) strains of *S. epidermidis* at a MOI of 1:10 (1DC: 10 Bacteria). The LPS group represents the positive control which corresponds to BMDCs stimulated with LPS. As negative controls, unstimulated BMDCs were used: incubation with only medium (M group) as a control for the WT and ΔT or medium with chloramphenicol (M+Cm group) as a control for the ΔT::T. Representative result of three independent experiments, each performed with duplicates. Bars correspond to mean plus SD. Data were analysed by one-way ANOVA with Tukey post hoc test and the asterisks indicate a statistically significant difference. * P<0.05. Cm, chloramphenicol. M, medium. WT, Wild Type. ΔT, mutant strain. ΔT::T, complemented strain.

4. *S. epidermidis* stimulation of human monocyte-derived dendritic cells and monocyte-derived macrophages

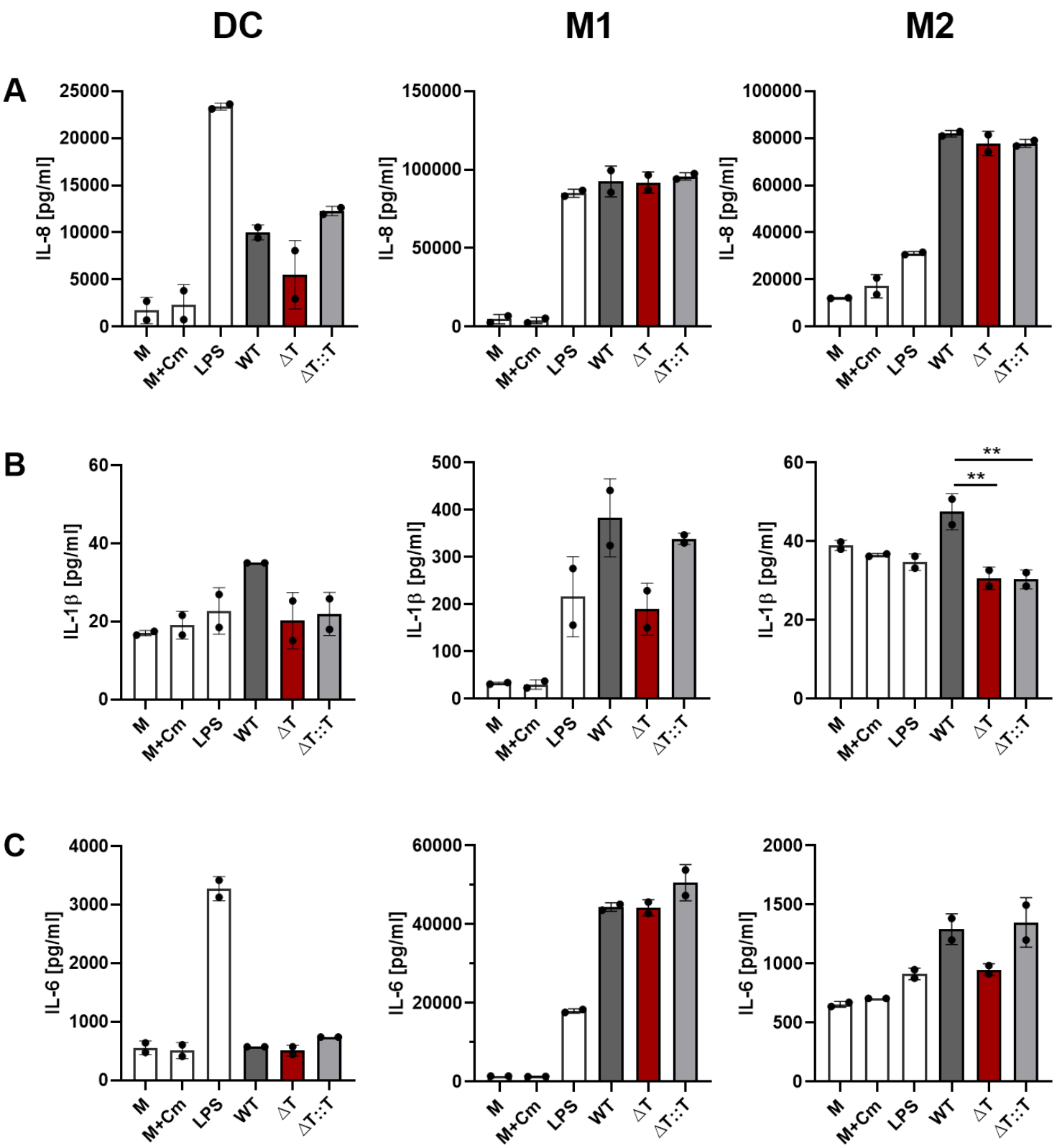
Monocytes ($CD14^+$ cells) are circulating cells capable of crossing blood vessels and differentiate into different cell types when exposed to specific cytokines. Human monocytes can be differentiated into M1 macrophages, by exposure to the inflammatory setting of Th1-type responses and protect against bacteria, and to M2 macrophages, by exposure to Th2-type responses (Zarif et al., 2016). Dendritic cells are another type of monocyte-derived immune cells. These cells can be polarized *in vitro* by stimulation with appropriate factors and used in human research.

4.1 Quantification of cytokines

To determine the cytokine production associated with MoDCs and MDM activation, the levels of IL-1β, IL-6, TNF-α, IL-12p70 and the anti-inflammatory cytokine IL-10 were quantified by ELISA in culture supernatants of MoDCs and MDM stimulated with *S. epidermidis* at a MOI of 1:10 (1 MoDC/MDM to 10 Bacteria) after 24h incubation (Fig.17). The production of the chemokine IL-8 (CXCL8) by these cells was also quantified. Culture supernatants of MoDCs and MDM stimulated with LPS were used as positive controls. The supernatants of MoDCs and MDM unstimulated (incubation with only medium as a control for the WT and mutant strains or medium with chloramphenicol as a control for the complemented strain) were used as negative controls.

In general, the M1 macrophages had a greater magnitude of response compared to the other cell types. There was less production of pro-inflammatory cytokines TNF-α and IL-12p70 by M1 macrophages when stimulated with the mutant strain compared to the stimulation with the WT. The production of IL-1β also seems to be reduced in this condition, although without statistical significance. Concerning M2 polarized macrophages, the IL-1β levels are significantly decreased in stimulation with the mutant strain, compared to stimulation with WT.

As for dendritic cells stimulated with *S. epidermidis*, there were no significant differences in cytokine production between the stimulation conditions with the mutant and WT strains. The IL-8 chemokine was produced in large amount, mainly by macrophages, although at no different amounts between strains.


DC
M1
M2
A
B
C
IL-8 [pg/ml]
IL-1β [pg/ml]
IL-6 [pg/ml]
M
M+Cm
LPS
WT
ΔT
ΔT::T
**
**

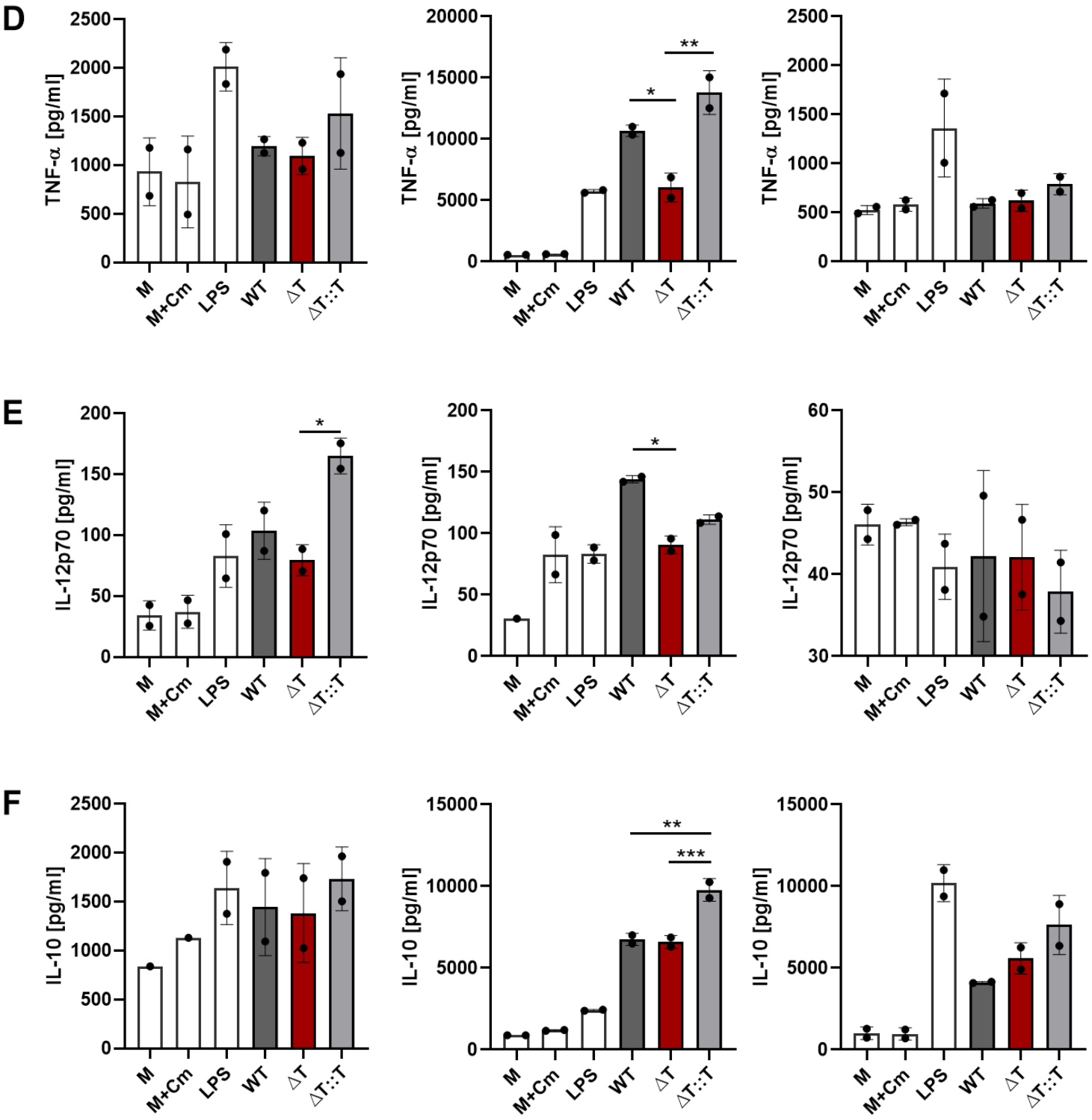


Fig. 17 - Quantification of cytokine levels in supernatants of MoDCs and MDM stimulated with *S. epidermidis*. MoDC and M1/M2 macrophages were stimulated with *S. epidermidis* at a MOI of 1:10 (1 MoDC/MDM: 10 Bacteria). After 24h incubation, the levels of the cytokines **(A)** IL-8, **(B)** IL-1β, **(C)** IL-6, **(D)** TNF-α, **(E)** IL-12p70 and **(F)** IL-10 produced by these cells were quantified in culture supernatants by ELISA. The LPS group represents the positive control which corresponds to culture supernatants of MoDC/MDM stimulated with LPS. As negative controls, culture supernatants from unstimulated MoDC/MDM were used: incubation with only medium (M group) as a control for the WT and ΔT or medium with chloramphenicol (M+Cm group) as a control for the ΔT::T. Representative result of three independent experiments, each performed with duplicates. Bars correspond to mean plus SD. Data were analysed by one-way ANOVA with Tukey post hoc test and the asterisks indicate a statistically significant difference. * P<0.05; ** P<0.01; *** P<0.001. Cm, chloramphenicol. M, medium. WT, Wild Type. ΔT, mutant strain. ΔT::T, complemented strain.

The results presented here with MoDC are not in agreement with those previously observed in the stimulation of mouse BMDC (Fig. 15). Here, the results demonstrate that the WTA does not seem to have much influence in terms of the cytokine profile produced by the human DCs. The magnitude of response is similar in the presence or absence of WTA. The recognition of the bacteria by mouse and human dendritic cells may be activating different signalling pathways.

In macrophages, the lack of WTA leads to a decrease in the production of certain pro-inflammatory cytokines in the condition of stimulation with the ΔT strain. The fact that the absence of WTA leads to a decrease of phagocytosis in RAW macrophages (Fig. 13 and 14) and less production of pro-inflammatory cytokines in human macrophages (Fig.17), suggests that these polymers are important to stimulate the functions of macrophages in the control of infection by this bacterium.

The differences in cytokines production by human dendritic cells and macrophages when stimulated with bacteria can be explained by the divergent roles of these cells in the immune response. Although most innate receptors are common to both types of cell, there are differences in signal transduction pathways after PRR recognition that are related to their distinct functions (Zanoni and Granucci, 2009).

4.2 Evaluation of human MoDC activation

To evaluate the maturation of MoDC upon infection with *S. epidermidis* strains, the surface expression of activation markers was determined by flow cytometry after 24h incubation. The gating strategy used to analyse the population of live dendritic cells is shown in Fig. 18A. The expression of activation markers HLA, CD80, CD83 and CD86 by MoDC is shown in the overlapping histograms in Fig. 18B and graphs of the Fig.18C. The expression of these markers in cells unstimulated and stimulated with LPS was evaluated as a control.

In the analysis of Fig. 18, it is possible to verify that part of the dendritic cell population is already stimulated before infection with *S. epidermidis*. This can be seen by the expression of the activation markers in cells not submitted to infection (shaded in grey in the histograms and M group in the graphs).

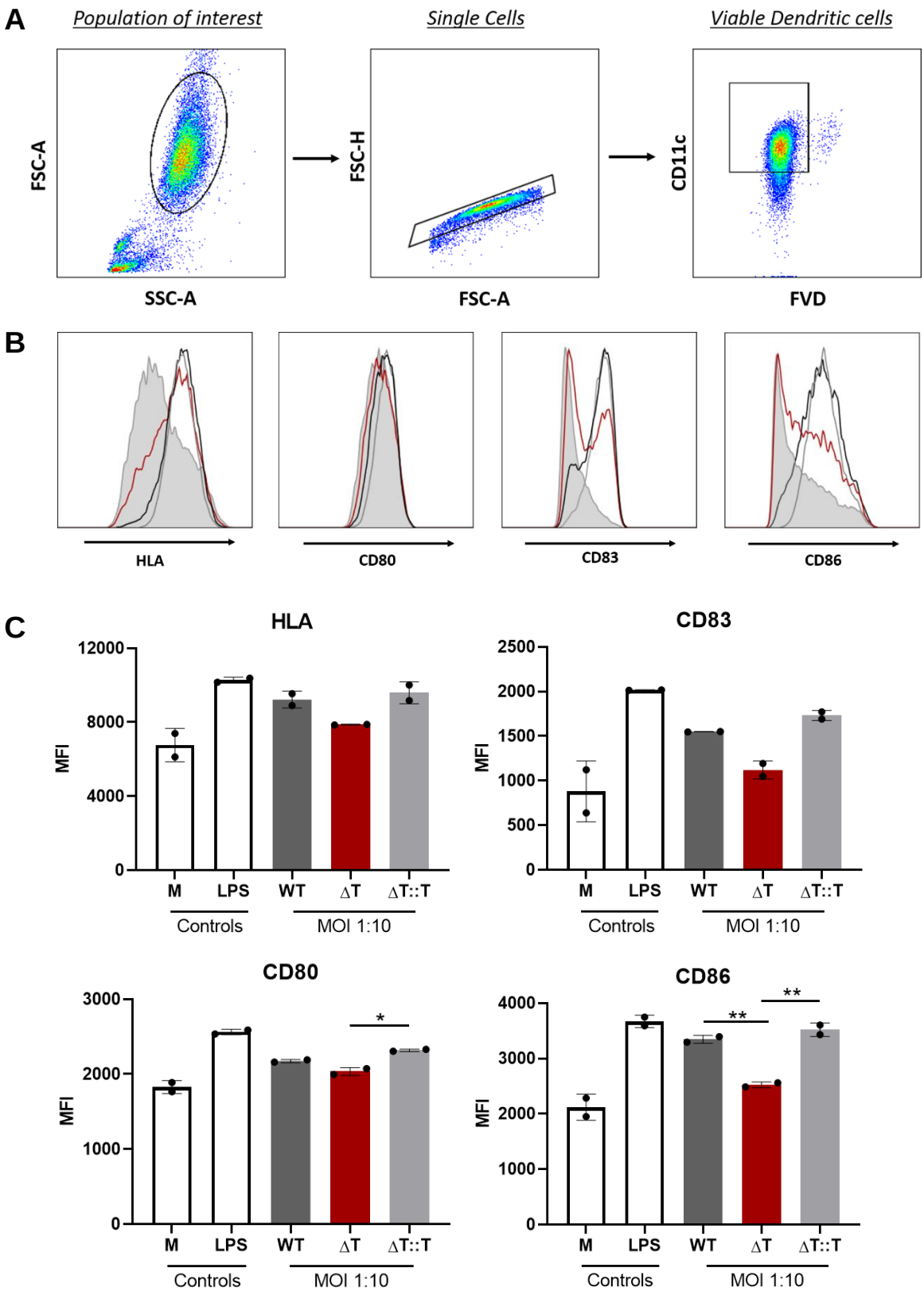


Fig. 18 – Maturation analysis of MoDCs stimulated with *S. epidermidis*. MoDCs were stimulated with *S. epidermidis* at a MOI of 1:10 (1 DC: 10 Bacteria) and the expression of surface activation markers were evaluated after 24h incubation. **(A)** The gating strategy to analyse the population of viable dendritic cells consisted in the selection of the population of single dendritic cells that do not label with FVD. **(B)** An overlay of histograms is shown for each activation marker (HLA, CD80, CD83 and CD86) that represents unstimulated MoDCs (shaded in grey) and MoDCs stimulated with the WT (black line), mutant (red line) and complemented (grey line) strains of *S. epidermidis*. **(C)** The expression of these activation markers in MFI units. The LPS group represents the positive control which corresponds to MoDCs stimulated with LPS. As negative controls, unstimulated MoDCs were used (M group, incubation with only medium). Representative result of three independent experiments, each performed with duplicates. Bars correspond to mean plus SD. Data were analysed by one-way ANOVA with Tukey post hoc test and the asterisks indicate a statistically significant difference. * P<0.05; ** P<0.01. M, medium. WT, Wild Type. ΔT, mutant strain. ΔT::T, complemented strain.

In general, the stimulation with the different *S. epidermidis* strains led to an increase in the expression of activation markers. Therefore, the bacterium is being recognized and activating dendritic cells. The biggest difference in expression of the surface markers was found for the co-stimulatory molecule CD86 which was significantly less expressed in cells stimulated with the mutant strain compared to stimulation with WT. Although the difference to the other markers is not significant, their expression has always been reduced in cells infected with the mutant.

Overall, the human dendritic cells seem to respond to infection with the mutant strain but in a less pronounced manner than in infection with the WT. Despite this, all strains of *S. epidermidis* seem to have the ability to activate dendritic cells and the capacity to induce antigen presentation. However, it would be interesting to analyse in different infection times to evaluate the activation kinetics of these cells.

5. Measurement of biofilm formation

As *S. epidermidis* biofilm formation is associated with the development of chronic infections, the impact of WTAs on biofilm growth was evaluated. To assess whether the absence of WTAs could compromise the biofilm production in *S. epidermidis*, a semiquantitative and quantitative measurement of the biofilms produced was performed. To achieve this, the optical density was measured and the CFU were counted for each mildly sonicated biofilm suspension. 48 hours-old biofilms were tested for each strain and the result of two independent measurements is shown in Fig. 19.

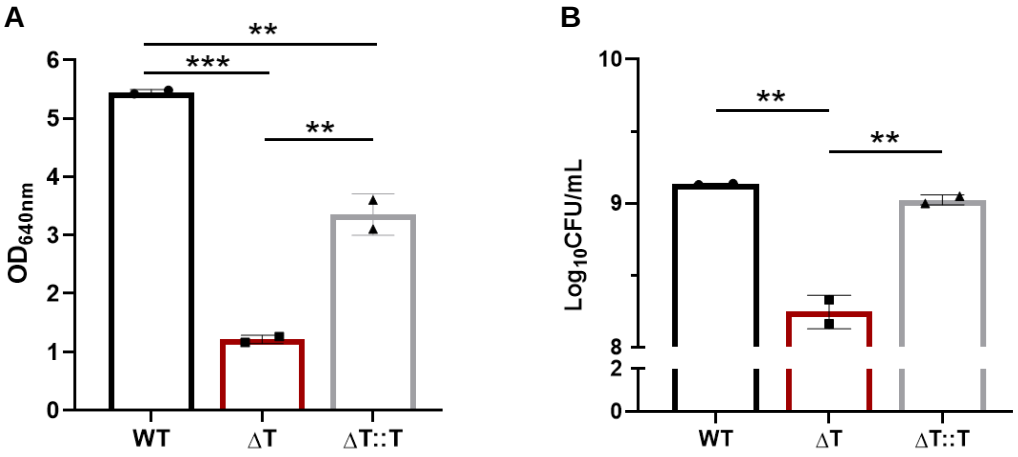


Fig. 19- Evaluation of *S. epidermidis* biofilm formation. Sonicated suspensions of biofilms with 48h of growth were used to compare biofilm production by different strains. The comparative analysis was performed by determining **(A)** OD_{640nm} and **(B)** CFU counts for two sonicated biofilm suspensions. Data were analysed by one-way ANOVA with Tukey post hoc test and the asterisks indicate a statistically significant difference. ** P<0.01; *** P<0.001. OD, optical density. WT, Wild Type. ΔT, mutant strain. ΔT::T, complemented strain.

Analysis of the biofilm production by the bacterial strains revealed that the *tagO* mutant displayed a reduced capacity to form biofilm *in vitro* compared to the WT strain, as can be seen by the low OD value and lower CFU counts (Fig. 19). In fact, the observation of the wells before the washing steps showed that the *tagO* mutant appears to form a “pellicle” of cells loosely attached to the polystyrene surface (Fig.20).

The “pellicle”, previously designated by Vergara-Irigaray *et al.* (2008), was easily detached when rinsed, revealing that *tagO* mutant exhibited poor adhesion to the plate. Thus, the results indicate that the absence of WTAs could compromise the initial adhesion, a critical step for the biofilm formation.

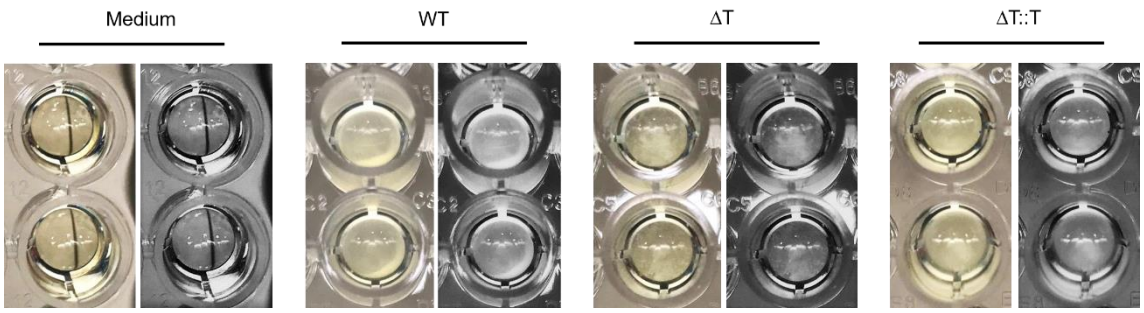


Fig. 20 – Comparison of biofilm formation by *S. epidermidis* strains grown in TSB supplemented with human AB serum and glucose for 48h at 37ºC and 80 rpm. Representative images showing two biofilms from each strain (1biofilm/well) and wells with only culture medium as a control in 96 well-plates. Duplicate images with different contrast are shown and taken before proceeding with the washing step. WT, Wild Type. ΔT, mutant strain. ΔT::T, complemented strain.

Our data were consistent with previous studies of *S. epidermidis* and *S. aureus tagO* mutants (Holland et al., 2011; Vergara-Irigaray et al., 2008). These studies also reported that *tagO* mutant, growing overnight in liquid culture, form macroscopic aggregates that rapidly settled at the bottom of the tubes (Holland et al., 2011; Vergara-Irigaray et al., 2008). The increased hydrophobicity of bacterial surface observed in *tagO* mutants (Holland et al., 2011; Kohler et al., 2009) is thought to contribute for an enhanced intercellular aggregation and limit the interactions with surfaces, which can consequently affect the biofilm formation (Holland et al., 2011; Vergara-Irigaray et al., 2008). Also, Holland et al., 2011 reported a decrease in the expression of the *icaADBC* operon and reduced PNAG production in *S. epidermidis tagO* mutant, which directly compromised the biofilm formation and indirectly by contributing to the increase in surface hydrophobicity (Holland et al., 2011).

CHAPTER 4: FINAL CONSIDERATIONS

WTA are abundant cell wall polymers that play numerous and varied roles in bacterial physiology and their interaction with the external environment. The use of mutants deficient in WTA biosynthesis allows specifically studying the impact of this polymer of interest. Here, we used a strain mutant lacking *tagO*, the first gene in the WTA biosynthetic pathway, which lacks WTA polymers. Even though this bacterium displays a range of defects, it shows a viable phenotype in laboratory conditions.

In the present work, the deletion of the *tagO* gene in *S. epidermidis* has shown to have pleiotropic effects. The results obtained for the *tagO* mutant were compared with those of the WT strain and correlate with the well-established functions for this polymer. Although prior research in *S. epidermidis* is limited or non-existent, studies in *S. aureus* have allowed, in part, broadening the available literature on the subject.

The characterization of bacterial growth and morphological analysis allowed to verify that the mutant strain has a compromised cell division, which is consistent with the role already reported for WTA in this process. The absence of WTA also appears to interfere with the ability of *S. epidermidis* to form a biofilm, possibly due to a difficulty for the mutant strain to establish initial adhesions to the plate surface.

The WTA polymers also appear to influence the interaction between the bacteria and the immune system. The lack of WTA seems to compromise the phagocytic process in RAW macrophages but, on the other hand, it leads to greater production of pro-inflammatory cytokines by mouse dendritic cells. Presumably, the WTA are important for the recognition of the bacteria by macrophages, but their presence can “mask” possible components of the cell wall capable of inducing pro-inflammatory responses in dendritic cells.

In human cells stimulated with *S. epidermidis*, while the WTA does not seem to have much influence on the cytokine profile produced by DCs, it leads to an increase in the production of certain pro-inflammatory cytokines by macrophages. Then, the presence of these polymers in the cell wall seems to be important to stimulate the functions of macrophages. The inconsistent results for mouse and human dendritic cells may possibly be due to differences in bacterial recognition between species.

The mutant strain complemented with the *tagO* gene (Δ*tagO::tagO*) was used as a control to verify if the observed phenotype for the mutant strain was related to the lack of WTAs. It would be expected that the complemented strain would present a phenotype and induce responses similar to those observed for the WT. However, there was some discrepancy between these two strains in the different trials. Until now, the only reasonable explanation for this disagreement has been a 100-fold increase in the expression of the *tagO* gene in the complemented strain compared to expression in the WT (França et al., unpublished). It would be important to assess how much this increase in gene expression is reflected in the level of polymer production. In *S. aureus*, strains with an increased WTA content were more active in induction of the skin abscess in an animal model than WTA^{low} strains (Wanner et al., 2017). Previously the same group reported that WTA contributes to $CD4^{+}$T cells activation through an MHC Class II-dependent mechanism (Weidenmaier et al., 2010). Then, it was suggested that the correlation between the amount of WTA and the abscess induction depends on the greater ability of WTA^{high} strains to induce $CD4^{+}$ T cell proliferation and IFN-γ production than WTA^{low} strains (Wanner et al., 2017). In agreement, production of IL-12p70, which leads to IFN-γ-mediated responses, was found increased in mouse dendritic cells infected with the complemented strain, as compared to WT (Fig. 15D).

Considering the obtained results, we can assume that WTA has some influence on the immune response to the bacterium developed by the host. These polymers seem to be important for the response elicited in macrophages, but on the other hand, they might compromise a response by dendritic cells which, consequently, has implications for the response of T cells. Although a role for this molecule in immune evasion can be thus hypothesized, it remains to unravel the changes that occur in the cell wall in its absence and whether the response that develops will be more effective against the bacteria. Since WTA represent a significant component of the cell wall of *S. epidermidis*, the removal of these polymers should have a major impact on the structure and composition of the cell wall. This is supported by the increase in cell size that seems to be occurring in their absence (Fig.12B) and by studies that show a greater sensitivity of the cell wall to external conditions (Holland et al., 2011; Vergara-Irigaray et al., 2008). The lack of WTA may have been compensated for other components of the cell wall and may have exposed certain components of the cell wall that were otherwise hidden from the immune system. However, more studies will be needed to help support our hypotheses.

CHAPTER 5: FUTURE PERSPECTIVES

Our research provides only a first indication for a role of wall teichoic acids in *S. epidermidis* establishment of infection in the host. A detailed characterization of the cell wall of these *S. epidermidis* strains would be crucial to assess whether the depletion of WTA has an impact on the proportion of other cell wall components. Relatedly, an immunoassay with antibodies specific for cell wall components would be equally important to assess whether WTA depletion interferes with the exposure of possible PAMPs to host immune system and if certain components may become more accessible for immune recognition. The link between the antibody and the respective component to be tested would be assessed by fluorescence microscopy or flow cytometry. Also, the incubation of immune cells with antagonist antibodies to certain receptors, before infection, may help to unravel the mechanism by which the different strains are being recognized. These studies could help to explain the data obtained here in phagocytosis and cytokine profile assays involving the *tagO* mutant. The production of intracellular ROS by macrophages could also be evaluated to elucidate whether WTAs influence this key mechanism that unfolds inside macrophages after phagocytosis.

A future research objective would be the characterization of the adaptive immune response that would be developed in the absence of WTA and to evaluate whether it could be more effective compared to that induced by WT. For this purpose, a TCR transgenic mouse system (OTII) could be used to determine the impact of *tagO* deletion in T helper cell polarization into different phenotypes (Th1, Th2, Th17). Naïve $CD4^+$T and dendritic cells would be incubated with the bacteria for a few days and then the polarized T cell phenotype evaluated. T cell surface markers, the cytokine profile present in culture supernatants and the intracellular expression of cytokines would be evaluated.

More data would be obtained relatively to the role of specific immune cell populations in host resistance/susceptibility to *tagO*-deficient bacteria using *in vivo* immunosufficient and immunodeficient mouse models.

To improve our knowledge on the importance of WTA in the infection caused by *S. epidermidis,* future research should focus on biofilms and their interaction with the innate immune system since this form of growth is the responsible for the persistence of the bacteria in the host and, therefore, for the chronic character attributed to this infection.

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