UNIVERSIDADE FEDERAL DE MINAS GERAIS INSTITUTO DE CIÊNCIAS BIOLÓGICAS DEPARTAMENTO DE BIOLOGIA GERAL PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA

Tese de Doutorado

Involvement of the inflammasome in the response of host cells during *Staphylococcus aureus* infection

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Belo Horizonte - Brasil
Rennes - France
2019

Elma Lima Leite

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Tese apresentada ao programa de Pós-Graduação em Genética do Departamento de Biologia Geral do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais como requisito parcial para obtenção do título de Doutora em Genética.

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ATA DA DEFESA DE TESE

Elma Lima Leite

Às quatorze horas do dia **20 de agosto de 2019**, reuniu-se, no Instituto de Ciências Biológicas da UFMG, a Comissão Examinadora de Tese, indicada pelo Colegiado do Programa, para julgar, em exame final, o trabalho intitulado: **"Involvement of the inflammasome in the response of host cells during Staphylococcus aureus infection "**, requisito para obtenção do grau de Doutora em **Genética.** Abrindo a sessão, o Presidente da Comissão, **Vasco Ariston de Carvalho Azevedo**, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra à candidata, para apresentação de seu trabalho. Seguiu-se a arguição pelos Examinadores, com a respectiva defesa da candidata. Logo após, a Comissão se reuniu, sem a presença da candidata e do público, para julgamento e expedição de resultado final. Foram atribuídas as seguintes indicações:

Prof./Pesq.	Instituição	CPF	Indicação
Vasco Ariston de Carvalho Azevedo	UFMG	283.171.225-49	APROVADA
Jean-Marc Chatel	INRA- France	18CU32060	APROVADO
Eric Guédon	INRA- France	19DHS79458	APROVADO
Aristóteles Góes Neto	UFMG	544.348.825-20	APROVADA
Gwénaël Jan	INRA- France	14AC20370	aprovada.
Anderson Mioyshi	UFMG	—	Aprovado.
Nadia Berkova	INRA- France	19KR74797	aprovada

Pelas indicações, a candidata foi considerada: APROVADA

O resultado final foi comunicado publicamente à candidata pelo Presidente da Comissão. Nada mais havendo a tratar, o Presidente encerrou a reunião e lavrou a presente ATA, que será assinada por todos os membros participantes da Comissão Examinadora.

Belo Horizonte, 20 de agosto de 2019.

Vasco Ariston de Carvalho Azevedo - Orientador _____

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"Involvement of the inflammasome in the response of host cells during Staphylococcus aureus infection "

Elma Lima Leite

Tese aprovada pela banca examinadora constituída pelos Professores:

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Belo Horizonte, 20 de agosto de 2019.

“*E, naquele dia, sendo já tarde, disse-lhes:*

Passemos para a outra margem”.

Marcos 4:35

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List of Abbreviations

ANOVA: Analysis of variance

ASC: Apoptosis-associated speck-like protein containing a caspase recruitment domain

ATP: Adenosine triphosphate

BHI: Brain Heart Infusion

CA-MRSA: Community-Acquired Methicillin-Resistant *S. aureus*

CARD: Caspase recruitment domain

DC: Dendritic cells

DMEM: Dulbecco's Modified Eagle Medium

DNA: Deoxyribonucleic acid

ELISA: enzyme-linked immunosorbent assay

FACS: Fluorescence-activated cell sorting

Fn: Fibronectins

FnBPA and FnBPB: Fibronectins Binding Proteins (A and B)

Gent100: Gentamicin 100 µg/mL

Gent25: Gentamicin 25 µg/mL

HA-MRSA: Hospital-Associated Methicillin-Resistant *S. aureus*

ICB: Institudo de Ciências Biológicas

ICE: IL-1-converting enzyme ou enzyme transformant IL-1

IFN-γ: interferon gamma

IL-1β: interleukin-1 beta

INRA: French National Institute for Agricultural Research

LDH: Lactate dehydrogenase

LPS: lipopolysaccharide

MOI: Multiplicity of infection

MRSA: Methicillin-resistant *Staphylococcus aureus*

MS: Mass spectrometry

MSCRAMMs: Microbial Surface Components Recognizing Adhesive Matrix Molecules

NK: Natural killers

NLR: NOD-like receptor

NOD: nucleotide binding oligomerization domain

PAMPs: Pathogen associated molecular pattern

PBMC: Peripheral blood mononuclear cells

PBS: Phosphate Buffered Saline

PMA: phorbol 12-myristate 13-acetate

PSM: Phenol-soluble modulins

PVDF: Polyvinylidene fluoride membrane

ROS: reactive oxygen species/espèces réactives de l'oxygène

RPMI-1640: Roswell Park Memorial Institute medium

***S. aureus*:** *Staphylococcus aureus*

FCS: Fetal Calf Serum

SCCmec: staphylococcal cassette chromosome mec

SCV: Small colony variant

SD: Standard deviation

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SE: Staphylococcal enterotoxins

TBS: Tris-buffered saline

Th: T helper cells

TLR: Toll-like receptor

TNF-α: Tumor Necrosis Factor-α

TPA: 12-O-tetradecanoylphorbol 13-acetate

UFMG: Universidade Federal de Minas Gerais

V: Volts

WB: Western Blotting

WT: Wild type

Resumo

Staphylococcus aureus (*S. aureus*) é uma bactéria Gram-positiva altamente adaptativa e versátil que pode causar uma ampla gama de doenças infecciosas em humanos ou animais. Nos estágios iniciais da infecção, a interação entre *S. aureus* e as células hospedeiras causa inflamação, cujo processo depende de vários mecanismos celulares complexos e de uma cascata de sinalização coordenada principalmente pelas citocinas e sua ativação. Os principais fatores que determinam o início da inflamação e sua progressão são *pathogen-/microbe-associated molecular patterns* (PAMPs/ MAMPs), *danger-associated molecular patterns* (DAMPs), *pattern recognition receptors* (PRRs), e o sistema imune inato e adaptativo. Inflammassomas, complexos de sinalização multiprotéicos que se agrupam após a detecção de PAMPs/DAMPs, são fatores-chave da resposta imune inata. A maioria dos inflamassomas é formado por um receptor NLR, uma proteína adaptadora ASC e uma Caspase-1 ativa. Uma vez montados, os inflamassomas atuam como plataformas ativadoras que promovem a maturação das citocinas pró-inflamatórias IL-1β e IL-18 em suas formas ativas. Seu papel em diferentes tipos de fagócitos profissionais durante a infecção por *S. aureus* tem sido extensivamente estudado. Por outro lado, o conhecimento sobre o envolvimento de inflamassomas em fagócitos não profissionais (por exemplo, células epiteliais, células endoteliais ...) é muito fragmentado. O objetivo deste trabalho foi estudar o papel da ativação do inflamassoma durante a infecção por *S. aureus* em fagócitos não profissionais. Adotamos um modelo de infecção por *S. aureus* desenvolvido em células osteoblásticas (linhagem MG-63). Análises de Western blot e ELISA foram usadas para controlar a ativação de caspase-1 e a maturação de IL-1β na interação de *S. aureus*/MG-63. Geramos células MG-63 deletadas do gene da caspase-1 (CASP1$^{-/-}$ MG-63) usando a abordagem CRISPR/Cas9. Isso nos permitiu demonstrar o envolvimento do inflamassoma na resposta imune inata a *S. aureus* por fagócitos não profissionais. Inesperadamente, mostramos que *S. aureus* prolifera nas células CASP1$^{-/-}$ MG-63, sugerindo que Casp1 (ou um fenômeno regulado pela Casp1) está envolvido na eliminação do *S. aureus* intracelular. Descobrimos que a formação do inflamassoma nas células MG-63 é mais tardia que nos fagócitos profissionais. Além disso, o uso de cepas de *S. aureus* (mutantes deletados) expressando ou não *Phenol-Soluble Modulins* (PSM) nos permitiu determinar o papel das PSMs como desencadeador. Assim, demonstramos que *S. aureus* é capaz de ativar a formação de inflamassomas em células não fagocíticas (MG-63) e que os PSMs estão envolvidos nesse fenômeno.

Palavras-chave: Osteoblástos, *Staphylococcus aureus*, inflammasomes, caspase-1, IL-1β

Abstract

Staphylococcus aureus (*S. aureus*) is a highly adaptive and versatile Gram-positive bacterium that can cause a wide range of infectious diseases in humans or animals. In the early stages of infection, the interaction between *S. aureus* and the host cells causes inflammation, the process of which depends on several complex cellular mechanisms and a signaling cascade coordinated mainly by cytokines and their activation. The key factors determining the initiation of inflammation and its progression are pathogen/ microbe-associated molecular patterns (PAMPs/MAMPs), danger-associated molecular patterns (DAMPs), pattern recognition receptors (PRRs), innate and adaptive immunity. Inflammasomes, multi-protein signaling complexes that assemble after sensing PAMPs/ DAMPs, are key players of the innate immune response. Most inflammasomes are formed by an NLR receptor, an ASC adapter protein, and active caspase-1. Once assembled, inflammasomes act as activating platforms that promote the maturation of pro-inflammatory cytokines IL-1β and IL-18 in their active forms. Their role in different types of professional phagocytes during *S. aureus* infection has been extensively studied. Conversely, knowledge about the involvement of inflammasomes in non-professional phagocytes (e.g. epithelial cells, endothelial cells...) is very fragmented. This work aimed to study the role of inflammasome activation during *S. aureus* infection in non-occupational phagocytes. We adopted a model of *S. aureus* infection developed on osteoblastic cells (MG-63 line). Western blot and ELISA analyses were used to control caspase-1 activation and IL-1β maturation in the *S. aureus* MG-63 interaction. We generated MG-63 cells deleted from the caspase-1 gene (CASP1$^{-/-}$ MG-63 cells) using the CRISPR/Cas9 approach. This allowed us to demonstrate the involvement of inflammasomes in the innate immune response to *S. aureus* by non-professional phagocytes. Unexpectedly, we have shown that *S. aureus* proliferates in CASP1$^{-/-}$ MG-63 cells, suggesting that Casp1 (or Casp1-regulated phenomenon) is involved in the elimination of intracellular *S. aureus*. We found that the formation of the inflammasome in MG-63 cells is later than in professional phagocytes. Besides, the use of *S. aureus* strains (deletion mutants) expressing or not Phenol-Soluble Modulins (PSM) allowed us to determine the role of PSM in this trigger. Thus, we have demonstrated that *S. aureus* can activate inflammasome formation in non-professional phagocytes (MG-63) cells and that PSM is involved in this phenomenon.

Key words: Osteoblasts, *Staphylococcus aureus*, inflammasomes, caspase-1, IL-1β

Résumé

Staphylococcus aureus (*S. aureus*) est une bactérie à Gram positif hautement adaptative et polyvalente qui peut causer un large éventail de maladies infectieuses chez l'homme ou l'animal. Lors des premières étapes de l'infection, l'interaction entre *S. aureus* et les cellules hôtes entraine une inflammation dont le processus dépend de plusieurs mécanismes cellulaires complexes et une cascade de signalisation coordonnée principalement par des cytokines et leur activation. Les facteurs clés déterminant l'initiation de l'inflammation et sa progression sont les *pathogen-/microbe-associated molecular patterns* (PAMPs/MAMPs), les *danger-associated molecular patterns* (DAMPs), les *Pattern Recognition Receptors* (PRRs), l'immunité innée et adaptative. Les inflammasomes, complexes de signalisation multiprotéines qui s'assemblent après la détection de PAMPs/DAMPs sont des facteurs clés de la réponse immunitaire innée. La plupart des inflammasomes sont composés d'un récepteur NLR, d'une protéine adaptatrice ASC et de la caspase-1 activée. Une fois assemblés, les inflammasomes agissent comme des plateformes activatrices qui favorisent la maturation des cytokines pro-inflammatoires IL-1β et IL-18 sous leurs formes actives. Leur rôle dans les différents types de phagocytes professionnels au cours de l'infection à *S. aureus* a été largement étudié. A l'inverse, les connaissances sur l'implication des inflammasomes dans les phagocytes non-professionnels (e.g. cellules épithéliales, endothéliales…) sont très parcellaires. L'objectif de ce travail était d'étudier le rôle de l'activation de l'inflammasome lors d'une infection à *S. aureus* sur des phagocytes non-professionnels. Nous avons adopté pour cela un modèle d'infection à *S. aureus* mis au point sur des cellules ostéoblastiques (lignée MG-63). Des analyses Western blot et ELISA ont été utilisées pour contrôler l'activation de Caspase-1 et la maturation de l'IL-1β lors de l'interaction *S. aureus* MG-63. Nous avons généré des cellules MG-63 délétées du gène caspase-1 (CASP1$^{-/-}$ MG-63 cells) en utilisant l'approche CRISPR/Cas9. Ceci nous a permis de démontrer l'implication des inflammasomes dans la réponse immune innée à *S. aureus* par des phagocytes non-professionnels. De façon inattendue, nous avons montré que *S. aureus* prolifère dans les CASP1$^{-/-}$ MG-63 cells, suggérant que Casp1 (ou les phénomènes régulés par Casp1) est impliquée dans l'élimination de *S. aureus* intracellulaire. Nous avons constaté que la formation de l'inflammasome dans les cellules MG-63 est cependant plus tardive que dans des phagocytes professionnels. De plus, l'utilisation de souches de *S. aureus* (mutants de délétion) exprimant ou non des *Phenol Soluble Modulins* (PSM) nous a permis de déterminer le rôle des PSM dans ce déclenchement. Ainsi, nous avons démontré que *S. aureus* est capable d'activer la formation d'inflammasome dans des cellules (MG-63), phagocytes non professionnels et que les PSM sont impliqués dans ce phénomène.

Mots clés: Ostéoblastes, *Staphylococcus aureus*, inflammasomes, caspase-1, IL-1β

Collaborations

This PhD thesis was prepared in collaboration between the Federal University of Minas Gerais (UFMG) (Belo Horizonte, Brazil) and the Institute of Agricultural Sciences, Agro-Food, Horticulture and Landscape (Rennes, France). The work presented in this manuscript was performed at the Laboratory Science & Technologie du Lait & de l'Oeuf (STLO) at the Institut National de la Recherche Agronomique (INRA), located in Rennes, France under the supervision of Dr. Nadia Berkova and co-supervision of Dr. Yves Le Loir and at the Laboratory of Cellular and Molecular Genetics in the Institute of Biological Sciences, Federal University of Minas Gerais under the supervision of Prof. Vasco Azevedo. In this context, the objective of my project was to investigate whether *Staphylococcus aureus* invasion can induce the activation of inflammasomes in non-professional phagocytes, using osteoblast-like cells as a model of cells infections and which *S. aureus* virulence factors are involved in inflammasomes activation during infection. To address these questions, we generated knock-out osteoblastic-like cells for the caspase-1 gene using CRISPR/Cas9-based genome editing in collaboration with Dr. Arthur Gautron and Dr. David Gilot, IGDR, Rennes, France and Pr. Petr Broz, University of Lausanne, Switzerland. Inflammasomes activation was investigated in infected WT MG-63 *vs* CASP1$^{-/-}$ MG-63 cells through immunological detection of active caspase-1 and active IL-1β. Moreover, bacterial clearance in WT MG-63 *vs* CASP1$^{-/-}$ MG-63 cells was investigated using gentamicin protection assay and immunofluorescence methodology. To decifer the role of principal *S. aureus* virulence factors in inflammasomes activation of infected osteoblasts, we used wild type and mutant strains of *S. aureus* kindly provided by Pr. Michael Otto, NIH, USA and Pr. Friedrich Goetz, Tubiengen University, Germany. Our work brings new knowledge on the pathogenic mechanisms of *S. aureus*, a major cause of chronic infection in humans and animals.

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Manuscript structure

This manuscript is subdivided into six major parts:

I. Chapter 1. Bibliographic Synthesis. A general introduction to the following topics: (I) microbiological aspects of *Staphylococcus* aureus, as well as diseases caused by this pathogen and associated pathogenic processes*;* phenol-soluble modulins (PSMs) and their role in pathogenesis; (II) the inflammasome, a primordial complex of innate immunity; (III) innate immunity receptors; (IV) molecular platform: the inflammasome.

II. Chapter 2. Problem statement of the project thesis.

III. Chapter 3. Literature review. Strain and cell type-specificity of host cell response to *Staphylococcus aureus* invasion.

IV. Chapter 4. Original article - Involvement of caspase-1 in inflammasomes activation and bacterial clearance in *S. aureus*-infected osteoblasts. In this part it will be described (the methodology, the results and the discussion) of the work.

V. Chapter 5. Results and general discussion

VI. Chapter 6. General conclusion and perspectives of the work performed during PhD project.

General introduction / Introduction générale

Staphylococcus aureus (*S. aureus*), is a Gram-positive bacterium capable of colonizing both human and animal hosts and have the potential to cause a wide range of diseases including pneumonia, bacteremia, sepsis, endocarditis, osteomyelitis, device-related infections, toxic-shock syndrome, brain abscesses, mastitis among other diseases (FITZGERALD, 2012; FOSTER, 1996; WERTHEIM et al., 2005). *S. aureus* is a predominant cause of bone and joint infections (BJI), a disease associated with high morbidity and high health costs worldwide (DEL POZO; PATEL, 2009; TUCHSCHERR; GERACI; LÖFFLER, 2017; WRIGHT; NAIR, 2010; ZIMMERLI; TRAMPUZ; OCHSNER, 2004). *S. aureus*-induced BJI can evolve into chronic infection and become highly refractory to antibiotic treatment (LOWY, 1998). This is probably due to the several strategies that this bacterium has developed to invade host cells and to survive in the intracellular environment (EIFF; PETERS; PROCTOR, 2001; MONTGOMERY; DAVID; DAUM, 2015; TUCHSCHERR; GERACI; LÖFFLER, 2017).

In recent years, considerable progress has been made in our understanding of how pathogens are recognized by the innate immune system that forms the first line of defense against infection (CARRILLO et al., 2017; NICHOLSON, 2016). The invasion of foreign microorganisms induces a spectrum of inflammatory responses in the infected host (**Fig. 1**). Generally, inflammation is classically described as an essential response of the host immune system that enables survival during infection or injury, and that maintains tissue homeostasis under a variety of noxious conditions (CHEN et al., 2017a; CRUVINEL et al., 2010; MEDZHITOV, 2010a; RANKIN; ARTIS, 2018).

The type of inflammatory response depends on the category of the inflammatory trigger. It was demonstrated that bacterial pathogens are recognized by receptors of the innate immune system, such as Toll-like receptors (TLRs), resulting in the induction of various inflammatory mediators such as inflammatory cytokines and chemokines, as well as prostaglandins, which act on target tissues (CARRILLO et al., 2017; CHEN et al., 2017a; LUIS MUÑOZ-CARRILLO et al., 2019; TURNER et al., 2014). The acute inflammatory response is finished when the triggering agent is eliminated, the infection is cleared, and the damaged tissue is repaired. If this initial inflammatory response does not eliminate the pathogens, as in a case of chronic infections or unrepaired tissue damage, the adaptive immune system comes into play. The cells of the adaptive immune system translocate to the site of infection and begin to eliminate pathogens and damaged cells and tissue (MEDZHITOV, 2008, 2010a). However, in some chronic infections, inflammation can persist without adaptive immunity (CRUVINEL et al., 2010).

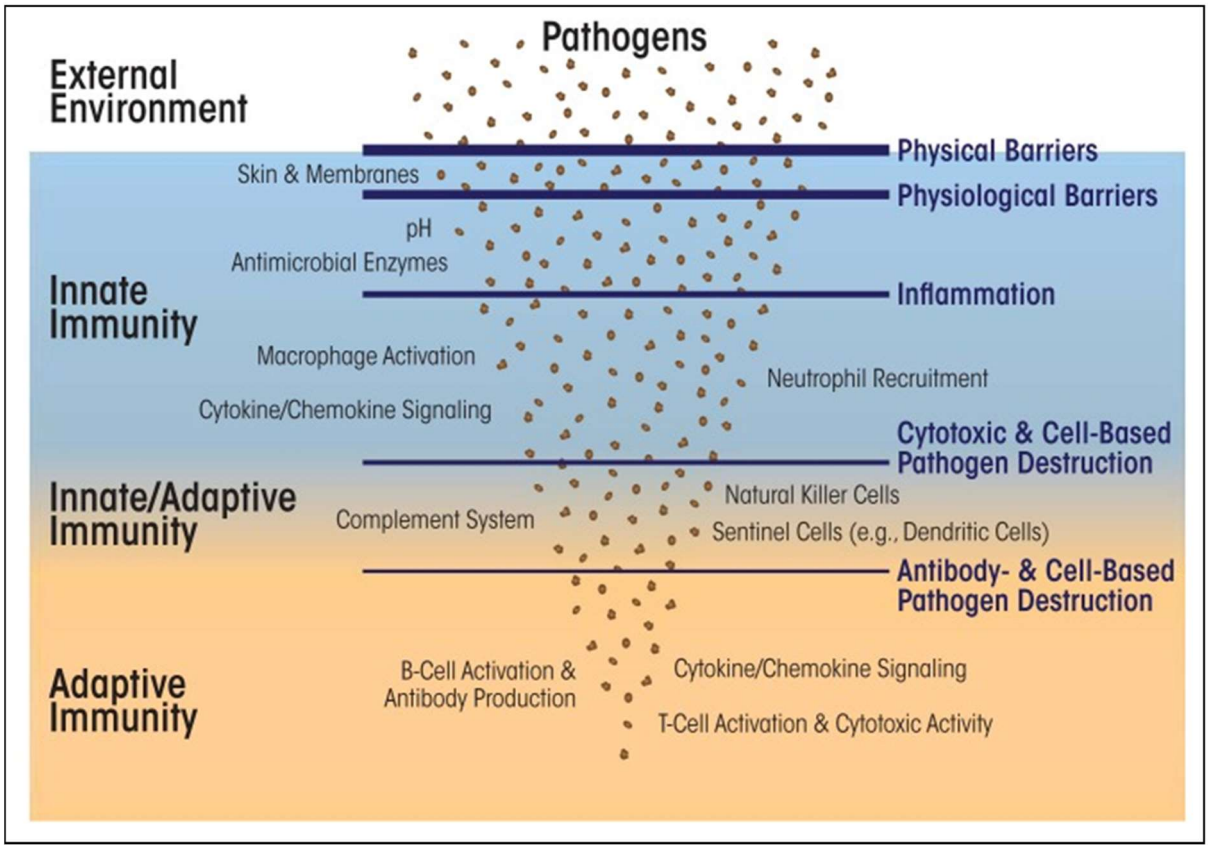

Figure 1: Overview of the immune system.

The innate response is characterized by being the first line of defense against infection in which leukocytes such as (e.g., neutrophils, monocytes, macrophages, etc.), are involved. These defense cells detect and attack other cells carrying pathogen-associated molecular patterns (PAMPs), and small proteins that signal pathogen invasion (i.e., cytokines and chemokines) or short peptides that directly attach to and restrict microbial pathogens. The cells of the adaptive immune system rely on T and B lymphocytes and proteins (i.e., antibodies) that detect and eliminate specific pathogens and also use cytokine/chemokine to signal and recruit other immune cells. Memory T and B lymphocytes have an essential role in immunity against pathogenic invasion. The complement system, along with natural killer cells and dendritic cells, straddles both innate and adaptive immunity. Adopted from (SPIERING, 2015).

Thus, the innate immune response plays a pivotal role in the defense against pathogens and is initiated through the pattern recognition receptors (PRRs). PRRs recognize pathogen/microbe-associated molecular patterns (PAMPs/MAMPs) and endogenous danger-associated molecular pattern (DAMPs), leading to the activation of host defense pathways that result in the clearance of the infection (CARRILLO et al., 2017; JANG et al., 2015; KUMAR; KAWAI; AKIRA, 2011; MOGENSEN, 2009; ROH; SOHN, 2018). Toll-like receptors (TLRs), a group of membrane-bound extracellular and endosomal receptors, sense the presence of infection through recognition of PAMPs. The innate immune response against microbes also involves a major inflammatory pathway known as inflammasomes (MARTINON; BURNS; TSCHOPP, 2002; SCHRODER; TSCHOPP, 2010).

Inflammasomes are multi-protein signaling complex that assembles after recognition of danger signals and/or pathogens by a family of cytosolic receptors called NLRs (nucleotide-binding domain leucine-rich repeat containing receptors) or PYHIN protein family members that consisting of immune sensors of intracellular DNA identified to activate inflammasomes (MARTINON; BURNS; TSCHOPP, 2002). Once assembled, inflammasomes initiate signaling by activation of downstream proteases, most notably caspase-1 and less frequently caspase-11, which then proteolytically mature and promotes the secretion of pro-IL-1β and pro-IL-18, cytokines strongly implicated in the pathology of BJI (BROZ; MONACK, 2013; LATZ, 2010; LATZ; XIAO; STUTZ, 2013a; MARTINON; BURNS; TSCHOPP, 2002; WILSON et al., 1994).

Furthermore, inflammasome activation triggers pyroptosis, an inflammatory form of cell death (BERGSBAKEN; FINK; COOKSON, 2009; YANG et al., 2019; YUAN et al., 2018). The role of NLRP1, NLRP3, NLRC4, NLRP6, NLRP7, NLRP12 and the PYHIN proteins AIM2 and IFI16 in assembling inflammasome and controlling the innate immune response against various microbes including different bacteria and the involvement of various bacterial factors have been intensively explored during the past decade (CANEPARO et al., 2018; LIN; ZHANG, 2017; SÁ; FESTA NETO, 2016; VLADIMER et al., 2013). Studies have shown that these inflammasomes form in response to various stimuli and are also subject to negative regulatory mechanisms, which cause the production of inflammatory cytokines to occur in a controlled manner after the onset of the inflammatory response (NAIK; DIXIT, 2010).

The NLRP3 inflammasome is the best characterized NLR so far. NLRP3, together with proteins ASC and caspase-1, form a cytoplasmic oligomeric complex known as the NLRP3 inflammasome, which plays a critical role in initiating innate immune responses, however, the molecular mechanisms of its activation are not fully understood (DAVIS; WEN; TING, 2011; DEIGENDESCH; ZYCHLINSKY; MEISSNER, 2018; MELEHANI et al., 2015). Assembly of the NLRP3 inflammasome has been shown to contribute to several pathologies like autoinflammatory syndromes, chronic inflammation, and metabolic diseases (DEIGENDESCH; ZYCHLINSKY; MEISSNER, 2018).

The role of inflammasomes formed in *S. aureus*-infected professional phagocytes that ingest and/or kill invasive bacteria has been extensively investigated. However, despite numerous works on the mechanism used by *S. aureus* to internalize into and survive in non-professional phagocytes, such as epithelial cells (KREMSEROVA; NAUSEEF, 2019; MA et al., 2019), the involvement of inflammasomes and processed IL-1β, which plays a crucial role in bone homeostasis in *S. aureus*-associated BJI, has not been investigated. This is of particular interest in the case of BJI as *S. aureus* is shown to internalize and to survive within the osteoblasts, generating chronic infections refractory to antimicrobial treatments. It is therefore

essential to develop new approaches to better understand the mechanisms by which staphylococci interact with osteoblasts and, in fine, generates damage to bones.

Staphylococcus aureus (*S. aureus*) est une bactérie à Gram positif capable de coloniser l'hôte humain ou animal et de provoquer un large panel d'infections allant de bénignes à potentiellement mortelles telles que pneumonie, septicémie, endocardite, ostéomyélite, infections associées aux dispositifs médicaux internes, syndrome de choc toxique, abcès, ou mammite entre autres maladies (FITZGERALD, 2012; FOSTER, 1996; WERTHEIM et al., 2005). *S. aureus* est notamment une cause fréquente d'infections des os et des articulations (BJI, pour l'anglais *Bone and Joint Infection*), maladie associée à une morbidité élevée et à d'importants coûts de santé dans le monde (DEL POZO; PATEL, 2009; TUCHSCHERR; GERACI; LÖFFLER, 2017; WRIGHT; NAIR, 2010; ZIMMERLI; TRAMPUZ; OCHSNER, 2004).

Les infections osseuses induites par *S. aureus* peuvent évoluer en infections chroniques, devenant très réfractaires aux traitements antibiotiques (LOWY, 1998). Cela est probablement dû aux différentes stratégies que cette bactérie à développer pour s'adapter et survivre à l'intérieur des cellules hôtes (EIFF; PETERS; PROCTOR, 2001; MONTGOMERY; DAVID; DAUM, 2015; TUCHSCHERR; GERACI; LÖFFLER, 2017).

Au cours des dernières années, notre compréhension de la manière dont le système immunitaire inné reconnaît les agents pathogènes a considérablement avancé (CARRILLO *et al.*, 2017; NICHOLSON, 2016). Une invasion de microorganismes étrangers induit un spectre de réponses inflammatoires chez l'hôte infecté (**Fig. 1**). L'inflammation est classiquement décrite comme une réponse essentielle de la réponse immunitaire de l'hôte qui permet la survie pendant une infection ou une blessure et maintient l'homéostasie tissulaire dans diverses conditions nocives (CHEN, L. *et al.*, 2017; CRUVINEL *et al.*, 2010; MEDZHITOV, 2010a; RANKIN; ARTIS, 2018).

Le type de réponse inflammatoire dépend de la catégorie du déclencheur inflammatoire. Il a été démontré que les bactéries pathogènes sont reconnues par les récepteurs du système immunitaire inné, tels que les *Toll-like receptors* (TLR), entraînant l'induction de divers médiateurs inflammatoires comme les cytokines et les chimiokines inflammatoires, ainsi que les prostaglandines, qui agissent sur les tissus cibles (CARRILLO *et al.*, 2017; CHEN, L. *et al.*, 2017; LUIS MUÑOZ-CARRILLO *et al.*, 2019; TURNER *et al.*, 2014). La réponse inflammatoire aiguë est terminée lorsque l'agent déclencheur de l'infection est éliminé et que les tissus endommagés sont réparés. Si cette réponse inflammatoire initiale n'élimine pas les agents pathogènes, comme dans le cas d'infections chroniques ou de lésions tissulaires non réparées, le système immunitaire adaptatif intervient. Les cellules du système immunitaire adaptatif migrent vers le site de l'infection et commencent à éliminer les agents pathogènes et

les cellules et tissus endommagés (MEDZHITOV, 2008, 2010a). Cependant, dans certaines infections chroniques, l'inflammation peut persister sans immunité adaptative (CRUVINEL *et al.*, 2010).

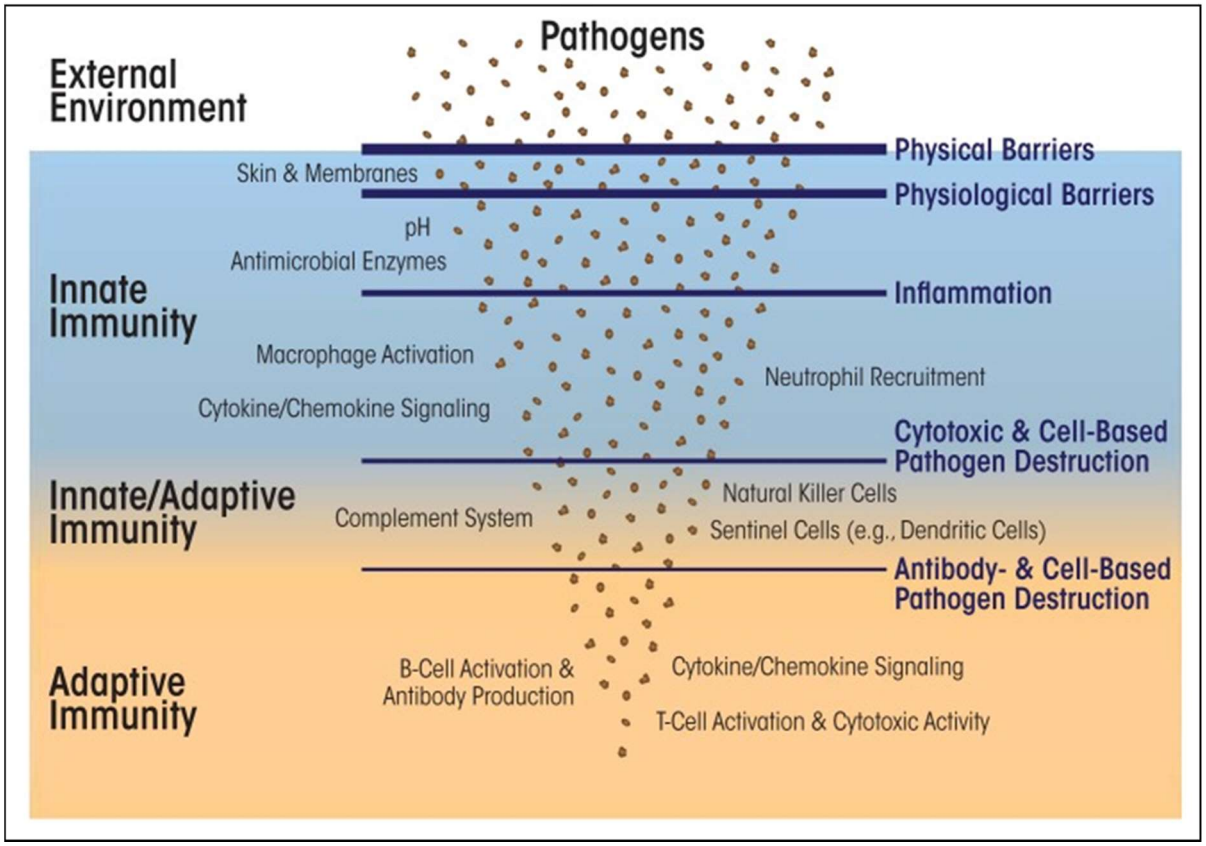

Figure 1: Vue d'ensemble du système immunitaire. La réponse innée est caractérisée par le fait qu'elle constitue la première ligne de défense contre l'infection dans laquelle interviennent des leucocytes tels que des neutrophiles, des monocytes, ou des macrophages. Ces cellules de défense détectent et attaquent d'autres cellules portant des structures *pathogen-associated molecular patterns* (PAMP) et de petites protéines qui signalent une invasion d'agents pathogènes (cytokines et chimiokines) ou de courts peptides antimicrobiens se liant directement aux agents pathogènes microbiens. Les cellules du système imunitaire adaptatif s'appuient sur des lymphocytes T et B et des protéines (les anticorps) qui détectent et éliminent spécifiquement des agents pathogènes et prodiosent également une cytokine/chimiokine afin de signaler et de recruter d'autres cellules immunitaires. Les lymphocytes T et B jouent un rôle essentiel dans l'immunité contre l'invasion pathogène. Le système du complément, ainsi que les cellules tueuses naturelles (NK) et les cellules dendritiques, interviennent à la fois dans l'immunité innée et adaptative. Reproduit de (SPIERING, 2015).

Ainsi, la réponse immunitaire innée joue un rôle central dans la défense contre les agents pathogènes et est initiée par les *pattern recognition receptors* (PRR). Les PRR reconnaissent les *pathogen/microbe-associated molecular patterns* (PAMPs/MAMPs) et les *endogenous danger-associated molecular pattern* (DAMPs), ce qui conduit à l'activation des voies de défense de l'hôte qui entraînent l'élimination de l'infection (CARRILLO *et al.*, 2017; JANG *et*

al., 2015; KUMAR, H.; KAWAI; AKIRA, 2011; MOGENSEN, 2009; ROH; SOHN, 2018). Les récepteurs Toll-like (TLR), un groupe de récepteurs extracellulaires et endosomaux liés à la membrane, détectent la présence d'une infection par la reconnaissance des PAMP. La réponse immunitaire innée contre les microbes implique également une voie inflammatoire majeure connue sous le nom d'inflammasomes (MARTINON; BURNS; TSCHOPP, 2002; SCHRODER; TSCHOPP, 2010a).

Les inflammasomes sont des complexes de signalisation multi-protéines qui s'assemblent après la reconnaissance des signaux de danger et/ou des agents pathogènes par une famille de récepteurs cytosoliques appelés NLR (*nucleotide-binding domain leucine-rich repeat containing receptors*) ou de membres de la famille de protéines PYHIN (MARTINON; BURNS; TSCHOPP, 2002). Une fois assemblés, les inflammasomes initient l'activation de protéases en aval, notamment la caspase-1 et la caspase-11, qui ensuite maturent protéolytiquement la pro-IL-1β et la pro-IL-18 et favorisent leur sécrétion cellulaire (BROZ; MONACK, 2013; LATZ, 2010; LATZ; XIAO; STUTZ, 2013a; MARTINON; BURNS; TSCHOPP, 2002; WILSON *et al.*, 1994).

De plus, l'activation de l'inflammmasome déclenche la pyroptose, une forme inflammatoire de mort cellulaire (BERGSBAKEN; FINK; COOKSON, 2009; YANG, YANG *et al.*, 2019; YUAN *et al.*, 2018). Le rôle de NLRP1, NLRP3, NLRC4, NLRP6, NLRP7, NLRP12 et des protéines AIM2 et IFI16 de PYHIN dans l'assemblage de l'inflammasome et le contrôle de la réponse immunitaire innée contre divers microbes, y compris différentes bactéries, est maintenant bien documenté. De même, l'implication de divers facteurs bactériens dans le déclenchement de l'inflammasome a été intensément explorés au cours des dernières années (CANEPARO *et al.*, 2018; LIN; ZHANG, 2017; SÁ; FESTA NETO, 2016; VLADIMER *et al.*, 2013a). Ces inflammasomes se forment en réponse à divers stimuli et sont également soumis à des mécanismes de régulation négative qui modulent la production de cytokines inflammatoires après le déclenchement de la réponse inflammatoire (NAIK; DIXIT, 2010).

L'inflammasome NLRP3 est le NLR le mieux caractérisé. NLRP3, ainsi que la protéine ASC et la Caspase-1, forment un complexe oligomérique cytoplasmique appelé inflammasome NLRP3, qui joue un rôle essentiel dans l'initiation de la réponse immunitaire innée. Cependant, les mécanismes moléculaires de son activation ne sont pas entièrement compris (DAVIS; WEN; TING, 2011; DEIGENDESCH; ZYCHLINSKY; MEISSNER, 2018; MELEHANI et al., 2015). L'assemblage de l'inflammasome NLRP3 a été associé à plusieurs pathologies telles que des syndromes auto-inflammatoires, l'inflammation chronique et certaines maladies métaboliques (DEIGENDESCH; ZYCHLINSKY; MEISSNER, 2018).

Le rôle des inflammasomes formés dans les phagocytes professionnels infectés par *S. aureus* qui ingèrent et/ou tuent les bactéries invasives a été étudié de manière approfondie.

Cependant, en dépit de nombreux travaux sur le mécanisme utilisé par *S. aureus* à internaliser dans et survivre dans les phagocytes non professionnels, comme les cellules épithéliales (KREMSEROVA; NAUSEEF, 2019; MA et al., 2019) l'implication des inflammasomes et de l'IL-1β traitée, qui joue un rôle crucial dans l'homéostasie osseuse dans le BJI associée à *S. aureus*, n'a pas encore été démontrée. Ceci est particulièrement intéressant dans le cas de BJI que *S. aureus* est représenté à internaliser et à survivre dans les ostéoblastes, générant des infections chroniques réfractaires aux traitements antimicrobiens. Il est donc essentiel de développer de nouvelles approches pour mieux comprendre les mécanismes par lesquels les staphylocoques interagissent avec ostéoblastes et, in fine, génère des dommages aux os.

Chapter 1: Bibliographic Synthesis

1. General characteristics of *Staphylococcus aureus*

1.1. Definition and morphology

Staphylococcus aureus (*S. aureus*) was described by Alexander Ogston and Louis Pasteur as cocci with a mean diameter of 0.5 and 1.5 µm, isolated from furuncle and abscesses in 1880 (OGSTON, 1881). The genus *Staphylococcus* belongs to the family Micrococcaceae, which is composed of Gram-positive bacteria (ROSYPAL; ROSYPALOVÁ; HOREJS, 1966). The name "*Staphylococc*" derives from the Greek words Staphyle (Grape-like clusters) and *aureus* (gold) (BROWN; GRILLI, 1998). *S. aureus* is immobile, non-spore-forming, produces coagulase and is often unencapsulated or has a limited capsule (OGSTON, 1881).

1.2. General cultural, biochemical characteristics and genome of *S. aureus*

S. aureus is an aerobic and facultative anaerobic bacterium. It readily grows on basal media and forms golden-yellow colonies on nutrient agar. It is positive for catalase, negative for oxidase and capable of fermenting mannitol (LOWY, 1998). *S. aureus* can grow in a wide range of temperatures. Its optimal growth temperature is 30º- 37ºC, but it can grow between 7º and 48.5ºC. Its optimum pH is 7.2 (but it can grow in a range of 4.2 – 9.3). It also grows in a wide range of sodium chloride concentration (7.5-10% NaCl) (LE LOIR; BARON; GAUTIER, 2003; SCHMITT; SCHULER-SCHMID; SCHMIDT-LORENZ, 1990). In blood agar, *S. aureus* forms beta-hemolytic colonies due to the production of hemolysins (α-toxin, β-toxin, γ-toxin and δ-toxin) (BLAIR, 1958; DINGES; ORWIN; SCHLIEVERT, 2000).

The first fully sequenced and annotated genomes were published in 2001 by Hiramatsu's group, comparing the genomes of two methicillin-resistant strains, Mu50 and N315 (KURODA et al., 2001). This opened the way to numerous other sequencing projects, which led to great insights into our understanding of this major pathogen. Genomic analyses of animal and human strains show that the whole genome is about 2.82 Mb in size. The genome of *S. aureus* contains 2.872 genes encoding 2.767 proteins. The GC content of the genome was found to be 32.7%. The core genome of *S. aureus* consists of about 80% of conserved genes between strains, encoding essential metabolic and regulatory functions, as well as surface proteins involved in adhesion and surface architecture (BABA et al., 2008; GILLASPY et al., 2006; LINDSAY; HOLDEN, 2004).

The other predicted genes make up an "accessory" genome, comprising a collection of mobile genetic elements (MGEs), including bacteriophages, chromosomal cassette and pathogenicity islands (KURODA et al., 2001; LINDSAY; HOLDEN, 2004, 2006). MGEs encode most of the virulence factors of *S. aureus*. The exchange of virulence factors that are transported by MGEs from a strain to another is a driving force in *S. aureus* evolution. The difference in the gene content of *S. aureus* strains is due to deletions of genes and insertions by MGEs.

Some regions of the central genome show inter-strain variability. The allelic variation of structural and functional genes of *S. aureus* arises from the random substitution of nucleotides, followed by selection, recombination and horizontal gene transfers (CROSSLEY et al., 2009; FENG et al., 2008; LINDSAY; HOLDEN, 2004). In addition to the chromosome, *S. aureus* strains can also contain plasmid elements encoding for resistance and virulence determinants (CROSSLEY et al., 2009; FITZGERALD et al., 2001; LINDSAY, 2010; LINDSAY; HOLDEN, 2004, 2006).

1.3. General pathogenesis and clinical diseases

1.3.1 Pathogenesis

The development of *S. aureus* infections consists of five different stages: bacterial colonization, followed by local infection, dissemination, metastatic infection and toxinosis (GNANAMANI; HARIHARAN; PAUL-SATYASEELA, 2017). *S. aureus* is often found in healthy carriage in mammals and birds. It is a frequent inhabitant of human retronasal ways, in which it may be part of the human microbiota without causing disease in the host (OTTO, 2010a).

S. aureus is a commensal and opportunistic pathogen that causes a wide range of acute infections (septicemia, bacteremia, pneumonia, meningitis) and chronic diseases of bones, joints, and skin in humans, ranging from mild to life-threatening infections (CHAMBERS, 1997; FITZGERALD, 2012; LOWY, 1998). Osteomyelitis is an inflammatory disease that accompanies bone destruction. It is commonly caused by bacteria. However, infections occurring through fungi or other parasites have also been reported (GOMES; PEREIRA; BETTENCOURT, 2013; URS et al., 2016).

S. aureus is the most common infectious causative organism of osteomyelitis with more than 70% of occurrences. In terms of prevalence, it is followed by Gram-negative bacilli, *Streptococcus pneumoniae* and *Streptococcus pyogenes* (CHIAPPINI; MASTRANGELO; LAZZERI, 2016). These bacteria adhere to the bone matrix leading to the formation of biofilms. In these biofilms, pathogens undergo complex metabolic changes that make these bacteria

less sensitive, both to the host immune response and to antibiotics. The complex nature of biofilm makes treatment difficult leading to long-term antibiotic therapy, and to high morbidity and elevated cure costs for patients and hospitals (CRAFT et al., 2019).

S. aureus is also a major animal pathogen. In dairy animals, such as cows and small ruminants, mastitis remains a major concern worldwide that affects animal health, and leads to huge economic losses in the dairy industry due to veterinary treatments, premature culling, and the change in milk quality (LESCOURRET; COULON; FAYE, 1995; PETON; LE LOIR, 2014; RAINARD et al., 2018). Although viruses, fungi or protozoa can cause mastitis, the most common causes are bacteria (AMIN; HAMOUDA; ABDEL-ALL, 2011; DEB et al., 2013). *Escherichia coli* is often associated with acute mastitis in bovine. In contrast, *S. aureus* most commonly causes subclinical mastitis that tends to become chronic in cows (GÜNTHER et al., 2017; SEARS; MCCARTHY, 2003; YOUNIS; JAVED; BLUMENBERG, 2016).

Subclinical mastitis accounts for about 80% of all economic losses associated with mastitis, related to reduced milk production and quality, as well as treatment and prevention costs (PETROVSKI; TRAJCEV; BUNESKI, 2006). Massive antibiotic use in veterinary medicine is regarded as a major problem in terms of the emergence and spread of antibiotic-resistant strains (FAIR; TOR, 2014; LI; WEBSTER, 2018; VENTOLA, 2015a). Prevention (e.g. anti-mastitis vaccine) is by far the best strategy. However, most vaccines tested to date do not allow the development of an efficient and protective defense against *S. aureus* mastitis (WALLEMACQ et al., 2012).

In human medicine, *S. aureus* causes huge problems such as nosocomial infections, notably because some strains are resistant to a wide variety of antimicrobials. Penicillin was discovered by Alexander Fleming in 1928 and was first administered to infected patients as a chemotherapeutic agent in 1941. The resistance to penicillin in *S. aureus* rapidly appeared in 1942, followed by more general mechanisms of resistance against the family of beta-lactams (CHAIN et al., 1940; FLEMING, 1929a; FLETCHER, 1984; RAMMELKAMP; MAXON, 1942; VENTOLA, 2015a). The resistance of *S. aureus* to beta-lactams and other types of antibiotics has contributed to a decrease in the number of effective drugs against this microorganism (LEVY; MARSHALL, 2004).

1.3.2 Virulence factors

The importance and success of *S. aureus* as a pathogen relies on the combination of toxins-mediated virulence, invasiveness and antimicrobial resistance (LE LOIR; BARON; GAUTIER, 2003), as well as cell-cell interaction, immune response evasion, and tissue damage (FENG

et al., 2008; GORDON; LOWY, 2008). The pathogenicity of this bacterium is due to both the diversity of its virulence factors and its ability to express its virulence in various host environments (PEACOCK et al., 2002).

The repertoire of *S. aureus* virulence factors is extensive and comprises secreted and structural products, including membrane proteins, that direct the process of pathogenesis (GORDON; LOWY, 2008). Due to the impact of this bacterium on human and animal health, the structure and function of its virulence factors, its interaction with the host and the molecular mechanisms involved in resistance to antibiotics have been extensively studied (HECKER et al., 2010; LINDSAY; HOLDEN, 2004).

Based on their mechanism of action and role in pathogenesis, staphylococcal virulence factors are classified as represented in **Table 1**. Among the virulence factors produced by this microorganism, the Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) are of particular importance in *S. aureus* colonization. MSCRAMMs form a group of molecules that bind to components of the extracellular matrix, such as collagen, fibronectin (Fn) and fibrinogen (Fg). Binding to these components facilitates bacterial internalization into the host cells and promotes tissue damage (WANN; GURUSIDDAPPA; HOOK, 2000).

The fibronectin binding proteins FnBPA and FnBPB of *S. aureus* are responsible for the Fn adhesion process into the host cell surface (FOSTER et al., 2014; FOSTER; HÖÖK, 1998; JOSSE; LAURENT; DIOT, 2017). Binding to Fn plays a major role in bacteria-cell interactions, but *S. aureus* also produces components able to bind to Fg and collagen (Hienz et al, 1996). The binding to Fg is mainly mediated by Clumping factor A and B (ClfA) and (ClfB) proteins, which allow bacterial adhesion to blood plasma and biomaterials exposed to human or animal blood (FOSTER; HÖÖK, 1998).

All these factors allow *S. aureus* to bind to the components of the extracellular matrix, facilitating the infection of several cell types, such as epithelial and immunological cells (OTTO, 2010a; PIETROCOLA et al., 2017). *S. aureus* also produces a variety of other secreted virulence factors including toxins (leucocidins, haemolysins, exfoliative toxins, enterotoxins, (TSST-1) - Toxic shock syndrome toxin -1), enzymes, and a group of factors encompassing the phenol-soluble modulins (PSM) (GNANAMANI; HARIHARAN; PAUL-SATYASEELA, 2017; OTTO, 2014a). PSMs may play an important role in the different stages of pathogenesis, including adhesion, invasion, persistence, evasion, and death of host cells (GORDON; LOWY, 2008; OTTO, 2014b).

The expression of virulence factors is tightly controlled in *S. aureus*. Following the establishment of the colonization, *S. aureus* represses the expression of surface proteins

associated with adhesion and internalization and produces toxins and other secreted virulence factors responsible for bacterial dispersion and, consequently, the symptoms of infections (NOVICK, 2003; NOVICK; GEISINGER, 2008). The different virulent factors expressed are controlled by a definite locus named the accessory gene regulator (*agr*) and the staphylococcal accessory regulator (*sar*) (JARRAUD et al., 2002).

The *agr* system is formed by a two-component system, which is responsible for the regulation of several virulence factors associated with the infectious processes (NOVICK; GEISINGER, 2008; OTTO, 2004). The *agr* operon is composed of four genes (*agrA*, *agrB*, *agrC* and *agrD*) that respectively produce AgrA, AgrB, AgrC and the auto-inducible peptide (AIP) (**Fig. 2**). Virulence expression is based on a quorum-sensing mechanism. During bacterial growth, AIP accumulates in the surrounding medium and when AIP concentration reaches a given threshold, it induces a shift of expression resulting in a repression of the genes encoding surface proteins and an induction of genes encoding secreted proteins (toxins). AIP, which is processed and secreted by AgrB, binds to the membrane receptor formed by AgrC, which phosphorylates or dephosphorylates AgrA. AgrA functions as a signal transducer of the AIP binding to the ArgC receptor, activating the expression of the *agr* operon and the *RNAIII* gene. The latter expresses the RNAIII, a regulator of several genes related to virulence in *S. aureus* (LE; OTTO, 2015; NOVICK, 2003; NOVICK; GEISINGER, 2008).

Factors	Characteristics	References
Helping attachment to host tissues		
Microbial Surface Components Recognizing adhesive matrix molecules (MSCRAMM)	Cell surface proteins which interact with host molecules such as collagen, fibronectin and fibrinogen, thus, facilitate the tissue attachment. Staphylococcal protein A, fibronectin-binding proteins A and B, collagen-binding protein and clumping factor A and B belong to this family. They are also involved in host immune evasion	Vazquez et al., 2011
Breaking/evading the host immunity		
Polysaccharide microcapsule	Resist the phagocytosis and killing by polymorphonuclear phagocyte	Nilsson et al., 1997
Protein A (SpA)	Binding of the Fcγ domain of immunoglobulin (Igs)	Hong et al., 2016
Panton-Valentine leukocidin (PVL)	PVL is found in most of community-associated MRSA (CA-MRSA). PVL consists of two subunits, LukS and LukF. LukS binds to human complement receptors C5aR and C5L2 and allows docking of LukF. The Luk subunits oligomerizes and form a pore lysing the cell.	Genestier et al., 2005 Voyich et al., 2006
α-Hemolysin	Disruption of epithelial or endothelial surfaces	Bhakdi, and Tranum-Jensen, 1991
Chemotaxis-inhibitory protein of S. aureus (CHIPS	Impediment of neutrophil chemotaxis	Postma et al., 2004
Tissue invasion		
Extracellular adherence protein (Eap)	Blockade of neutrophil adherence to endothelia	Edwards et al., 2012
Proteases, lipases, nucleases, hyaluronatelyase, phospholipase C, metalloproteases (elastase), and	These extracellular enzymes cause tissue destruction and,	
Induces toxinosis		
Staphylococcal Enterotoxins (SE)	There are more than 25 known SEs. SEs bind MHC class II receptors on T-cells and hyperstimulate the T-cells. S. aureus produces battery of enterotoxins which are potent gastrointestinal exotoxins. The Staphylococcal food poisoning is an intoxication which results from consumption of foods containing sufficient amount of preformed enterotoxins	Argudin et al., 2010
Toxic shock syndrome toxin -1 (TSST-1)	TSST-1 and some of enterotoxins are called as pyrogenic toxin super antigens. TSST-1 causes toxic shock syndrome especially in menstrual women	Dinges et al., 2000
Exfoliative toxins A and B	Serine proteases which selectively recognize and hydrolyze desmosomal proteins in the skin. ETs cause staphylococca-scalded skin syndrome, a disease predominantly affecting infants	Bukowski et al., 2010

Table 1: Virulence factors of *S. aureus* and their characteristics. Adapted by (GNANAMANI; HARIHARAN; PAUL-SATYASEELA, 2017).

Cytotoxins and enzymes can be seen as a means to convert host molecules into nutrients for bacterial growth or to cleave antibiotic molecules (OTTO, 2014a). Hemolysins (α, β, γ, and δ), nucleases, proteases, lipases, hyaluronidase, and collagenase can indeed provide nutrients for the bacterium from the host material. Besides, enzymes such as β-lactamases are responsible for inhibiting the activity of antibiotics derived from β-lactam rings. Among the toxins produced by *S. aureus*, superantigens are potent and non-specific immune response inducers (MAKHLIN et al., 2007; TOKAJIAN et al., 2011). Also referred to as pyrogenic toxins, they include staphylococcal enterotoxins (more than 25 SEs have been described so far) and toxic shock syndrome toxin-1 (TSST-1) (CHOI et al., 1989; DINGES; ORWIN; SCHLIEVERT, 2000; KRAKAUER, 2019; MARRACK; KAPPLER, 1990).

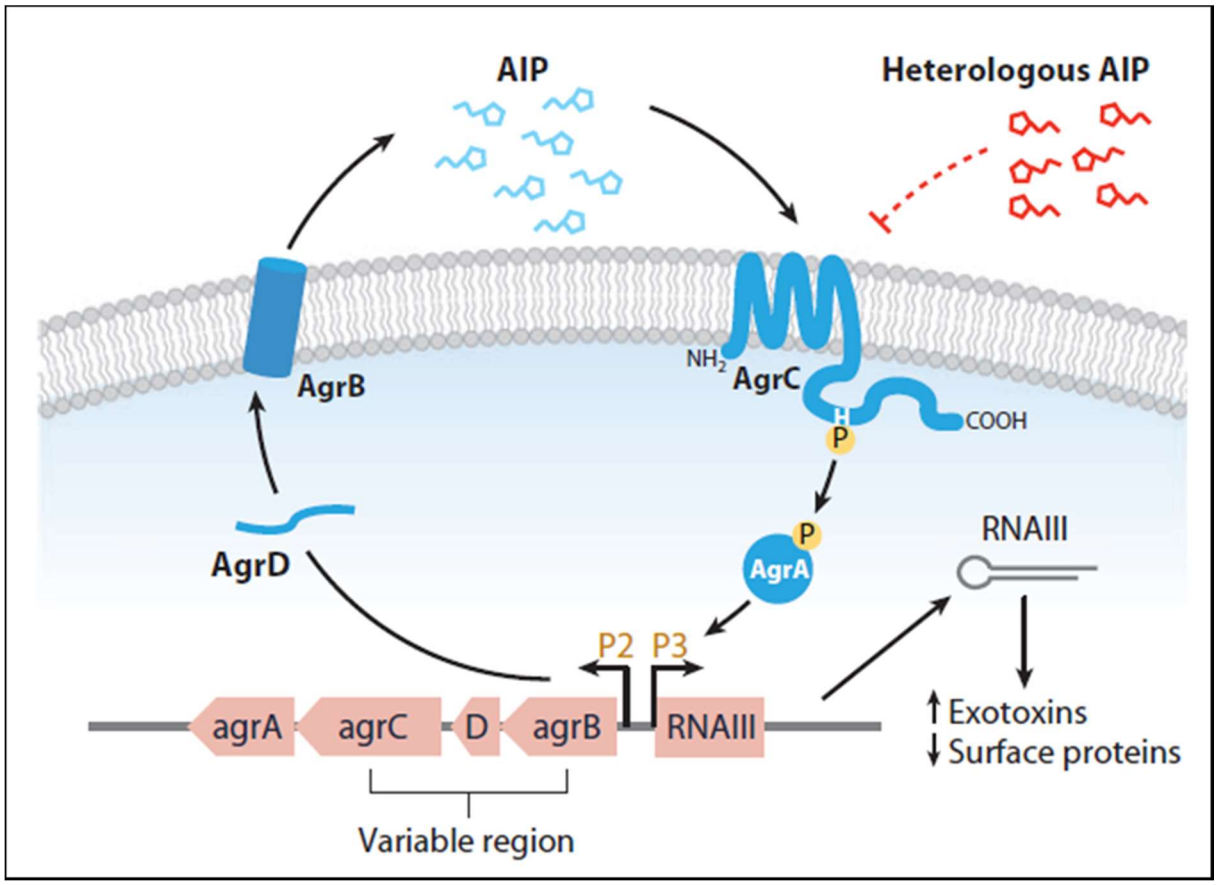

Figure 2: Accessory gene regulatory system (Agr).

Quorum sensing system agr encodes gene products that ultimately promote further transcription of agr locus in a process called autoactivation. According to (NOVICK; GEISINGER, 2008).

1.3.3 Phenol-soluble modulins (PSMs)

In 1999, Seymour Klebanoff described the so-called Phenol-soluble modulins (PSM), a group of molecules contained in the supernatant of *Staphylococcus epidermidis,* isolated by size exclusion chromatography with the use of a solvent containing phenol (MEHLIN; HEADLEY;

KLEBANOFF, 1999; OTTO, 2004). PSMs are part of a family of short amphipathic, α-helical peptides, which are produced by various staphylococcal strains and which have great importance in the virulence process of *S. aureus* (**Fig. 3**) (GONZALEZ et al., 2014; OTTO, 2014b; PESCHEL; OTTO, 2013).

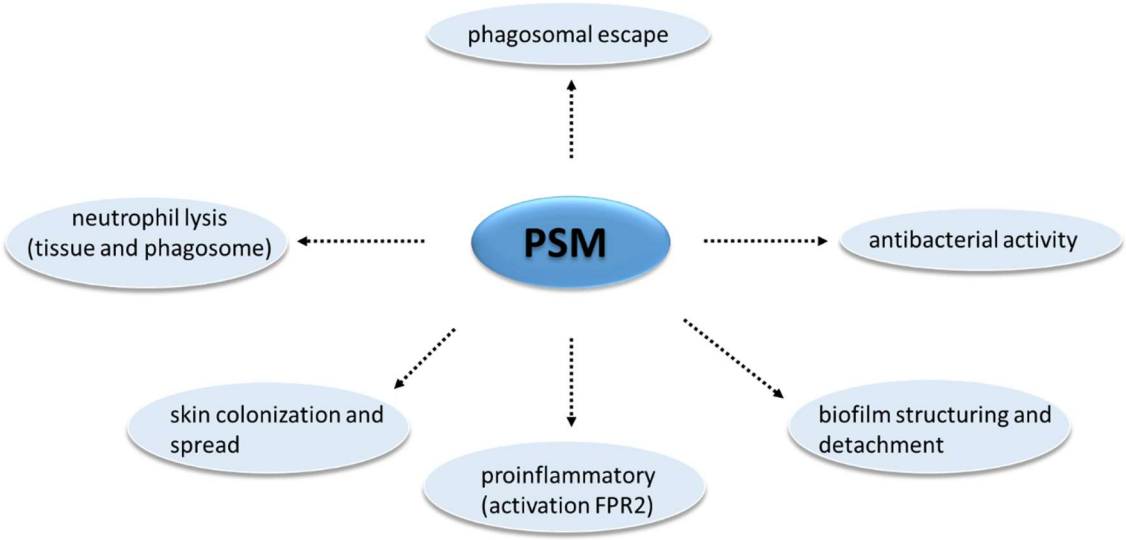

Figure 3: Overview of phenol-soluble modulin activities in *S. aureus*.

PSMs participate in the formation of biolfims. PSMs may be antimicrobial agents, they also act to induce cell lysis, cytokine production through the FPR2 receptor, and participate in the *S. aureus* dispersion process.

PSMs were described as a proinflammatory "complex" of seven PSMs, including the shortest α type with approximately 20-25 amino acids in length (four PSMα1-PSMα4 peptides), the longest β type with approximately 43- 45 amino acids (PSMβ1 and PSMβ2) and δ-toxin peptides ranging in length from 25 to 26 amino acids (CHEUNG et al., 2014, 2016; MEHLIN; HEADLEY; KLEBANOFF, 1999). The PSM–encoding genes scattered at different locations in the genome. The PSMα1-PSMα4 genes are encoded in the PSMα operon. The PSMβ1 and PSMβ2 peptides encoded in the PSMβ operon and the δ-toxin is encoded within the RNAIII sequence (**Fig. 4**) (CHATTERJEE et al., 2011; OLIVEIRA; BORGES; SIMÕES, 2018).

Likewise several other major staphylococcal exotoxins, PSMs are positively regulated by *agr*, the global regulatory quorum sensing system (GOMES-FERNANDES et al., 2017; QUECK et al., 2008; XU et al., 2017). The PSMs are *agr*-regulated through AgrA, not by RNAIII, which modulates several other virulence genes in *S. aureus* (MARROQUIN et al., 2019; PESCHEL; OTTO, 2013; TAN et al., 2018).

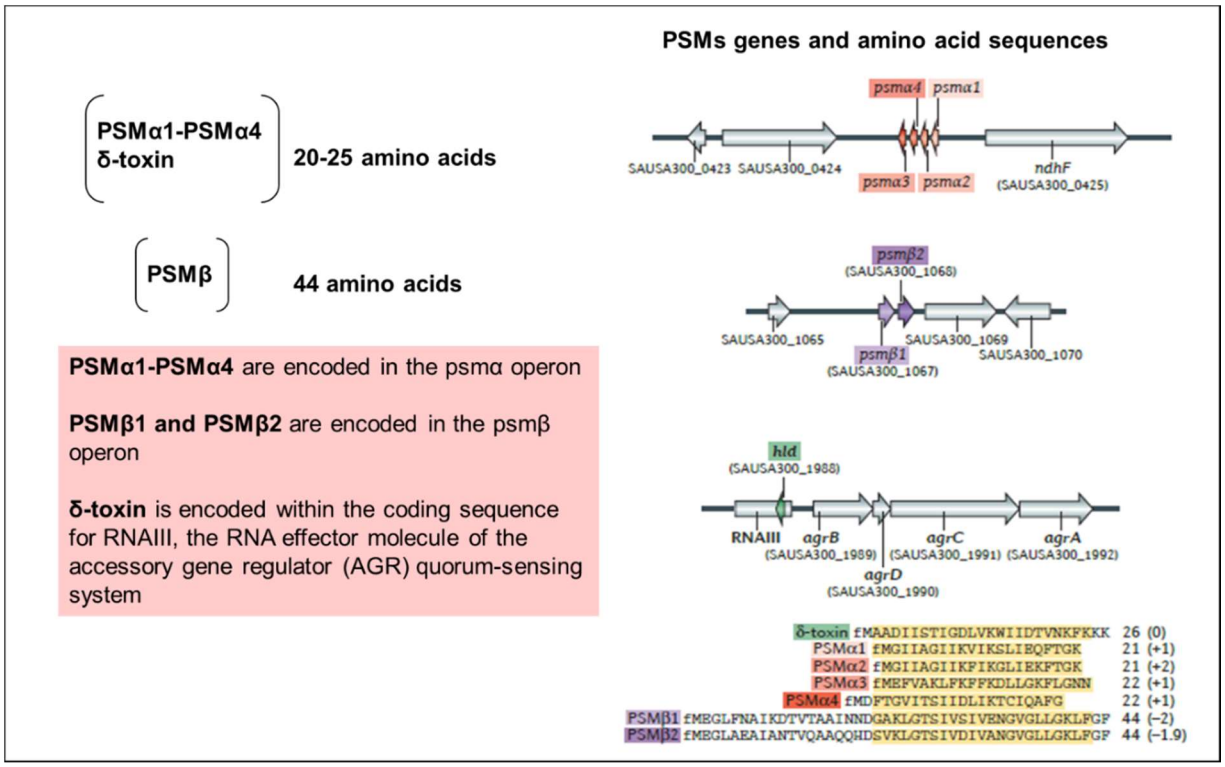

Figure 4: Phenol-soluble modulins peptides (PSMs) define the virulence potential of *S. aureus.*

PSMs have a pronounced ability to lyse human leukocytes and other cell types, to stimulate an inflammatory response and the formation of biofilm (CHEUNG et al., 2014, 2016; PESCHEL; OTTO, 2013; TOWLE et al., 2016).

Their importance in the pathogenesis was reported by Voyich et al. who showed in 2005 that, in the CA-MRSA strains (LAC and MW2), PSMs participate in *S. aureus* virulence, in a model of skin infections and bacteremia in mice (PESCHEL; OTTO, 2013). Specifically, PSMα group induces a stronger inflammatory response compared to PSMβ, whereas the PSMβ group and the δ-toxin did not significantly influence the inflammation process. PSMα group increased the amount of neutrophils and monocyte chemotaxis, a critical step in the establishment of *S. aureus* infection, in addition to elevating leukocyte death *in vitro* (WANG et al., 2007). The dissemination capacity of *S. aureus* and *S. epidermidis*, associated with biofilm formation, is directly related to the production of PSMs (LE; OTTO, 2015; OTTO, 2017; QUECK et al., 2008; TSOMPANIDOU et al., 2013; WANG et al., 2011).

The ability to induce cellular lysis is the most relevant feature of these PSMs (CHEUNG et al., 2014). The action of the PSMs is not specific and is independent of receptors. It affects almost all eukaryotic membranes leading to cell lysis, which is probably due to a disturbance of the plasma membrane (CHEUNG; DUONG; OTTO, 2012; KRETSCHMER et al., 2010). Other *S. aureus* toxins, such as α-toxin or Panton-Valentine Leukocidin (PVL), specifically lyse polymorphonuclear neutrophils (PMNs) (DIEP et al., 2010; LÖFFLER et al., 2010; OLIVEIRA;

BORGES; SIMÕES, 2018). Although this group of peptides is linked to the cellular lysis process, only a portion of the PSMs is responsible for this activity (CHEUNG et al., 2014). *S. aureus* PSMα3 and *S. epidermidis* PSMδ exhibit the most evident cytolytic activities among PSMs, whereas PSMα1 and PSMα2 are moderately cytolytic (CHEUNG; DUONG; OTTO, 2012; CHU et al., 2018; DA et al., 2017; OTTO, 2017).

Among other activities, PSMs induce neutrophil chemotaxis, oxygen radical production, intracellular calcium influx, and release of proinflammatory cytokines such as Interleukin-8 (IL-8), a member of the CXC subfamily of chemoattractant cytokines (HENKELS et al., 2011; PARKER et al., 2016; WANG et al., 2007). The ability of the PSMs to induce an inflammatory response classify them as pathogen-associated molecular patterns (PAMPs), which are a group of microbial molecules that alert the host organism when a pathogen infection occurs (BIANCHI, 2007; CHEUNG et al., 2014). In addition, PSMs have been shown to be capable to decreasie the expression of proinflammatory cytokines such as IL-32, IL-6 and IL-8 (DEPLANCHE et al., 2016).

2. The inflammasome, a primordial complex of innate immunity

2.1. Mechanisms of the inflammatory response

The immune system is formed by a series of effector mechanisms which can destroy a variety of invading pathogenic organisms belonging to four main groups fungi, bacteria, parasites, and viruses (CARRILLO et al., 2017; MESQUITA JÚNIOR et al., 2010; NICHOLSON, 2016).

The immune system is composed of two types of responses: antigen-specific adaptive immune response and the innate immune response recognizing microbial associated molecular patterns (MAMP's) (RYU, 2017). Innate immune response described as the organism's first line of defense is of primary importance in the immediate recognition and elimination of invasive microorganisms (ESPINOSA; RIVERA, 2016). The innate immune response consists basically of physical and chemical barriers, reactive oxygen species (ROS), innate immune cells, and soluble mediators such as the complement system, innate antibodies, and associated cytokines and recognizes pathogen-associated molecular patterns (PAMPs) (CARRILLO et al., 2017; CRUVINEL et al., 2010).

PAMPs are recognized by pattern recognition receptors (PRRs), expressed primarily in cells of the innate immune system (KUMAR; KAWAI; AKIRA, 2011; MOGENSEN, 2009). PRRs can also recognize host molecules that contain damage-associated molecular patterns (DAMPs), these molecules are released when a pathogenic organism is able to multiply in the host as a

result of the infection process or as a result of a tissue injury (sterile inflammation), these microorganisms are usually absent from the extracellular environment (GRAZIOLI; PUGIN, 2018; LAND, 2015; RANI et al., 2017). To initiate inflammation, coordinated action between PAMPs/MAMPs and DAMPs is required. Inflammation is induced by both the recognition of foreign molecules and by cell damage (DI VIRGILIO, 2013; GASTEIGER et al., 2017; ROH; SOHN, 2018).

The inflammatory pathway is composed of inducers, sensors, inflammatory mediators and target tissues in which mediators act (MEDZHITOV, 2010a). In the last decades, two types of inflammatory conditions have been identified: acute and chronic reaction (MEDZHITOV, 2010a; ZHOU; HONG; HUANG, 2016). The acute inflammatory response is caused by infection or tissue injury leading to the migration of neutrophils and monocytes/macrophages from the blood to the tissue (CHU et al., 2018; CRUVINEL et al., 2010; SELDERS et al., 2017; TAKEUCHI; AKIRA, 2010). This inflammatory response involves receptors (PRRs) located on the surface of resident macrophages or mast cells, and their activation leads to the production of inflammatory mediators such as cytokines and chemokines (CHEN et al., 2017a; CRUVINEL et al., 2010).

During the inflammatory response, leukocytes are activated and induce the release of toxic substances contained in their granules like reactive oxygen species (ROS) and reactive nitrogen species (RNS) (CHERTOV et al., 2000; MEDZHITOV, 2008; NGUYEN; GREEN; MECSAS, 2017). When pathogens can escape from the innate immune system, the adaptive system comes into action (GASTEIGER et al., 2017; NICHOLSON, 2016). To maintain homeostasis, cell death acts continuously as a type of inflammatory response (ARBOGAST; GROS, 2018). Four types of cell death are known: apoptosis and pyroptosis are related to the action of caspases, whereas autophagy and necrosis act as an alarm system that indicates that the cell can no longer cope with aggression (DELOU; BIASOLI; BORGES, 2016; FAN; ZONG, 2012; YANG et al., 2015). Thus, the error in the elimination of the infectious agent by the immune system can lead to a persistent inflammatory process inducing a chronic inflammatory process contributing to a variety of chronic inflammatory diseases (CHEN et al., 2017a; CRUVINEL et al., 2010; FERRERO-MILIANI et al., 2006).

2.2. Inducers of the inflammatory response

Inflammatory inducers are endogenous or exogenous signals that initiate an inflammatory response through immune system receptors , such as Toll-like receptors (TLRs), leading to

the production of mediators such as interleukins and chemokines that act on target tissues (CARRILLO et al., 2017; MEDZHITOV, 2010a).

Exogenous inducers may be of microbial origin or not, such as toxic compounds and foreign bodies, allergens, and may be classified in PAMPs and virulence factors such as pore-forming exotoxins produced by Gram-positive bacteria, which are detected by the NLRP3 inflammasome (NACHT - leucine-rich repeat- and pyrin-domain-containing protein) (GREANEY; LEPPLA; MOAYERI, 2015; LATZ, 2010; MARIATHASAN et al., 2006; MEDZHITOV, 2008). These exogenous inducers are not restricted to pathogens, but may be generated from an alternative detection pathway, through components induced by damaged tissues or cell death (MEDZHITOV, 2008). Endogenous inducers result from stressed, damaged, or malfunctioning tissues involved in acute inflammatory responses and tissue injury (CHEN et al., 2017a; MEDZHITOV, 2008). Another class of endogenous inducers related to chronic inflammatory responses in particular includes uric acid crystals and oxidized lipoproteins (MEDZHITOV, 2008).

2.3. Mediators of the inflammatory response

Mediators of the inflammatory response are either secreted by the cells or are derived from plasma proteins, and may circulate in the blood plasma as inactive precursors and have higher concentrations during the acute phase of the inflammatory process (ABDULKHALEQ et al., 2018; CARRILLO et al., 2017). These mediators can be classified according to their biochemical characteristics into seven groups: amines and vasoactive peptides, fragments derived from complement components, lipid mediators, cytokines, chemokines and proteolytic enzymes (CRUVINEL et al., 2010; MEDZHITOV, 2008). Several mediators have a direct effect on the target tissues, acting, for example, in the recruitment of leukocytes and by inducing the production of other mediators (CHEN et al., 2017a; MEDZHITOV, 2010b).

The production of inflammatory mediators, such as cytokines, plays a crucial role, which is regulated by anti-inflammatory signals. Cytokines are the main mediators of the inflammatory response and are small proteins secreted by cells involved in the regulation of inflammation, tissue repair and hematopoiesis (CARRILLO et al., 2017).

Cytokines act in various biological processes, and are involved in conserved signaling pathways responsible for controlling various types of cellular response, such as apoptosis and cell differentiation (CARRILLO et al., 2017; CHEN et al., 2017a). There are over fifty cytokines organized into several classes, which are classified into groups: Interleukins, tumor necrosis factors (TNF), interferons (IFN), colony stimulating factors (CSF), transforming growth factors

(TGF) and chemokines (CARRILLO et al., 2017). Among the proinflammatory cytokines are: TNF, IL-1, IL-12, IL-18 and IFN-γ; and among the anti-inflammatory cytokines are IL-4, IL-10, IL-13 and TGF-β (CARRILLO et al., 2017). However, the family of interleukin (IL) -1 ligands and receptors stands out among cytokines due to the important role they play in acute and chronic inflammations (DINARELLO, 2009a, 2018a).

2.4. IL-1β

2.4.1 The maturation of IL-1β

The concept of interleukin (IL) appeared in 1979 and has defined a protein capable of acting as a communication signal between different populations of leukocytes (A. DINARELLO, 2017; DINARELLO, 2009a). IL-1 family members are responsible for severe attacks in the context of systemic or local inflammation and contribute for many chronic diseases (CHEN et al., 2017a). Inflammatory processes related to a dysregulated production of IL-1 are involved in the pathophysiology of several common diseases such as atherosclerosis, osteoarthritis, metabolic syndromes and type 2 diabetes (type 2 diabetes or type 2 diabetes mellitus) (A. DINARELLO, 2017). However, numerous biological properties of the IL-1 family are still non-specific (DINARELLO, 2018a).

Currently, there are 11 members of the IL-1 family (IL-1α, IL-1β, IL-1Rα, IL-18, IL-33, IL-36α, IL-36β, IL-36γ, and IL- IL-38) (GUAN; ZHANG, 2017), and 10 members of the IL-1 family of receptors (IL-1R1 to ILR10) (BORASCHI et al., 2018). Among the IL-1 family, IL-1β, also known as IL-1F2, is one of the most characterized. It is considered as a key pro-inflammatory cytokine, mainly produced by myeloid cells, inducing the physiological, hematological, metabolic and immunological characteristics of the host after infection, trauma or immunological activation (DINARELLO, 2018a).

The gene structure, expressional regulation, and function of IL-1β are evolutionarily well conserved (PALOMO et al., 2015), and gene encoding it is not constitutively expressed (DINARELLO, 2018a; YIN et al., 2009; ZHU; KANNEGANTI, 2017). Thus, in response to endogenous or exogenous inflammatory agents, such as Toll-like receptor (TLRs) ligands, which are responsible for the recognition of a host of PAMPs, IL-1β is synthesized as a pro-cytokine (pro-IL-1β), having a molecular weight of 31-kDa. Pro-IL-1β is then cleaved by caspase-1 at two sites related to the sequence: Asp27-Gly28 (site 1) and Asp116-Ala117 (site 2) with a molecular weight of 17.5-kDa, resulting in mature IL-1β generation (AFONINA et al., 2015; AMARANTE-MENDES et al., 2018; CAMERON et al., 1985; CERRETTI et al., 1992;

DINARELLO, 2018a; GUARDA; SO, 2010; KWAK et al., 2016; SCHRODER; TSCHOPP, 2010). Pro-IL-1β accumulates in the cytosol (SEMINO et al., 2018; STOFFELS et al., 2015).

IL-1β maturation can occur through two possible levels of activation: One induced by NF-κB-mediated leading to pro-IL-1β expression and the second option by of the proteolytic cleavage of caspase-1 and subsequent maturation of pro-IL-1β (NAIK; DIXIT, 2010; YANG et al., 2019). Studies indicate that caspase-8 might be able to cleave pro-IL-1β during immune responses (LATZ; XIAO; STUTZ, 2013b).

The cleavage and thus maturation of IL-1β also appear to take place out of the cell and could involve other proteases that can cleave IL-1β but are present in the extracellular inflammatory environment (GIULIANI et al., 2017; LOPEZ-CASTEJON; BROUGH, 2011; STEHLIK, 2009). Extracellular processing of pro-IL-1β is not novel and other proteases derived from neutrophils and mast cells have been implicated to be present in pro-inflammatory environments (AFONINA et al., 2015; BENT et al., 2018; DAUTOVA et al., 2018). Several studies have reported that exosomes were involved in the transfer of fully functional cytokines and growth factors (COSSETTI et al., 2014; DAUTOVA et al., 2018; WEBBER et al., 2010).

2.4.2 Excretion of IL-1β

The excretion process of IL-1β is not fully understood. Several secretory pathways have been proposed: exocytosis of granules derived from lysosomes; the release of exosomes derived from multi-vesicular bodies and the release of microparticles from the plasma membrane (ANDREI et al., 1999; DI VIRGILIO, 2013).

IL-1β secretion is also strongly associated with autophagy, an unconventional process (DANIELS; BROUGH, 2017). This process is independent of the endoplasmic reticulum and the Golgi apparatus and involves the autodigestion function of the cells by autophagy, which leads to the release of cellular content (DANIELS; BROUGH, 2017; KURODA et al., 2001). The role of autophagy in IL-β secretion is complex. Several studies suggest a tonic inhibition of IL-1β secretion both by the degradation of pro-IL-1β and by the negative regulation of inflammasome NLRP3 (HARRIS et al., 2011). However, additional research proposes that mature IL-1β is actively packaged in vesicles and secreted after autophagy stimulation (DUPONT et al., 2011; GUAN; ZHANG, 2017).

2.4.3 The regulation of IL-1β

IL-1β participates in most events involved in the activation and regulation of inflammatory response (CHEN et al., 2017a). IL-1β can stimulate other cytokines such as IL-6, TNF-α, IL-1α, granulocyte macrophage colony-stimulating factor (GM-CSF), and granulocyte-colony stimulating factor (G-CSF) (BHATTACHARYA et al., 2015; DINARELLO, 2018a; SÖDERQUIST et al., 1995). It is also implicated in the release of neutrophils (polymorphonuclear cells), activation of lymphocytes, differentiation of Th17 cells and activation of dendritic cells (BENT et al., 2018; COSTA et al., 2019; PELLETIER et al., 2010).

IL-1β also plays a role in the secretion of vascular endothelial growth factor (VEGF), reactive oxygen species (ROS) and reactive nitrogen species (RNS) (PAPIEWSKA-PAJĄK et al., 2017; ROBERTS et al., 2010). IL-1β is an essential cytokine for the host immune system as it also participates in the activation and differentiation of T cells, B cells and natural killer (NK) cells (CHEN; KANG; FU, 2018; CRUVINEL et al., 2010; LUIS MUÑOZ-CARRILLO et al., 2019). This proinflammatory mediator is generated at lesion sites and coordinates the recruitment of leukocytes to neutralize and phagocyte pathogens, being one of the first cytokines to be secreted during the early stages of inflammation (CHEN; KANG; FU, 2018; CRUVINEL et al., 2010; SCHRODER; TSCHOPP, 2010).

Furthermore, IL-1β, IL-6 and tumor necrosis factor α (TNFα) are considered the most important cytokines of inflammation in bone physiology and pathology (KWAN TAT et al., 2004; ROMAS; GILLESPIE; MARTIN, 2002). The balance of cell activity, such as osteoblasts that are located on the bone surface and are derived from mesenchymal progenitors (BLAIR, 1998), and osteoclasts that are multinucleated cells derived from the myeloid lineage (BLAIR et al., 1989), has been reported as essential for the maintenance of bone homeostasis and its renewal. Any imbalance in the relative levels of activity of these two cell types may induce bone pathology (HENDERSON; NAIR, 2003). *S. aureus* infection can cause such imbalance, mediated in part by the inflammatory response (RASIGADE et al., 2013).

Osteoblasts and osteoclasts interact permanently. The osteoblasts can identify different danger signs and modulate the differentiation and activity of osteoclasts (HENDERSON; NAIR, 2003; MATSUO; IRIE, 2008). A key interaction in the regulation of osteoclastogenesis occurs between the receptor activator of nuclear factor kappa B (RANK) expressed by osteoclast precursors and its ligand-expressed RANKL by osteoblasts (LIU; ZHANG, 2015; LORENZO; HOROWITZ; CHOI, 2008; ROGGIA et al., 2001; ROMAS; GILLESPIE; MARTIN, 2002).

In this context, IL-1β stands out as a strong stimulator in vitro and in vivo of bone resorption through the regulation of RANKL that stimulates osteoclastogenesis (LUO et al., 2018;

RUSCITTI et al., 2015). Thus, IL1β plays a predominant role in the regulation of bone remodeling, and that any disturbance of the expression levels of this cytokine may lead to an imbalance in bone remodeling homeostasis in favor of osteoclastogenesis and bone resorption activity and bone mineralization and production by osteoblasts, eventually leading to the destruction of bone tissue (DINARELLO, 2009b; RASIGADE et al., 2013; YANG et al., 2019).

2.5. Other cytokines

IL-1α or (IL-1F1), like IL-1β, was identified in 1974, both bind to the same IL-1 receptor (IL-1R) and are potent proinflammatory cytokines that are constitutively expressed in many cell types (DINARELLO, 2010). However, little is known about this cytokine in comparison to IL-1β. IL-1α, unlike IL-1β, is also active in its precursor form and is present intracellularly in healthy tissues (DINARELLO, 2010, 2018a; WERMAN et al., 2004). Depending on the activator, IL-1α secretion is dependent on inflammation (DI PAOLO; SHAYAKHMETOV, 2016).

IL-1α processing induces proteolytic cleavage of pro-IL-1α in mature IL-1α. However, this cleavage is dependent on calpains which are Ca2-dependent proteases present on the cytosolic side of the cell membrane (CARRUTH; DEMCZUK; MIZEL, 1991; FETTELSCHOSS et al., 2011; TAPIA et al., 2019). Another study demonstrated that IL-1α surface expression is independent of caspase-1, whereas mature IL-1α secretion requires the activation of the inflammasome and caspase-1 (FETTELSCHOSS et al., 2011; GROSS et al., 2012). According to (DINARELLO, 2010), intracellular IL-1α is also associated with chromatin playing a dynamic role in the nucleus of living cells (DINARELLO, 2010). During the apoptotic process, this intracellular cytokine is concentrated in dense nuclear foci and is not released along with the cytoplasmic content but remains inactive. However, the precursor of IL-1a is released from cells that undergo necrosis by becoming active (CHEN et al., 2007; COHEN et al., 2010).

IL-18 (IL-1F4 or previously IFN-gamma inducing factor) was first described in 1989 and is another proinflammatory cytokine belonging to the IL-1 family (DINARELLO, 2018a; GIRARD-GUYONVARC'H et al., 2018). The maturation stage of IL-18 is mediated by caspase-1 and the inflammasome, and has a similar structure to IL-1β (DINARELLO, 2018b; FENINI; CONTASSOT; FRENCH, 2017). IL-18 is constitutively expressed in cells of the immune system but also in keratinocytes and epithelial cells (NAKANISHI, 2018). This cytokine plays a key role in T-cell stimulation and is an important inducer of IFN-γ. Th1 and T cells are involved in the Th17 cytokine production by T cells, NK cells, basophils and mast cells (NAKANISHI, 2018; SILVESTRE; SATO; REIS, 2018; YASUDA; NAKANISHI; TSUTSUI, 2019).

In addition to IL-1β and IL-18, caspase is also able to regulate the activity of two other cytokines: IL-33 and IL-37. IL-33, also called IL-1F11, is a pro-inflammatory cytokine that has been recently added to the IL-1 family (AFONINA et al., 2015; CHERRY et al., 2008). It is a constitutive intracellular cytokine that is produced as pro-IL-33 and, after undergoing cleavage, is secreted into the extracellular medium as mature IL-33 (YANG et al., 2015). This cleavage occurs via proteases such as cathepsin G and elastase type and independently of caspase-1 (LEFRANÇAIS et al., 2012; TALABOT-AYER et al., 2009). However, studies have also shown that IL-33 can also be cleaved by Caspase-1 in vitro (CAYROL; GIRARD, 2018; TALABOT-AYER et al., 2009).

IL-33 is expressed at high levels in the nucleus of various cell types of the immune system, including non-professional phagocytes such as endothelial, epithelial and fibroblast cells in humans and mice (CAYROL; GIRARD, 2018; CHEN; KANG; FU, 2018; SOKOL; LUSTER, 2015). IL-33 is structurally related to IL-1b and IL-18, responsible for activating Th1/Th17 lymphocytes; however, it is biological functions mainly result in the production of IL-5 and IL-13 (DINARELLO, 2010, 2018b; OBOKI et al., 2010).

IL-37 or IL-1F7 is a recently described cytokine belonging to the IL-1 family which exhibits anti-inflammatory activity (BOUTET; NERVIANI; PITZALIS, 2019). In 2001, cloning of the coding genes located on chromosome 2 was described by Kumar and colleagues 2000 (KUMAR; KAWAI; AKIRA, 2011). There are five isoforms of the IL-37 gene encoding five isoforms (IL-37a, IL-37b, IL-37c, IL-37d and IL-37e), however, to date the IL-37b isoform is the most characterized (BOUTET; NERVIANI; PITZALIS, 2019). IL-37 has a similar receptor and structure to IL-18. However, to date it is not known whether its activity occurs via caspase-1 cleavage or whether it is active as a precursor (BOUTET; NERVIANI; PITZALIS, 2019; DINARELLO, 2018a). In a study developed by Ellidson and colleagues in 2017, additional sites of IL-37 cleavage predicted in silica have been shown to include cathepsin K, elastase-2 and matrix metalloproteinase (MMP) -9 cleavage sites (BOUTET; NERVIANI; PITZALIS, 2019; ELLISDON et al., 2017).

3. Innate immunity receptors

3.1. Pattern recognition receptors (PRRs)

The innate immune system is a sophisticated system, which relies on its ability to detect invading microbes, tissue damage or stress through evolutionarily conserved receptors (JANEWAY, 1989; MEDZHITOV; JANEWAY, 2000). It has a large number of well-conserved

microbial structures, which are known as pathogen-associated molecular patterns (PAMPs), recognized by pattern recognition receptors (PRRs) (CARRILLO et al., 2017; JANG et al., 2015; MOGENSEN, 2009). However, these receptors are less numerous than those of the adaptive immune system. The recognition of PAMPs by a PRR activates a signaling pathway leading to an inflammatory response that induces secretion of cytokines and chemokines and, consequently, the activation of an adaptive immune response (CARRILLO et al., 2017; JANG et al., 2015; MOGENSEN, 2009). PRRs are expressed primarily by cells of the innate immune system such as macrophages, monocytes, dendritic cells, and neutrophils. However, other cell types are able to express PRRs example of epithelial cells (JANG et al., 2015).

Historically, the theory that cells of the immune system can detect and respond to all molecular patterns of pathogens has been developed by Charles Janeway (JANEWAY, 1989). However, this model failed to explain how the immune system could distinguish between commensal and pathogenic bacteria. Thus, Matzinger proposed an alternative theory about the innate immune system that said that the system did not distinguish between what is proper (cells of the organism) and what is not itself (agents foreign to the organism), but rather respond to the signs of danger that are emitted by the body's cells, inducing an inflammatory response in response to these danger signals (MATZINGER, 1994; PRADEU; COOPER, 2012).

Currently, four families of PRRs are known to operate cooperatively to recognize endogenous microbial pathogens or endogenous danger signals: toll-like receptor system (TLRs) and C-type lectin receptors (CLRs), which are transmembrane receptors that monitor the extracellular environment, as well as the interior of the endosomes, and the intracellular receptors like [RIG-I-like receptors (RLRs) and nucleotide-binding domain leucine-rich repeats (NLRs)] that act on the cytosol (BAUERNFEIND; HORNUNG, 2013; TEMPLETON; MOEHLE, 2014). Among the transmembrane receptors, the TLRs were first discovered and therefore are the most studied class of receptors (AGIER; PASTWIŃSKA; BRZEZIŃSKA-BŁASZCZYK, 2018; JANG et al., 2015; KAWAI; AKIRA, 2009). TLRs can be expressed in various cell types, such as monocytes, macrophages, B and T lymphocytes, dendritic cells, neutrophils, NK cells, epithelial cells, endothelial cells, and Mast cells. Currently, 10 TLR genes have been identified in humans (TLR1–TLR10) and 12 (TLR1–TLR9, TLR11–TLR13) in mice (GOULOPOULOU; MCCARTHY; WEBB, 2015; KIELIAN, 2009).

TLRs can be classified according to their cellular location, for example the TLRs located on the plasma membrane are TLR 1, 2, 4, 5, 6, and 11 whereas the TLRs located in the endosomes are TLR 3, 7, 8, 9 and 13 (KAWAI; AKIRA, 2009). Broadly, TLRs can detect signs of danger such as peptides, lipopeptides, glycopeptides, glycolipids and nucleic acids (KAWAI; AKIRA, 2009). NLR receptors, along with TLRs, cooperate to recognize and respond to pathogens and activate pro and anti-inflammatory mechanisms (CARRILLO et al., 2017).

CLRs recognize specific polysaccharides and glycopeptides (ARTIGAS et al., 2017; WOLFERT; BOONS, 2013). RLRs are cytoplasmic receptors and respond primarily to the presence of RNA viruses and together with the NLRs are cytosolic proteins that probe intracellular microbial molecules and danger signs (RAYMOND et al., 2017).

3.2. NOD-like receptors (NLRs)

NLRs are a family of receptors located on the cytosol that recognize PAMPs. They are pattern recognition receptors that stand out as one of the major receptors involved in regulating the innate immune response. NLRs cooperate along with TLRs and act on the regulation of inflammatory and apoptotic responses, being found in professional phagocytes such as macrophages and dendritic cells and non-professional phagocytes such as epithelial cells (JANG et al., 2015).

The NLRs can be characterized by three structural domains:a leucine-rich repeat (LRR); a central nucleotide-binding domain/NOD (NACHT); and a caspase activation and recruitment domains (CARD), pyrin domain (PYD), or baculovirus inhibitor of apoptosis protein repeat (BIR) (BAUERNFEIND; HORNUNG, 2013). In humans, there are 22 known NLRs characterized by N-terminals composed of five receptor subfamilies, which resulted in the splitting of NLR family members into two main subfamilies: the NLRC - NLR family CARD domain-containing family (NOD1, NOD2, NLRC3, NLRC4 and NLRC5) and NLRP - NACHT, LRR, and the PYD domain-containing family (NLRP1-14), which accompanies three other small subfamilies NLRA (CIITA), NLRB (NAIP – neuronal apoptosis inhibitory protein) and NLRX (NLRX1) (BAUERNFEIND; HORNUNG, 2013) (**Fig. 5**).

The best characterized NLRs are NOD1 (NLRC1), NOD2 (NLRC2), NLR family containing a CARD domain 4 (NLRC4 - previously known as IPAF), and NLR family pyrin domain containing 3 (NLRP3) (DI VIRGILIO, 2013). Almost two decades ago, research began on the role of NLR receptors as inflammation mediators surrounding the discovery of a multiprotein complex called the inflammasome. This inflammasome is the basis of this work and will be described in the next topic.

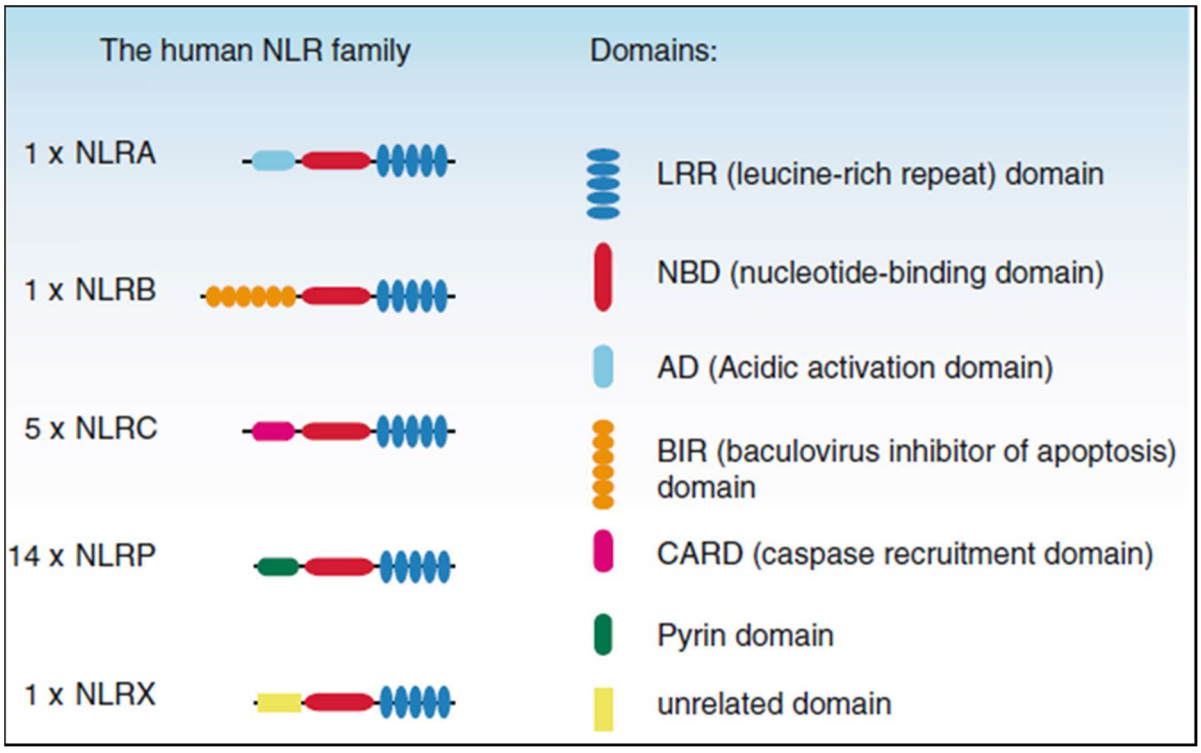

Figure 5: Scheme of NLR receptor subfamilies and their structure in humans.

NLRA (CIITA), NLRB (NAIP), NLRC (NOD1, NOD2, NLRC3, NLRC4 and NLRC5), NLRP3 (NLRP1, NLRP2-9, NLRP11-14 and NLRP10) and NLRX (NLRX1). According to (BAUERNFEIND et al., 2011).

4. Molecular platform: The inflammasome

Inflammasomes are multi-protein signaling complexes that assemble after recognition of danger signals and/or pathogens by a family of cytosolic receptors called NLRs (nucleotide binding domain and leucine-rich repeats containing receptors) or PYHIN protein family members. Once assembled, inflammasomes initiate signaling by activation of downstream proteases, most notably caspase-1 and caspase-11, which then proteolytically mature pro-IL-1β, pro-IL-18, and pro-IL-33, and promote their secretion from the cell. Most inflammasomes also use an adaptor molecule known as ASC (Apoptosis-associated speck-like protein) (**Fig. 6**) (GROSS et al., 2012; MARTINON; TSCHOPP, 2004).

After the activation of caspase-1, the cleavage of pro-IL-1β and pro-IL-18 occurs, producing their active forms IL-1β and IL-18, which stimulate inflammatory response (GROSS et al., 2012). Furthermore, activation of Caspase-1 via the inflammasome may induce pyrophosis, a type of inflammatory cell death (MIAO; RAJAN; ADEREM, 2011). The inflammasomes play an important role in the regulation of host immune response in response to different stimuli, whether endogenous or exogenous (MARTINON; BURNS; TSCHOPP, 2002).

Currently, five members of the NLRP family capable of forming the inflammasome have been described: NLRP1, NLRP2, NLRP3, NLRP6 and NLRP12. However, NLRB and NLRC4, are also able to form the inflammasome together with AIM2 (AIM2-Like receptor, ALR) (LAMKANFI; DIXIT, 2014; MARTINON; BURNS; TSCHOPP, 2002; SÁ; FESTA NETO, 2016; SCHRODER; TSCHOPP, 2010).

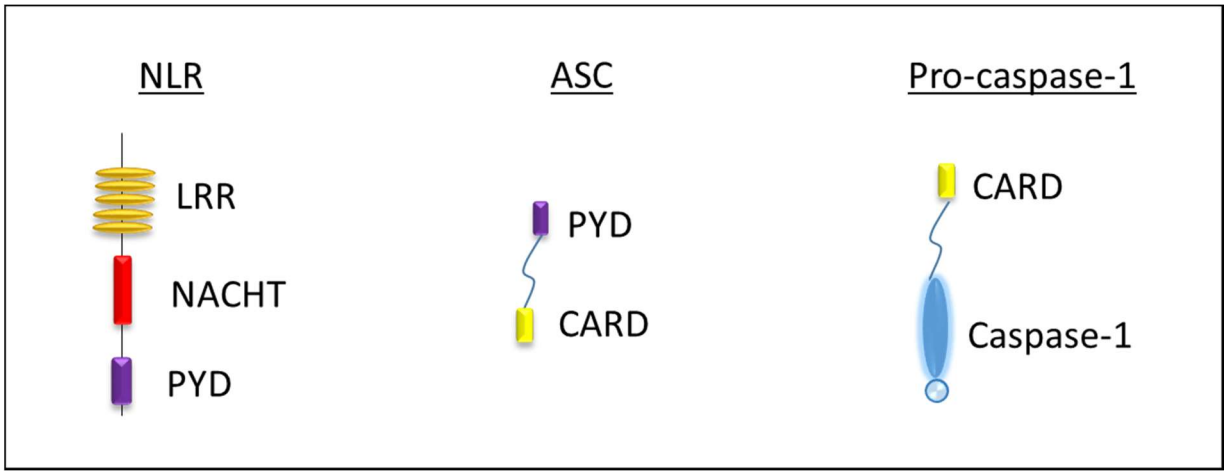

Figure 6: Scheme of the domain structure of the NLRP3 inflammasome components.

NLR (the nod-like receptor) is composed of a C-terminal LRR domain, a NACHT domain and an N-terminal PYD. ASC is composed of a PYD and a CARD, and Pro-caspase-1 has a CARD and caspase-1 (subunits p20 and p10) which is activated within the inflammasome multiprotein complex. Adapted by (INOUYE et al., 2018).

4.1 The Caspase-1 protein

In 1980, caspase-1 was initially discovered to play its role in the maturation of pro-caspase-1 that exists in the inactive form called (proform or zymogen) named ICE (Interleukin-1 converting enzyme or transformant IL-1 enzyme) due to its activity of pro-IL-1β processing in its mature form IL-1β (AFONINA et al., 2015). However, the mechanism involved in its activation was only well elucidated from the year 2002 when Tschopp and Martinon discovered the inflammasome (MARTINON; BURNS; TSCHOPP, 2002). Caspase-1 is present in the cellular cytoplasm in an inactive form of 45 kDa (pro-caspase-1), that consist, of an N-terminal CARD domain and a domain containing subunits (p20) and (p10) in C- terminal contains the small catalytic subunit of the enzyme (NAIK; DIXIT, 2010; SCHRODER; TSCHOPP, 2010; SHAMAA et al., 2015).

However, the functions of caspase-1 are not exclusively cytokine maturation. It also participates in the control of a type of programmed cell death called pyroptosis(DI VIRGILIO, 2013; GROSS et al., 2012). According to Petr Broz and colleagues (2011), the long form of

caspase-1 induces pyroptosis after the assembly of the NLRC4 inflammasome in *Salmonella typhimurium* and *Legionella pneumophila* (BROZ; MONACK, 2011). These results show that the re-expression of the non-cleaved form of caspase-1 in macrophages that were deficient for the caspase-1 gene is able to induce cell death, but with reduced IL-1β secretion (BROZ; MONACK, 2011). Also, interestingly, caspase-1 is able to regulate anti-inflammatory signaling pathways, for example its catalytic activity is responsible for cleaving and activating IL-37 (DINARELLO, 2018a; WANG, 2018).

4.2 Non-canonical activation of inflammatory caspases

Caspase-8 (Cysteinyl aspartic acid-protease 8) is an early protease of the extrinsic apoptosis pathway, a form of programmed cell death (ELMORE, 2007; MASUMOTO et al., 1999). Caspase-8 is implicated in a non-canonical type of activation and is directly involved in the cleavage and activation of IL-1β, inducing inflammatory responses (KETELUT-CARNEIRO et al., 2018; LATZ; XIAO; STUTZ, 2013a).The assembly of the non-canonical caspase-8 inflammasome occurs via ASC protein, without interaction with cytosolic receptors (CHUNG et al., 2016; LATZ; XIAO; STUTZ, 2013b; MASUMOTO et al., 1999). However, the interaction between caspase-8 and ASC occurs via their DED (Death effector domain) and PYD domains (FU et al., 2016).

Caspase-11 (non-canonical) is composed of an N-terminal and C-terminal CARD and can activate the NLRP3 inflammasome by directly binding intracellular lipopolysaccharide (LPS) to the CARD domain in macrophages infected with *Escherichia coli, Vibrio cholera, Legionella pneumophila, Salmonella typhimurium* and other Gram-negative bacteria that enter the cytosol (murine caspase-11, caspase- 4 and caspase-5 are orthologs of caspase-11 in humans) (JOZALA et al., 2013; MUNDAY et al., 1995; YI, 2018). Caspase-11 can also activate the NLRP3 inflammasome by reducing intracellular potassium levels, causing the release of ATP and leading to the maturation of IL-1β and IL-18, in addition to playing an important role in pyroptosis via the direct cleavage of gasdermin D (RÜHL; BROZ, 2015). Caspase-11 and caspase-1 act in concert in the inflammatory process of the host (YI, 2018).

4.3 The ASC protein

The ASC adapter protein is a 22 KDa protein composed of (179-195) amino acids, which is composed of an N-terminal PYD domain that regulates its self-association and interactions with the NLRP3 protein and a C-terminal CARD domain. Its main function is the activation of

caspase-1 (MASUMOTO et al., 1999), thus, as a function of its concentration, the ASC protein modulates the activation of pro-caspase-1 and maturation of IL-1β (STEHLIK, 2009). This protein plays an essential role in the activation of all inflammasomes, being implicated in the secretion of IL-1β, IL-18, and IFN-γ (KUMAR; KAWAI; AKIRA, 2011).

Studies have shown that, in the structure of the NLRP3 and AIM2 inflammasomes, their PYD domain is able to induce a prion-like polymerization of the PYD domain of ASC, being confirmed through visualization by cytometry and immunofluorescence analyses (CAI et al., 2014; DICK et al., 2016; RÜHL; BROZ, 2015). The exposed ASC filaments cause caspase-1 recruitment via CARD-CARD interaction (DICK et al., 2016). Recently, studies have shown that the formation of ASC specks can occur due to the accumulation of cells dying by pyroptosis in the microenvironment (STUTZ et al., 2013). According to Broz, P. (2011) the formation of ASC specks is independent of the activity of caspase-1, however it is necessary that oligomerization of ASC in large stable insoluble aggregates occurs that are released to the extracellular medium via pyroptosis leading to activation of inflammasomes (BROZ; MONACK, 2011; DICK et al., 2016).

4.4 The NLRP1 inflammasome

The NLRP1 inflammasome was the first member of the NLR family to be described, however, it is the least studied (MARTINON et al., 2006). The NLRP1 gene is unique in humans whereas in murines there are three homologous genes: Nlrp1a, Nlpr1b and Nlrp1c, which are highly polymorphic among different mouse strains (BROZ; MONACK, 2011; SÁ; FESTA NETO, 2016). The NLRP1 inflammasome is composed of the NLRP1 protein from C-terminal to N-terminal, CARD, a domain with function to find (FIIND), LRR, NACHT, and PYD (**Fig 4**). However, the PYD domain is only found in human NLRP1 (CHAVARRÍA-SMITH; VANCE, 2015; LOPEZ-CASTEJON; BROUGH, 2011). The presence of the CARD domain suggests that NLRP1 can interact directly with caspase-1 and caspase-5, leading to maturation of IL-1β and IL-18 (FRANCHI et al., 2009; MARTINON; BURNS; TSCHOPP, 2002).

NLRP1b was identified as inducing the secretion of IL-1β and IL-18 and killing rat macrophage cells in response to Bacillus anthracis lethal toxin (LeTx) (**Fig. 4**) (NEWMAN et al., 2010). LeTx is a bipartite toxin composed of an antigen-binding protective (PA) subunit and a part of the catalytic lethal factor (LF) (KUSHNER et al., 2003). LF is a zinc metalloprotease that cleaves and inactivates all mitogen activated protein kinases (MAPKs) (AGRAWAL; PULENDRAN, 2004; NEWMAN et al., 2010; RANSON; KUNDE; ERI, 2017).

Thus, NLRP1 is associated with the activations of pyroptosis and caspases-2 and 9 in the complex called apoptosome. The apoptosome is a protein complex formed by the association of cytochrome C to the Apaf-1 protein and pro-caspase-9 during the intrinsic apoptosis signaling pathway (BRATTON; SALVESEN, 2010).

In addition, human NLRP1 was shown to recognize muramyl-dipeptide (MDP), a peptideoglycan composite, it may induce changes in conformation leading to the activation of Caspase-1 (**Fig. 7**) (LATZ; XIAO; STUTZ, 2013b; MARTINON; BURNS; TSCHOPP, 2002; MARTINON; TSCHOPP, 2004). However, the mechanism of activation is indirect and depends on ionic changes between the intracellular and extracellular medium suggesting a link between the activation of NLRP1 inflammasome and the NLRP3 inflammasome (DI VIRGILIO, 2013).

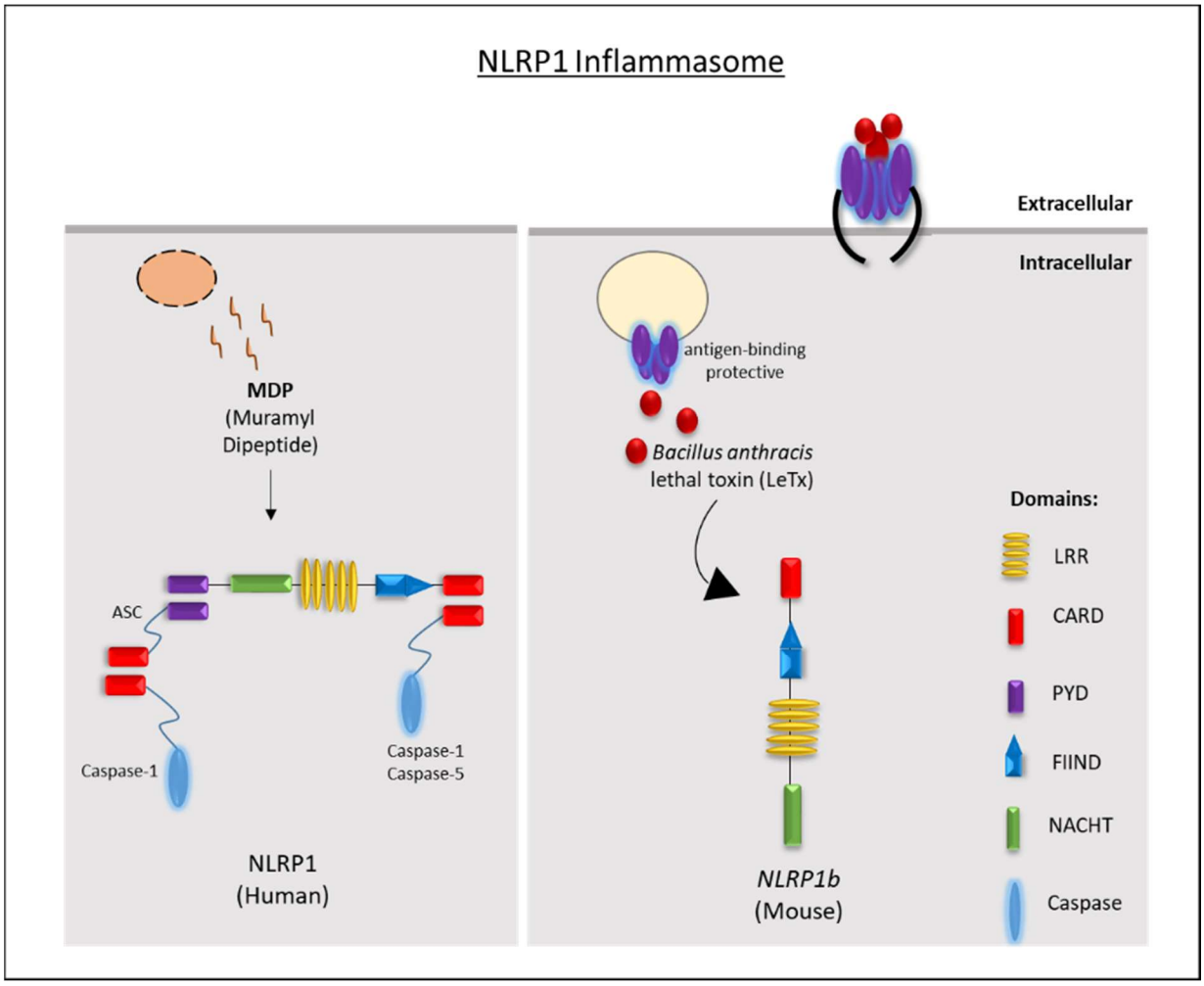

Figure 7: Human NLRP1 responds to muramyl dipeptide, while Anthrax lethal toxin triggers murine Nlrp1b. Adapted by (BROZ; MONACK, 2011).

4.5 The NLRP3 inflammasome

The inflammasome NLRP3 (also known as cryopyrin and NALP3) is the most extensively characterized member of the NLR family due to the diversity of its activation stimuli and its association with a wide range of inflammatory pathologies (LATZ; XIAO; STUTZ, 2013a). The NLRP3 inflammasome contains a structure of 3 separate domains; an N-terminal pyrin domain (PYD), a nucleotide-binding and oligomerization domain (NATCH) and a C-terminal leucine-rich repeat (LRR). However, unlike other NLRs, the NLRP3 inflammasome does not have (CARD), thus requiring the adapter protein (ASC) to bind to pro-caspase-1 (**Fig. 6**) (BAUERNFEIND; HORNUNG, 2013; MARTINON; TSCHOPP, 2004).

The NLRP3 inflammasome can be activated by PAMPs and damage-associated molecular patterns (DAMPs) such as Sendai virus, influenza A virus, adenovirus, *Staphylococcus aureus, L. monocytogenes, E. coli, Mycobacterium marinum, S. flexneri, Neisseria gonorrhoe, Candida albicans*, bacterial RNA, ATP, pore forming toxins, and structural aggregates such as monosodium urate and calcium pyrophosphate (SeeTable 2) (ALLEN et al., 2009; BAUERNFEIND et al., 2011; DUNCAN et al., 2009; GROSS et al., 2009; ICHINOHE; PANG; IWASAKI, 2010; JOLY et al., 2009; KANNEGANTI et al., 2006; MARIATHASAN et al., 2006; MARTINON et al., 2006; MURUVE et al., 2008).

However, the activation medium of the NLRP3 inflammasome is still controversial due to the large number of substances that can trigger its formation (**Table 2**). The most accepted theory regarding the NLRP3 inflammasome activation mechanism, supported by several studies, include potassium efflux out of the cell, reactive oxygen species (ROS), cathepsin release into the cytosol after lysosomal destabilization, and cardiolipin or release of mitochondrial DNA (GRAZIOLI; PUGIN, 2018; VANAJA; RATHINAM; FITZGERALD, 2015; WEI et al., 2019).

The activation of the NLRP3 inflammasome can occur by three different. *i)* Canonical pathway (Canonical NLRP3 inflammasome activation requires two parallel and independent steps: transcription and oligomerization), described in detail below. *ii)* Non-canonical (caspase-11-dependent NLRP3 activation. In particular, Gram-negative bacteria (i.e., *Escherichia coli, Salmonella typhimurium*), activate the TLR4–MyD88 and toll/IL-1 receptor homology-domain-containing adapter-inducing interferon-β (TRIF) pathways, with a consequent nuclear translocation of NF-κB, which in turn promotes the transcription of IL-1β, IL-18 (PELLEGRINI et al., 2017). *iii)* Alternative pathways (This alternative pathway is independent of K efflux and leads to a low and gradual IL-1β response without pyroptosome and depends on TRIF, RIPK1, FADD, and caspase-8 signaling pathway).

NLRP3 Inflammasome	Stimulus	Class	Reference
	Sendai virus	Virus	Kanneganti et al, 2006
	Influenza A		Allen et al, 2009
	Adenovirus		Muruve et al, 2008; Barlan et al, 2011
	Staphylococcus aureus	Bacteria	Mariathasan et al, 2006; Muñoz-Planillo et al, 2009
	Listeria monocytogenes		Meixenberger et al, 2010; Theisen et al, 2016
	Escherichia coli		Yen et al, 2015
	Mycobacterium marinum		Koo et al, 2008
	Neisseria gonorrhoeae		Duncan et al, 2009
	Candida albicans	Fungus	Gross et al, 2009; Joly et al, 2009
	Silica	DAMP	Hornung et al, 2008
	Monosodium uric acid crystals	DAMP	Martinon et al, 2006
	Lipopolysaccharide (LPS)	PAMP	Hong and Yu, 2018
	Nigericin	PAMP	Meixenberger et al, 2010
	ATP	DAMP	Mariathasan et al, 2006
	Listerolysin O	PAMP	Mariathasan et al, 2006
	Bacterial RNA	PAMP	Vanaja et al, 2014
	Hemolysins	PAMP	Muñoz-Planillo et al, 2009
	Amyloid beta	DAMP	Halle et al, 2008
	Hyaluronan	DAMP	Yamasaki , et al, 2009

Table 2: List of activators known to activate of the NLRP3 inflammasome.

The canonical pathway requires 2 steps: priming and activation. A first signal called "Priming" is initiated when a PAMPs or DAMPs bind to a TLR receptor through (LPS), or after signaling via endogenous cytokine receptors such as IL-1R or through tumor necrosis factor (TNF-alpha) (**Fig. 8**) (GRAZIOLI; PUGIN, 2018; MOGENSEN, 2009). This signal is required for the transcription, translation, and unubiquitination of NLRP3 to induce pro-IL-1β expression via activation of the transcription factor (NF-κB) (KINOSHITA et al., 2015; LIU et al., 2017; QIAO et al., 2012). This priming signal allows activating K63-specific deubiquitinase (BRCC3), a JAMM domain-containing Zn2+ metalloprotease leading to the unsubiquitination of NLRP3, a step necessary for its activation (HE; HARA; NÚÑEZ, 2016; PY et al., 2013).

The non-canonical pathway of the NLRP3 inflammasome activation," which depends on caspase-11 in mice (human orthologs are caspase 4 and caspase 5), has been described to be pivotal in the maintenance of intestinal immune homeostasis (KAYAGAKI et al., 2013; PELLEGRINI et al., 2017). Activation of the non-canonical pathway of caspase-11 in response to gram-negative bacteria was explained when it was discovered in recent years that LPS, which is a component of the outer membrane of gram-negative bacteria, delivered intracellularly by transfection, electroporation or by bacteria that reach the cytoplasm signals, independent of TLR4, binding directly to caspase-11 (BROZ et al., 2012; KAYAGAKI et al., 2013; RATHINAM; VANAJA; FITZGERALD, 2012). Since its discovery, the unconventional canonical pathway of inflammasome activation has been the subject of intense research.

The third mechanism of activation for the NLRP3 inflammasome in human monocytes is described as an "alternative pathway" and is similar to the canonical pathway (GAIDT; HORNUNG, 2017). This pathway depends on the binding of LPS via TLR4. However, contrary

to what happens with the canonical pathway, the efflux of potassium is not necessary (GAIDT; HORNUNG, 2017). Thus, the Fas-associated death domain (FADD), receptor-interacting protein kinase-1 (RIPK1) and caspase-8 act downstream of TLR4 receptor signaling via interleukin-1 receptor-domain-containing adapter-inducing interferon-β (TRIF) to activate NLRP3 (GROSLAMBERT; PY, 2018; HE; HARA; NÚÑEZ, 2016). However, there is no evidence of ASC speck formation and caspase-1 or pyroptosis in this pathway, although both ASC and caspase-1 are required for inflammasome formation (GAIDT; HORNUNG, 2017; GROSLAMBERT; PY, 2018).

Besides, in the literature three different mechanisms are described that result in the activation of the inflammasome NLRP3, however, these signals are not exclusive to the NLRP3 inflammasome: K^+ efflux, cathepsin B release into the cytosol after lysosomal destabilization and ROS production (STUTZ et al., 2013; YANG et al., 2019).

K^+ efflux is a common mechanism for the activation of the NLRP3 inflammasome. Perregaux and Gabel (1994) have shown that in murine macrophages stimulated with LPS, potassium ionophores such as nigericin promote IL-1β secretion (PERREGAUX; GABEL, 1994). Followed by other studies about the importance of K^+ efflux in the activation of the NLRP3 inflammasome (GIULIANI et al., 2017; MUÑOZ-PLANILLO et al., 2013; PICCINI et al., 2008). This efflux is induced in response to various stimuli such as ATP, nigericin and several extracellular crystals, all known to decrease cytosolic K^+ levels through the P2X7 receptor (GIULIANI et al., 2017). These pore channels promote potassium efflux, resulting in low concentrations of intracellular K^+, causing conformational changes in NLRP3 thereby activating the NLRP3 inflammasome (HE; HARA; NÚÑEZ, 2016; MUÑOZ-PLANILLO et al., 2013). Thus, the drop in K^+ concentration in the cytosol is proposed as a common trigger for the activation of the NLRP3 inflammasome (HE; HARA; NÚÑEZ, 2016; MUÑOZ-PLANILLO et al., 2013).

Particles such as alum or silica are phagocytosed by cells of the immune system and activate the NLRP3 inflammasome through the degradation of the phagolysosomal membrane causing the release of Cathepsin B, a protease that is able to bind directly to the NLRP3 receptor and induce the activation of inflammasome (LATZ; XIAO; STUTZ, 2013a). Furthermore, this particulate material may trigger a K^+ efflux, which is dependent on phagocytosis and is required for the activation of the NLRP3 inflammasome (MUÑOZ-PLANILLO et al., 2013; YANG et al., 2019).

The production of ROS in the cellular cytoplasm of macrophages and monocytes occurs via a mitochondrial dysfunction caused due to cellular stress in response to a stimulus such as ATP, silica or monosodium urate (MSU) (LAWLOR; VINCE, 2014; ZHOU et al., 2011). Dostert and collaborators, (2008) propose that the release of ROS produced by NADPH oxidase is

responsible for the activation of the NLRP3 inflammasome based on studies with chemical inhibitors (DOSTERT et al., 2008a).

4.6 The AIM2 inflammasome

AIM2 (absent in melanoma 2) is the first non-NLR receptor capable of forming an inflammasome (VANAJA; RATHINAM; FITZGERALD, 2015). AIM2 is part of the PYHIN-200 family, characterized by an N-terminal domain (PY) and a C-terminal domain (HIN200). AIM2 does not contain a CARD domain and therefore requires ASC recruitment through its PYD for the activation of the inflammasome, this step is necessary for there to be a PYD-PYD domain binding of AIM2 with caspase-1 (HE; HARA; NÚÑEZ, 2016) (**Fig 9**). Activation of the inflammasome occurs directly after stimulation by IFN-γ or double-stranded DNA (dsDNA) via HIN200, leading to the recruitment of ASC and caspase-1 by AIM2 (FERNANDES-ALNEMRI et al., 2007; SCHRODER; TSCHOPP, 2010; VANAJA; RATHINAM; FITZGERALD, 2015). AIM2 is able to detect from 80 base pairs of virus DNA such as mouse cytomegalovirus to cytosolic bacteria such as *Francisella tularensis* and *Listeria monocytogenes,* acting against these pathogens and promoting cell death in order to restrict bacterial growth (RATHINAM; FITZGERALD, 2016; RATHINAM; VANAJA; FITZGERALD, 2012).

4.7 The NLRC4 inflammasome

The NLRC4 inflammasome or ICE-protease activating factor (IPAF) is formed by the NLRC4 protein and caspase-1 (DI VIRGILIO, 2013; DUNCAN; CANNA, 2018). NLRC4 is composed of an N-terminal CARD domain interacting directly with caspase-1. However, although NLRC4 possesses a CARD domain, ASC is required to amplify the platform's operation and its activation (**Fig. 9**) (LAGE et al., 2014; VANCE, 2015).

Bacterial PAMPs are responsible for the formation of the NLRC4 inflammasome which is activated via NAIP (NLR family-apoptosis inhibitory protein) which binds to specific bacterial ligands and is co-assembled with NLRC4 (ZHAO et al., 2011). However, other bacteria such as *Salmonella typhimurium, Legionella pneumophila, Shigella flexneri* and *Pseudomonas aeruginosa*, are also known activators of this inflammasome (BROZ; MONACK, 2011).

In NAIP1 mice it is bound to the bacterial secretory system type III (T3SS), protein needle, NAIP2 to T3SS basal protein, and NAIP5 and NAIP6 bacterial flagellin, which is its most characterized activator (BAUERNFEIND et al., 2011; MIAO et al., 2006; RAYAMAJHI et al., 2013; REN et al., 2006; ZHAO et al., 2011). However, in humans, NAIP1 was only found bound

to the T3SS protein needle (YANG et al., 2018). Thus, after the junction of NAIP and its ligand, oligomerization with NLRC4 and ASC can be accomplished by initiating the assembly of the inflammasome (VANCE, 2015).

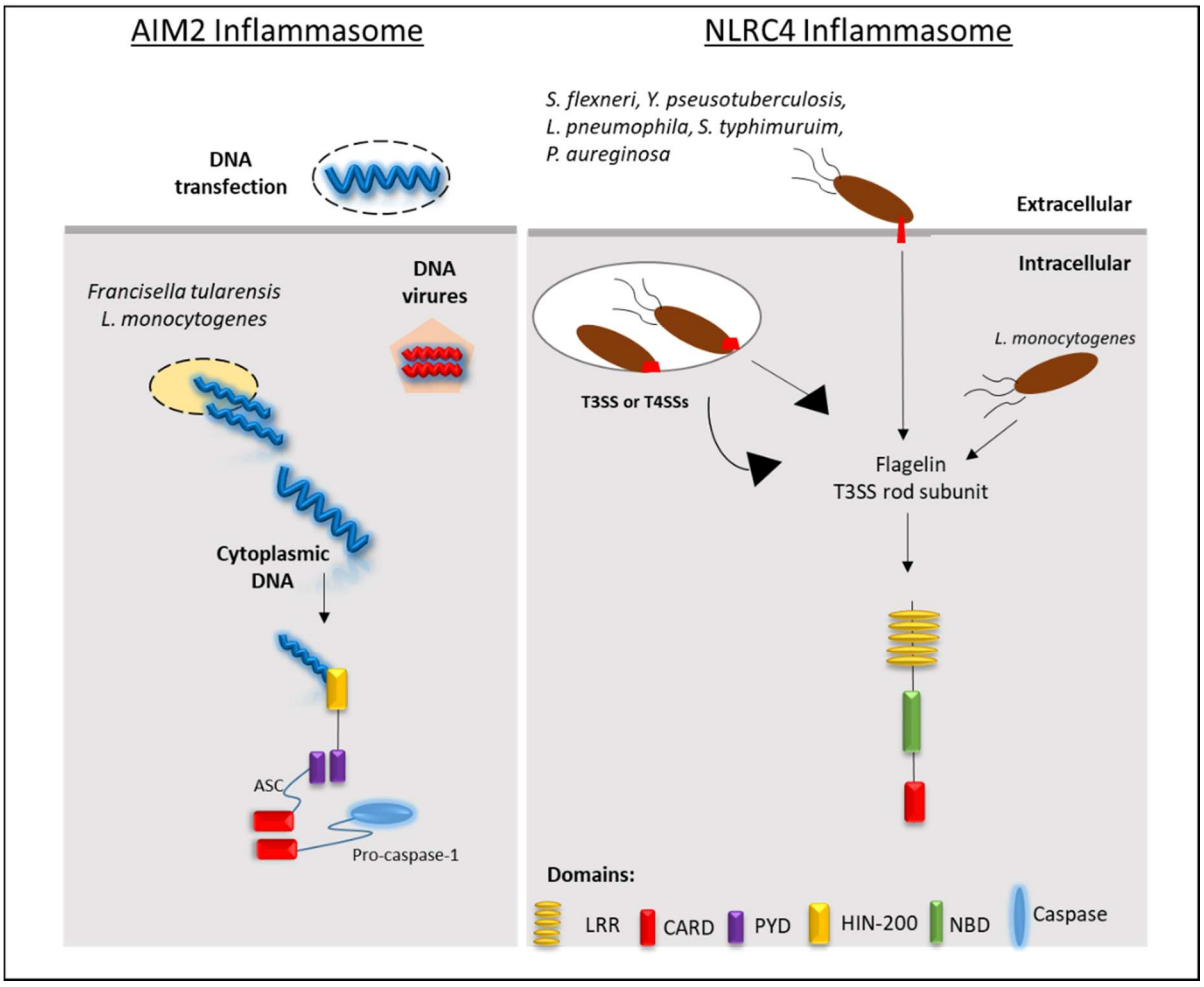

Figure 8: AIM2 and NLRC4 inflammasomes are activated by specific PAMPs.

AIM2 functions as a cytosolic DNA sensor, detecting DNA introduced by transfection, infection with the cytosolic bacterial pathogens *F. tularensis* or *L. monocytogenes* or DNA viruses. NLRC4 detects flagellin or the T3SS rod subunit in the cytosol. Adapted by (BROZ; MONACK, 2011).

4.8 Others inflammasomes

The NLRP6 inflammasome is implicated in the homeostasis of the intestinal flora and digestive tract of the host (DI VIRGILIO, 2013; OKUMURA; TAKEDA, 2017). The NLRP6 receptor expressed in cells of the immune system or epithelial cells is able to form inflammasome (ELINAV et al., 2011; LEI; NAIR; ALEGRE, 2015). In the intestinal epithelium, NLRP6, plays a

protective role in preserving the intestinal flora, but on the other hand can play a detrimental role by aggravating bacterial infections (DI VIRGILIO, 2013; YIN et al., 2019).

In a study by Elinav and colleagues (2011), it was shown that the absence of NLRP6 in mice causes a change in the composition of their microbiota, leading to an increase bacterial phyla Bacteroidetes (Prevotellaceae) and TM7, and a decrease of *Lactobacillus* ssp. (ELINAV et al., 2011). This activity is suggestive of the role of this inflammasome in the regulation of the microbiota.Furthermore, NLRP6/KO mice, when compared to wild-type mice, were shown to secrete less IL-18 and were more likely to develop tumors in a chemically induced DSS (Dextran-sodium sulfate) model of colitis, (CHEN et al., 2017a; ELINAV et al., 2011; MAO et al., 2018).

The NLRP7 inflammasome is responsible for the maturation and release of IL-1β and IL-18 after stimulation by microbial lipopeptides, being the cause of septic shock in mice (DI VIRGILIO, 2013; KHARE et al., 2012). NLRP7, ASC and caspase-1 form the complex molecule, however, the mechanism of action of this inflammasome does not involve the production of ROS, but the cathepsin B and K^+ efflux pathways (DI VIRGILIO, 2013; RADIAN et al., 2013).

The NLRP12 inflammasome participates in the recognition of the bacterium Yersinia pestis and promotes the release of IL-1β and IL-18, as well as the secretion of IFN-γ by the activation of lymphocytes, being also associated with the inhibition of NF-kB (SILVEIRA et al., 2017; VLADIMER et al., 2013). The interaction with the adapter protein ASC is required for the assembly the NLRP12 inflammasome, supporting the inclusion of NLRP12 as part of the inflammasome family (DI VIRGILIO, 2013).

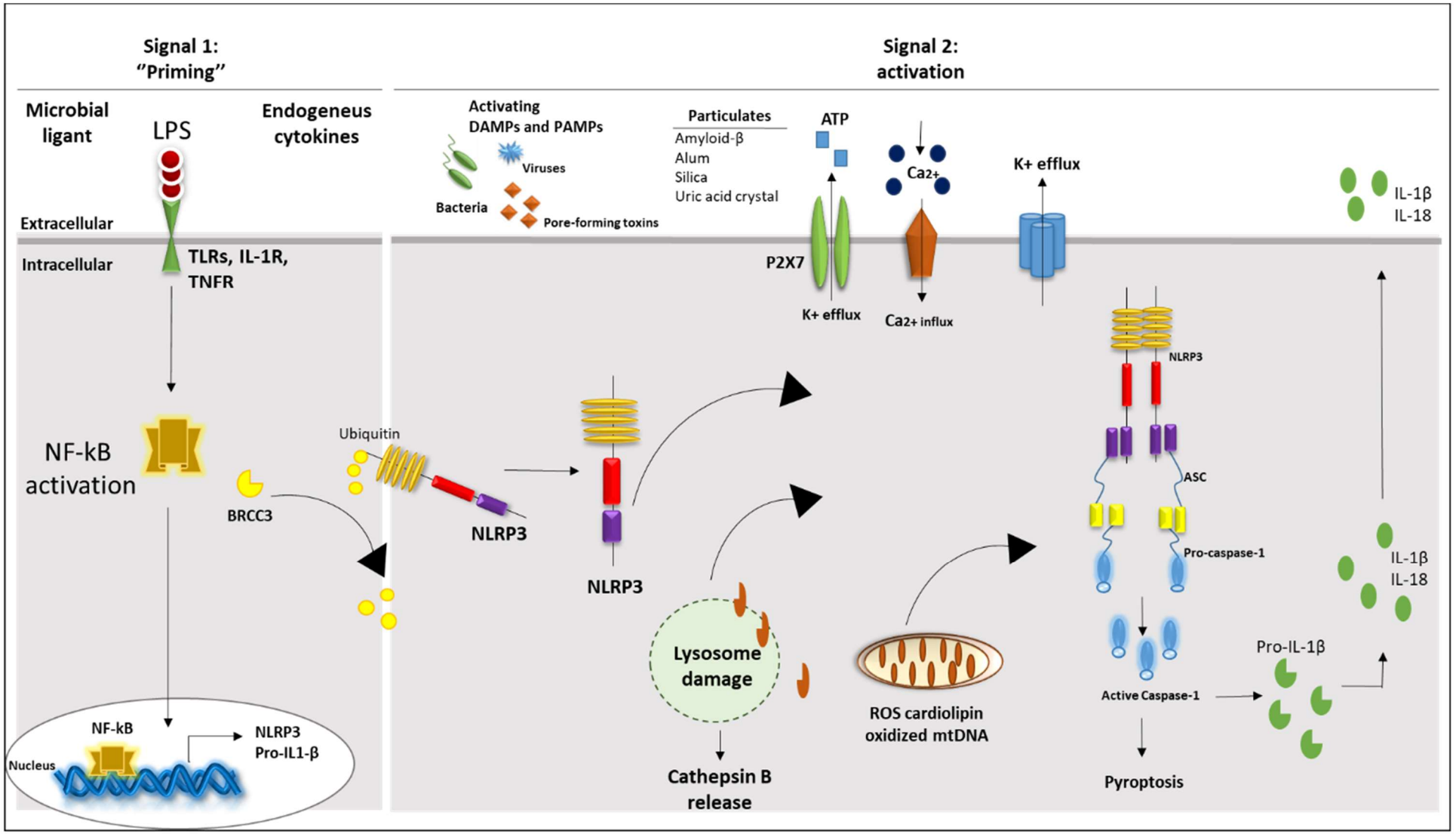

Figure 9: NLRP3 inflammasome activation mechanism.

Figure 8: **NLRP3 inflammasome activation mechanism**. The activation of a TLR allows the release of the transcription factor NF-kB, responsible for the synthesis of IL-1β and NLRP3 expressed in its basal state. The first signal called "priming" also allows for the activation of BRCC3, leading to the unubiquitination of NLRP3, a step necessary for its activation. The assembly of the NLRP3 inflammasome can occur through three known signals: 1) release of cathepsin B through lysosomal rupture; 2) ionic fluid via pore opening of the plasma membrane; 3) ROS synthesis. Activation of NLRP3 leads to its oligomerization and to the recruitment of ASC and pro-caspase-1, causing autoproteolysis and Caspase-1 activation. After activation, Caspase-1 cleaves and induces the release of IL-1β and IL-18 and may also induce pyroptosis.

4.9 Inflammasome and pathologies

The inflammasome plays an important role in relation to various diseases. Recently, studies have highlighted the characteristics of a group of pathologies related to the recurrent activation of the inflammasome, such as gout or pseudogout, caused by intracellular deposition of MSU crystals or CPPD (Calcium pyrophosphate deposition), as well as familial pathologies related to mutations or variants of the expression of inflammasome-forming receptors (DI VIRGILIO, 2013).

In humans, autoimmune diseases such as vitiligo, characterized as a chronic inflammatory disease in which melanocytes are destroyed, leading to skin depigmentation, rheumatoid arthritis, psoriasis, and autoimmune thyroiditis are related to a variant of the NLRP1 protein coding gene, thus the inflammasome plays an important role in regulating the adaptive immune response (CIRACI, 2017; MARIE et al., 2014).

Cryopyrinopathies, also known as periodic syndromes associated with cryopyrin (CAPS), are inflammasomopathies caused by autosomal dominant mutations in the gene encoding NLRP3. They are described as three types of inflammasomopathies: familial cold-induced autoinflammatory syndrome (FCAS), of milder gravity, Muckle-Wells syndrome (MWS) with an intermediate phenotype, and the most severe form is the neonatal onset multisystem inflammatory disorder (NOMID) (DI VIRGILIO, 2013; KASTNER; AKSENTIJEVICH; GOLDBACH-MANSKY, 2010).

Mutations that give rise to these inflammatory syndromes cause constitutive activation of NLRP3 in monocytes inducing an excessive production of IL-1β (BONAR et al., 2012; MAO et al., 2018). Other diseases are associated with inflammasomes as multiple sclerosis (GOVERMAN, 2009), Alzheimer's disease (HENEKA; GOLENBOCK; LATZ, 2015), Parkinson's disease model (SHULMAN; DE JAGER; FEANY, 2011), atherosclerosis

(ROBBINS; WEN; TING, 2014), type 2 diabetes (DONATH; SHOELSON, 2011), and obesity (DONATH; SHOELSON, 2011).

To date, frequent episodes of generalized inflammation occur without evidence of an apparent stimulus, even because the host's own immune system functions as a trigger for the pathological process. Thus, understanding how inflammasome works and its regulation process is fundamental for the development of adequate therapeutic strategies focused on the resolution of inflammation.

Chapter 2. Problem statement of the project thesis

Rationale of the PhD project

Advances on several research fronts have significantly broadened our knowledge about the inflammation triggers and modulation of the host inflammatory response. The recent discovery of the inflammasomes protein complex has been of great importance in the fields of immunology and medicine. Such importance is due to both the identification and characterization of the molecular mechanisms that link the cell stimulation (by pathogens, physical or chemical lesions) to the activation of early immune cell responses in the host.

Concerning the inflammation process by bacterial pathogens, *Staphylococcus aureus* is a major bacterial human pathogen that colonizes the nares and skin and frequently invades the soft tissues and bloodstreams. *S. aureus* has numerous mechanisms to evade and subvert the immune system, causing a variety of invasive and superficial infections (TONG et al., 2015). This is observed, for example, in diseases such as bone & joint infections (BJI), particularly in the presence of orthopedic devices (SAAVEDRA-LOZANO et al., 2017). The treatment of BJI infection is particularly challenging and time-consuming, leading to enormous health care costs (DEL POZO; PATEL, 2009; ZIMMERLI; TRAMPUZ; OCHSNER, 2004). Despite surgical treatment and adequate antimicrobial therapy, BJIs are linked to a high percentage of relapses, often leading to the development of chronic disease (FERRY et al., 2018; PEETERS et al., 2016). While many investigations are focused on antibacterial strategies, few efforts are directed to acquire knowledge about the role of the immune system in fighting against this infection.

The role of the inflammasomes formed in professional phagocytes such as macrophages and non-professional phagocytes, such as epithelial cells infected by *S. aureus* and other microorganisms, has been extensively investigated (FLANNAGAN; HEIT; HEINRICHS, 2015; KREMSEROVA; NAUSEEF, 2019; MA et al., 2019). Several types of cells including monocytes, macrophages, neutrophils, and dendritic cells (DCs) express the various PRRs that recognize PAMPs and DAMPs, which leads to the prompt activation of pro-inflammatory cytokines IL-1β and IL-18 (CARRILLO et al., 2017; FULLARD; O'REILLY, 2015; SCHRODER; TSCHOPP, 2010).

Many studies clarified the mechanisms responsible for the activation of pro-caspase-1 and the maturation of IL-1β. Once activated, IL-1β plays a crucial role in chronic inflammatory diseases, metabolic disorders, and cancer. Besides, several inflammatory diseases associated with increased bone resorption have been well reported (DE VRIES et al., 2019; HARDY; COOPER, 2009; ISEME et al., 2017; LACATIVA; FARIAS, 2010). IL-1β, as well as other

inflammatory cytokines (e.g. IL-6 and TNFα), play an important role in bone remodeling. *S. aureus* infection can trigger the local and systemic production of these three cytokines through host PRRs (RASIGADE et al., 2013). The increased production of these cytokines then leads to an imbalance in the homeostasis of bone turnover in favor of the activity of osteoclastogenesis and bone resorption (FENG; MCDONALD, 2011; PEETERS et al., 2016; RASIGADE et al., 2013).

Studies have shown that the invasion and persistence of *S. aureus* in host cells described as "non-professional phagocytes" is documented in many cell types, including epithelial cells, endothelial cells and keratinocytes (KINTARAK et al., 2004; RASIGADE et al., 2013). However, the interactions between osteoblastic cells and *S. aureus* remains comparatively understudied. In this context, we hypothesized that persistent infection by *S. aureus* involves the transcriptional reprogramming of osteoblastic cells to tolerate the asymptomatic transport of intracellular bacteria, leading to altered immune response, more specifically to the modification of the NLRP3 inflammasome activation and subsequent processing of the cytokine pro-inflammatory IL-1β.

Therefore, to analyze the activation of the inflammasome, the activation of pro-caspase-1 that undergo proteolytic processing to produce 2 subunits that dimerize to form the active caspase-1 needs to be monitored. Furthermore, monitoring the active caspase-1 (comprising the p20 and p10 subunits) and their effect on IL-1β induction is essential. To demonstrate that IL-1β is indeed activated by the inflammasome were required the generation of a deletion mutant Cas1$^{-/-}$ MG-63 using the CRISPR/Cas9 gene editing system. Thus, this work was divided into two parts:

- In the first part of the study, a model is required to allow investigating the involvement of the NLRP3 inflammasome in *S. aureus* infection. The knockout caspase-1 gene in MG-63 cells will be through CRISPR/cas9 gene editing approach. Subsequently, the activation of caspase-1 in wild type (WT) MG-63 and mutant cell line CASP1$^{-/-}$ MG-63 cells will be monitored (**Fig. 10-A**).

- In the second part of the study, the production of a soluble active form of IL-1β will be monitored in WT MG-63 vs CASP1$^{-/-}$ MG-63 cells in an *in vitro* cellular model of chronic infection using long-term infections (from 6 h to 11 days) of osteoblasts with different strains of *S. aureus* (**Fig. 10-B**).

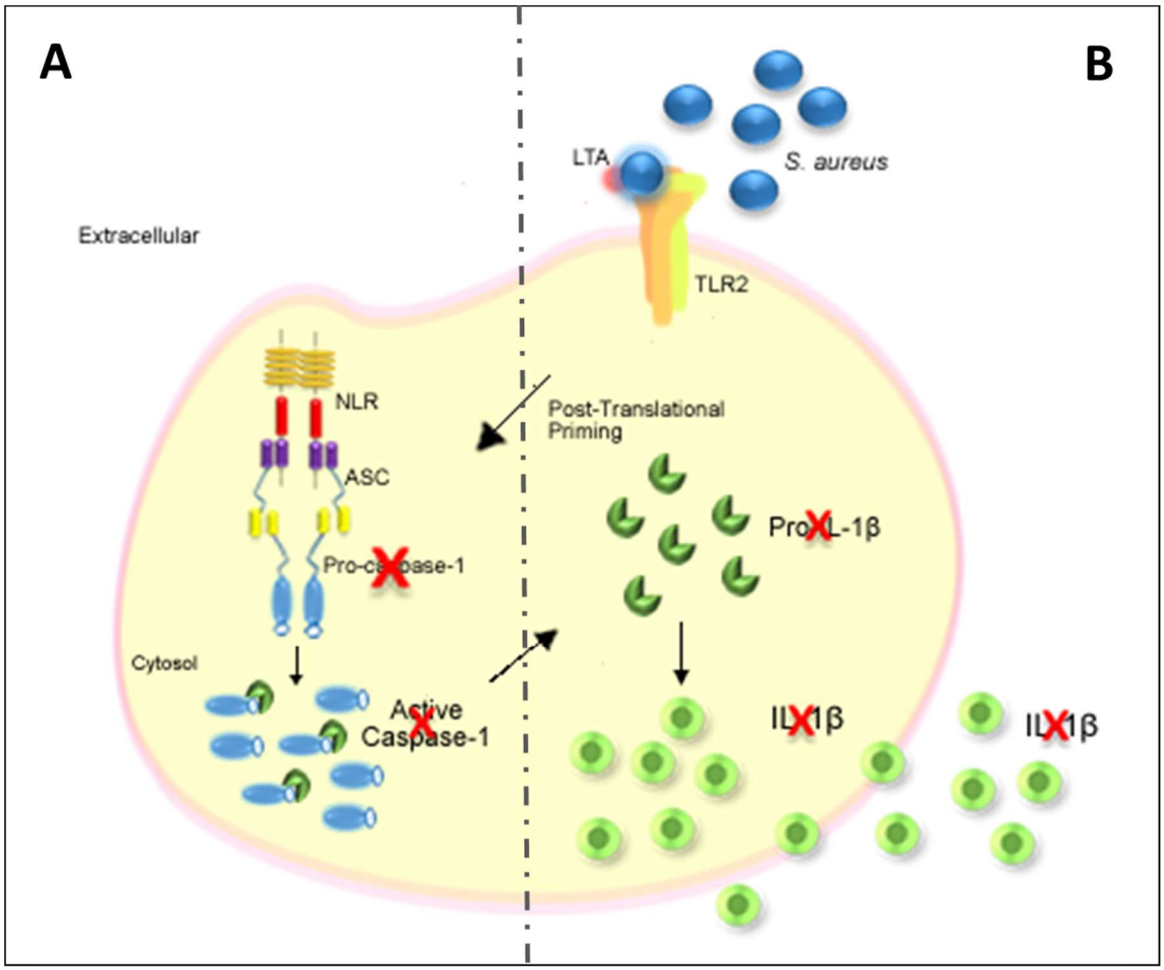

Figure 10: Schematic of the proposed objectives for the first and second part of the thesis.

Pertinence du projet de Thèse

Les avancées sur plusieurs fronts de recherche ont permis de considérablement améliorer notre connaissance des déclencheurs et des modulateurs de l'inflammation. La découverte récente des inflammasomes a été d'une grande importance dans les domaines de l'immunologie et de la médecine. Cette importance est due à l'identification et à la caractérisation des mécanismes moléculaires qui lient la stimulation cellulaire (par des agents pathogènes, des lésions physiques ou chimiques) à l'activation des réponses cellulaires immunitaires précoces chez l'hôte.

Concernant le processus d'inflammation par des bactéries pathogènes, *Staphylococcus aureus* est un agent pathogène humain majeur qui colonise les narines et la peau et envahit fréquemment les tissus mous et le sang. *S. aureus* dispose de nombreux mécanismes pour contourner et neutraliser le système immunitaire, provoquant diverses infections invasives et superficielles (TONG et al., 2015). Cela se voit, par exemple, dans des maladies telles que les infections des os et des articulations, en particulier en présence d'appareils orthopédiques (SAAVEDRA-LOZANO et al., 2017). Le traitement de cette infection est particulièrement difficile et prend du temps, avec des coûts énormes en soins de santé (DEL POZO; PATEL, 2009; ZIMMERLI; TRAMPUZ; OCHSNER, 2004). En dépit d'un traitement chirurgical et d'un traitement antimicrobien adéquat, ces infections donnent lieu à un pourcentage élevé de rechutes à court terme, conduisant souvent au développement d'une maladie chronique (FERRY et al., 2018; PEETERS et al., 2016). De nombreuses études sont axées sur les stratégies antibactériennes. On en sait moins sur le rôle du système immunitaire dans la lutte contre ce type d'infection.

Le rôle des inflammasomes formés dans les phagocytes professionnels tels que les macrophages et les phagocytes non professionnels, tels que les cellules épithéliales infectées par *S. aureus* et d'autres micro-organismes, a fait l'objet de nombreuses études (FLANNAGAN; HEIT; HEINRICHS, 2015; KREMSEROVA; NAUSEEF, 2019; MA et al., 2019). Plusieurs types de cellules, notamment les monocytes, les macrophages, les neutrophiles et les cellules dendritiques (CD) expriment les différents PRR reconnaissant les PAMP et les DAMP, ce qui conduit à l'activation rapide des cytokines pro-inflammatoires IL-1β et IL-18 (CARRILLO et al., 2017; FULLARD; O'REILLY, 2015; SCHRODER; TSCHOPP, 2010).

De nombreuses études ont clarifié les mécanismes responsables de l'activation de la pro-caspase-1 et de la maturation de l'IL-1β. Une fois activée, l'IL-1β joue un rôle crucial dans les maladies inflammatoires chroniques, les troubles métaboliques et le cancer. En outre,

plusieurs maladies inflammatoires associées à une résorption osseuse accrue ont été bien rapportées (DE VRIES et al., 2019; HARDY; COOPER, 2009; ISEME et al., 2017; LACATIVA; FARIAS, 2010). L'IL-1β, ainsi que d'autres cytokines inflammatoires (par exemple, IL-6 et TNFα), jouent un rôle important dans le remodelage osseux. L'infection à *S. aureus* peut déclencher la production locale et systémique de ces trois cytokines par le biais de PRR hôtes (RASIGADE et al., 2013). La production accrue de ces cytokines entraîne alors un déséquilibre dans l'homéostasie du remodelage osseux au profit de l'activité de l'ostéoclastogenèse et de la résorption osseuse (FENG; MCDONALD, 2011; PEETERS et al., 2016; RASIGADE et al., 2013).

Des études ont montré que l'invasion et la persistance de *S. aureus* dans les cellules hôtes décrites comme des "phagocytes non professionnels" sont documentées dans de nombreux types de cellules, notamment les cellules épithéliales, les cellules endothéliales et les kératinocytes (KINTARAK et al., 2004; RASIGADE et al., 2013). Cependant, les interactions entre les cellules ostéoblastiques et *S. aureus* restent relativement peu étudiées. Dans ce contexte, nous avons émis l'hypothèse que l'infection persistante par *S. aureus* implique la reprogrammation transcriptionnelle de cellules ostéoblastiques afin de tolérer le portage asymptomatique des bactéries intracellulaires, conduisant à une altération de la réponse immunitaire et plus spécifiquement à une modification de l'activation des inflammations et du traitement ultérieur de l'IL-1β.

Afin de mettre en évidence l'activation de l'inflammasome, l'activation de la pro-caspase-1 qui subit un traitement protéolytique pour produire 2 sous-unités qui se dimérisent pour former la caspase-1 active doit être surveillée. En outre, il est essentiel de surveiller la caspase-1 active (comprenant les sous-unités p20 et p10) et leur effet sur l'induction de l'IL-1β. Pour démontrer que l'IL-1β est effectivement activée par l'inflammasome, il a été nécessaire de générer un mutant de délétion du gène caspase-1, Cas1$^{-/-}$MG-63 sur des cellules MG-63 de type ostéoblaste en utilisant le système d'édition de gène CRISPR/Cas9. Ainsi, ce travail a été divisé en deux parties:

- Dans la première partie de l'étude, nous avons développé un modèle qui nous permettrait d'étudier l'implication de l'inflammasome NLRP3 dans l'infection à *S. aureus*. Tout d'abord, nous avons effectué l'inactivation du gène de la Caspase-1 sur des cellules MG-63, en utilisant l'approche CRISPR/cas9. Ensuite, l'activation de la Caspase-1 dans des cellules de type sauvage (WT) par rapport à des cellules (CASP1$^{-/-}$ MG-63) sera surveillée (**Fig. 10-A**).

- Dans la deuxième partie de l'étude, la production d'une forme active soluble d'IL-1β sera surveillée dans les cellules WT MG-63 vs CASP1$^{-/-}$MG-63 dans un modèle cellulaire *in vitro* d'infection chronique utilisant des infections à long terme (de 6 h à 11 jours) d'ostéoblastes avec différentes souches de *S. aureus* (**Fig. 10 -B**).

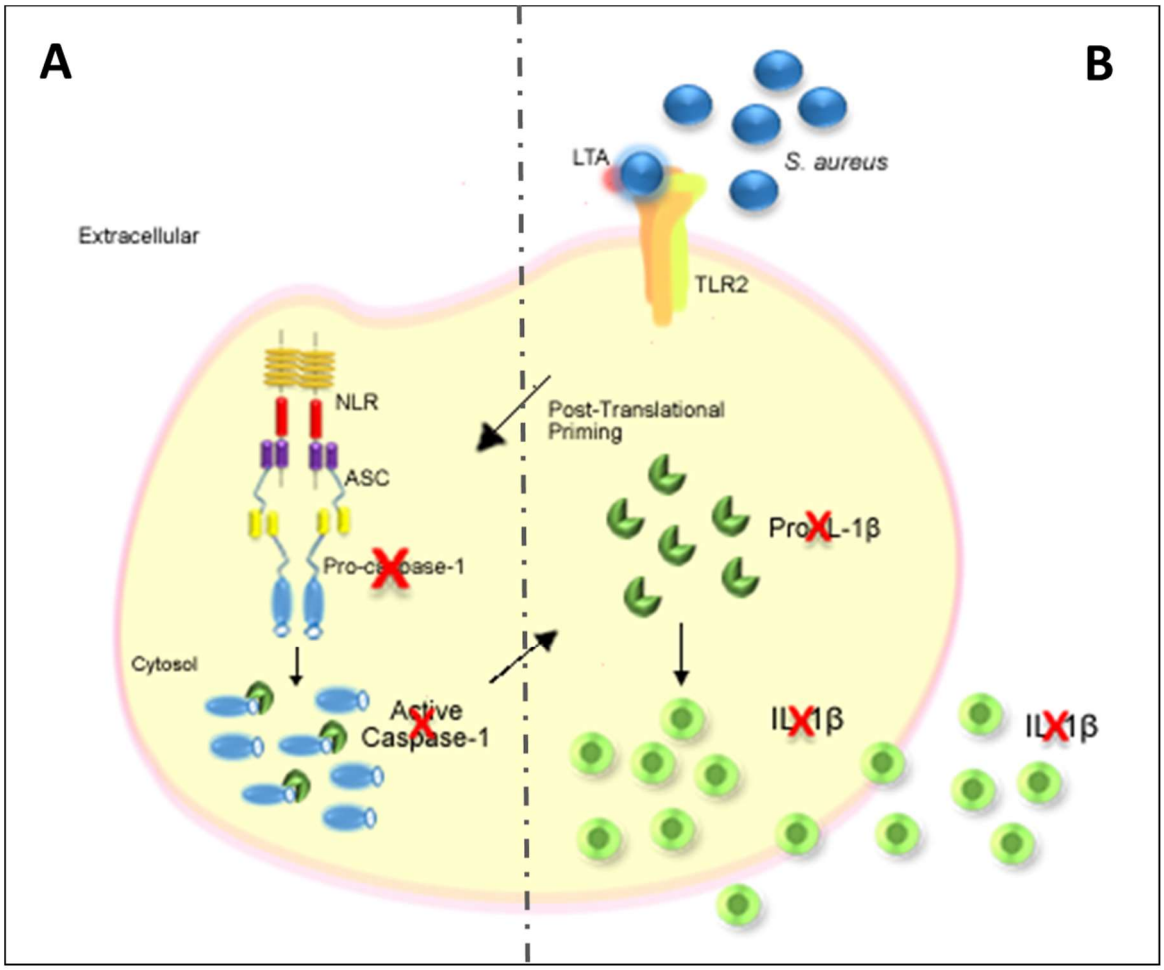

Figure 10: Schéma des objectifs proposés pour la première et la deuxième partie de la thèse.

Aim of the PhD project

This research aims to investigate whether *S. aureus* infection activates inflammasomes in non-professional phagocytes, such as human osteoblast-like cells, and which *S. aureus* virulence factors are involved in inflammasomes activation during infection.

Objectif du projet de thèse

Le but de cette recherche est de déterminer si l'infection à *S. aureus* active les inflammasomes dans les phagocytes non professionnels tels que les cellules ressemblant à des ostéoblastes humains et quels facteurs de virulence de *S. aureus* sont impliqués dans l'activation de l'inflammasomes lors de l'infection.

Chapter 3. Literature review. Strain and cell type-specificity of host cell response to *Staphylococcus aureus* invasion.

MINI REVIEW

Strain and cell type-specificity of host cell response to *Staphylococcus aureus* invasion.

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Abstract

Staphylococcus aureus is recognized as a facultative intracellular pathogen that can invade and persists in different cell types like professional phagocytes (macrophages, neutrophils), and non-professional phagocytes (epithelial, endothelial cells, fibroblasts, osteoblasts). Following the host cell invasion process via expression of adhesins by *S. aureus* can lead to different cellular responses of host cells depending on the type strains of *S. aureus* and the host cell type. The focus of this review is to present a guide to recent research outlining the variety of intracellular fates of *S. aureus* and their interaction with host cell types.

Keywords: *Staphylococcus aureus*, invasion, intracellular persistence.

1. Introduction

Staphylococcus aureus is a versatile Gram-positive bacterium and an opportunistic pathogen able to cause a wide range of infections in both humans (Chambers, 1997; Fitzgerald, 2012; Lowy, 1998), and animals (Peton and Le Loir, 2014). It has long been considered a strictly extracellular pathogen but it is now also regarded as a facultative intracellular pathogen (Fraunholz and Sinha, 2012). The mechanisms leading to internalization and underlying the intracellular persistence of *S. aureus* are mostly carried out using one strain in interaction with one model cell line, representative of a given cell type or tissue.

S. aureus uses a plethora of virulence factors to accommodate a diversity of niches in its various hosts. The array of virulence factors varies from a strain to another and, therefore, *S. aureus* strains do not all interact with the host cells in the same fashion and they do not all induce the same cellular response. Similarly, not all host cells respond in a same way to interaction with *S. aureus*. This can be due to cell types (professional and non-professional phagocytes). However, this can also be observed with different cell lines for a given cell type. Although these variations are more and more documented in the literature, as far as we know, they have not been reviewed yet. In this review, we address the variability of strain-specific traits as well as that of the host cells leading to a wide range of cell responses as outcomes of *S. aureus*-host cell interactions. We particularly focus on reports that experimentally addressed these issues.

2. *S. aureus*, a versatile opportunistic pathogen

S. aureus strains greatly vary in terms of virulence factors, which explains their ability to live as commensal bacteria, or adopt a pathogenic lifestyle and induce manifestations of disease as extracellular bacteria (in impetigo, abscesses, furuncles, septicemia, necrotizing pneumonia), as biofilm formers (catheter-induced infective endocarditis, atherosclerosis), or as intracellular pathogens. In the latter case, regarded as an immune-evasive strategy, *S. aureus* survives within its host cells and escapes detection by professional phagocytes, which leads to persistent infections.

S. aureus strains also greatly vary at the genotypic level with regard to their host origin. This was shown through Pulsed-field Gel Electrophoresis (PFGE) analysis (Alves et al., 2009; Hennekinne et al., 2003), or Multilocus sequence typing (MLST) approach, with some clonal complexes that are mostly found associated to a given host. Although all clonal complexes (CC) are found in human host, CC1, CC5 and CC130 are predominant in cattle, CC133 in

small ruminants, CC1, CC5, CC9 and CC97 in swine, CC8 and CC9 in horse, and CC5 in poultry (Guinane et al., 2010; Pantosti, 2012). These host-specific traits are also retrieved at the genomic level (Ben Zakour et al., 2008), and at the molecular level. Some *S. aureus* virulence factors that are indeed specifically present or preferentially active on their target in a given host as demonstrated e.g. for bovine variant of von Willebrand factor-binding protein (Viana et al., 2010), equine variant of staphylococcal complement inhibitor (SCIN) (de Jong et al., 2018), or ovine variants of staphylococcal enterotoxin type C (Marr et al., 1993).

3. *S. aureus* adhesion and internalization

Host cells invasion (i.e. adhesion onto and internalization into host cells) is a strategy used by certain bacterial microorganisms to evade the immune system (Alexander and Hudson, 2001). Bacterial adhesion to the host cell surface is the first step of the host cell invasion reviewed in (Sinha and Herrmann, 2005). *S. aureus* expresses an array of specific molecules, adhesins, which allow the bacterium to adhere to the matrix of host cells (Clarke and Foster, 2006). These adhesion factors are either covalently linked to the *S. aureus* envelope and called microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) or secreted in the surrounding medium and called secretable expanded repertoire adhesive molecules (SERAM) (Foster and Höök, 1998). *S aureus* uses MSCRAMMs to adhere to the cell, directly or via bridging ligands, i.e. cellular eukaryotic proteins with affinity for both host cell receptors and MSCRAMMs (Alexander and Hudson, 2001; Dziewanowska et al., 2000). Fibronectin-binding proteins (FnBPs) are reportedly the most important factor for host cell invasion and are therefore extensively studied for their role in staphylococcal infection (Alexander and Hudson, 2001). Their action is mediated via fibronectin bridging between host integrin α5β1 and staphylococcal surface proteins, FnBPs A and B (Dziewanowska et al., 1999; Kintarak et al., 2004; Sinha et al., 2000). In human osteoblasts and bovine mammary epithelial cells, fibronectin can bind via the α5β1 integrin leading to the fixation of *S. aureus* and favoring its internalization by non-professional cells (Ahmed et al., 2001; Dziewanowska et al., 1999).

Bacterial invasion followed by the infection process in various cell types have in common that the uptake mechanism requires an intact cytoskeleton (Menzies and Kourteva, 1998). During the internalization of *S. aureus,* the involvement of cytoskeletal elements was demonstrated, such as actin microfilaments (Ellington et al., 1999; Jevon et al., 1999). The integrin-linked kinase (ILK) activity plays an important role in the internalization of *S. aureus* in epithelial cells (Wang et al., 2006). The subsequent activation of ILK occurs after interaction with the

cytoplasmic domains of β integrins that occurs due to the interaction among α5β1 and the cytoskeleton (Wang et al., 2006).

Moreover, studies have shown that rearrangement focal adhesion leads to the remodeling of the actin cytoskeleton by the loss of focal adhesion kinase (FAK), including the adaptor protein paxillin and vinculin. The rearrangement is accompanied by a centripetal movement of *S. aureus* on the host cell surface (Fraunholz and Sinha, 2012; Schröder et al., 2006). Besides, the focal adhesion protein tensin, vinculin, and zyxin are recruited to the site of bacterial uptake (Agerer et al., 2005; Wright and Nair, 2010). Figure 1-A shows the mechanisms involved in the invasion of *S. aureus* into non-professional phagocytes cells.

It has been shown that when FnBPs interact directly with human heat shock protein 60 (Hsp60) on the membranes of human and bovine epithelial cells, the internalization efficiency is maximal (Dziewanowska et al., 2000). Hirschhausen and colleagues (2010) demonstrated that *S. aureus* autolysin (Atl) was identified to function as adhesin/invasin with the heat shock protein, Hsc70 as the direct cell receptor (Hirschhausen et al., 2010). The Alt-dependent internalization involves direct interaction with Hsc70 or an indirect interaction through the Fn connected to integrin α5β1 (Hirschhausen et al., 2010).

The extracellular adherence protein (Eap) is a multifunctional protein consisting of 4 to 6 tandem-repeat domains that stimulate the adherence of staphylococci to fibroblasts and endothelial cells (Fraunholz and Sinha, 2012; Hussain, 2002; Palma et al., 1999). However, the host cell receptor is not yet known. Besides, Weidenmaier and colleagues (2004) have shown that wall teichoic acid (WTA) plays an important role in nasal colonization by *S. aureus* and that under certain conditions are important in adherence to endothelial cells. However, their host cell-binding partners have not yet been identified, but there is evidence that a scavenger receptor family is involved in WTA binding (Weidenmaier et al., 2004, 2005, 2008). Figure 1-B shows a schematic presentation of the different receptors involved in *S. aureus* internalization in non-professional phagocytes.

4. Cell response to *S. aureus* infection is strain-dependent

S. aureus is a highly variable and versatile pathogen. Its variability relies in the set virulence factors borne by *S. aureus* strains and in the strains capability to adapt, grow and express their virulence in a given context. Such variability affects each step of the pathogenesis, from adhesion onto host cells to the host cell response. A very first step of bacteria-host cell interaction is the bacterial adhesion onto the cell surface, whose efficiency drives the

internalization rate of the strains (Haggar et al., 2003). Various cell types can indeed internalize *S. aureus* and infected cells can serve as reservoir and vehicle for the systemic dissemination of *S. aureus*. Once internalized, *S. aureus* capacity to adopt a small colony variant phenotype is also dramatically important in its persistence within the host cells. Of course, these differences in terms of interaction capacities result in variations in terms of host cell response and infection outcome.

5. Phenotypic modifications also alter the *S. aureus*-host cell interaction

S. aureus small colony variants (SCVs) are linked to bacterial persistence (Lowy, 1998). The intracellular persistence of *S. aureus* reviewed by (Garzoni and Kelley, 2009; Sendi and Proctor, 2009), has been described for a variety of host cells and pathogen strains. The relationship between *S. aureus* SCVs and persistent infection was first reported in a small clinical series in 1995 (Proctor et al., 1995). Once internalized, *S. aureus* can adopt a SCV phenotype is characterized changes in transcriptome and proteome, which are phenotypically associated with slow growth rates, reduced metabolic activity, increased resistance to antibiotics and anti-bacterial host strategies such as the oxidative burst. SCV is often non-hemolytic, non-cytotoxic (Proctor et al., 1995; Tuchscherr et al., 2011, 2017). *S. aureus* SCVs were found to produce fewer lytic enzymes, thereby allowing them to persist within the host cells (Proctor et al., 2006). Additionally, SCVs of *S. aureus* even have been shown to survive and grow within host cell phagosomes (Schröder et al., 2006; Tuchscherr et al., 2011), and their extended intracellular survival is probably involved in the chronicity of some *S. aureus* infections.

Besides, SCV also largely avoid the activation of the host immune system (Tuchscherr et al., 2010, 2011). Wild-type *S. aureus* induces the production of IL-1β, IL-6, and IL-12 and of tissue remodeling factors, which dramatically differs from immune responses to SCVs, characterized by the induction of TLR2 signaling (Ou et al., 2016).

SCV phenotype results from various point mutations in genes involved in heme, or menadione biosynthesis, in oxidative phosphorylation, in cytochrome c assembly or thymidylate synthase (thyA), which often result in an altered electron transport chain [reviewed in (Proctor et al., 2006)]. These point mutations are reversible and account for reversion to the wild-type phenotype. Transposable elements may also be involved in SCV phenotype, which is also reversible in this case (Kleinert et al., 2017). SCV phenotype switching may thus result from multiple pathways. As of yet, strain-specific behavior in terms of the ability to adopt an SCV phenotype has not been described in the literature. Although it is an important feature in *S.*

aureus-host cell interactions, with consequences on the cell response, one cannot conclude about variability concerning SCV phenotype.

In veterinary medicine, *S. aureus* is notably responsible for mastitis in dairy ruminants and strain-specific features were also identified, which had differential effects on bovine mammary epithelial cells (bMEC). The bMEC are the most abundant cell type of lactating parenchyma and they not only express the full repertoire of TLRs but are also highly immune competent. When challenged with the Gram-negative pathogen *Escherichia coli* (*E. coli*), bMEC strongly expresses a wide range of immune factors including cytokines and chemokines, membrane protecting factors and bactericidal components. This vigorous activation is driven by the TLR signaling activation triggered by the *E. coli* LPS. TLR signaling activates the NF-κB complex of transcription factors, which in turn, activate immune gene expression. This strongly contrasts with *S. aureus* intramammary infections, which fail to activate any TLR signaling in bMEC and, hence, elicit only a weak immune response (Petzl et al., 2018). However, *S. aureus* can efficiently colonize the mammary epithelium and numerous strain-specific features have been identified in the first steps of the intramammary infection. Notably, the capabilities of *S. aureus* to adhere to and to internalize into bMEC strongly vary in a strain-dependent fashion (Bouchard et al., 2013; Peton et al., 2014).

Some *S. aureus* strains can reproducibly induce subclinical and persistent mastitis whether other strains induce clinical and non-persistent mastitis in experimental animal models. These clinical outcomes correlate with genetic features such as lesser toxin production and better adhesion to bMEC *in vitro* for the strains that induce mild mastitis (Le Maréchal et al., 2011; Peton et al., 2014). The host immune response is also different, with a stronger inflammatory response in the early steps of the infection for non-persistent strains, and differential expression of cytokines in the curse of the infection (Pereyra et al., 2017).

6. Strain-specific ability to adhere and internalize

A comparative study of 120 *S. aureus* strains isolated from bovine mastitis milk samples belonging to lineages CC97, CC151 and ST136, showed no significant differences between the lineages in the ability to adhere to or internalize within bMEC although there were significant differences between individual isolates. In most cases, adherence to bMEC was correlated with the ability to bind bovine fibronectin, although isolates from CC151 could not bind bovine fibronectin in vitro, but adhered to bMEC in a fibronectin-independent manner (Budd et al., 2016). Adhesion to and internalization into bMEC were also compared in RF122 and Newbould 305, two bovine mastitis *S. aureus* strains, and were correlated to genome

analysis. Newbould 305 had better adhesion and internalization rates than RF122 and this correlated well with a longer fibronectin-binding protein (FnBP) compared to RF122 FnBP, as predicted from genome sequence (Bouchard et al., 2012; Peton et al., 2014).

7. Strain-specific cytotoxicity

Directly after host cell invasion, the bacteria can induce strong inflammatory effects and cytotoxic. Once internalized into the host cells, *S. aureus* cytotoxicity appears to be characteristic of individual strains rather than a general feature. Krut et al. (2003) observed that among 23 randomly collected clinical *S. aureus* isolates, a majority were killed inside keratinocytes and fibroblasts. This shows that the uptake of *S. aureus* is a means of cell-autonomous host defense for non-professional phagocytes. However, seven independent isolates survived intracellularly and induced significant cytotoxicity for host cells. Their results suggest that these characteristics are stable and genetically determined (Krut et al., 2003). Invasion of human keratinocytes by *S. aureus* and such *S. aureus* intracellular in human keratinocytes was shown to be haemolysin-independent and followed by features of apoptotic and necrotic cell death (Mempel et al., 2002). Such cytotoxic strains can escape degradation by the endolysosomal pathway. These dramatic differences in terms of cytotoxic and non-cytotoxic strains may partly explain the inter-strain differences in terms of dissemination of *S. aureus* infection.

The internalization of *S. aureus* in osteoblasts cells leads to the secretion of cytokines, chemokines and growth factors that induce the activation of the immune system (Turner et al., 2014). It has been demonstrated that the interaction between *S. aureus* and osteoblastic cells increase the production of IL-6, CCL2 (MCP-1), CCL3 (MIP-1α) and CCL5 (RANTES), as well as up-regulated of CXCL8 (IL-8) and CXCL2 (MIP-2α) (Dapunt et al., 2014; Josse et al., 2015; Wright and Friedland, 2004). Besides, primary osteoblasts showed increased expression of the CCL5 and CXCL11 gene compared to the corresponding cell line after internalization with *S. aureus* 6850 and SH100, demonstrating that the internalization process induces a different response in primary cells and cell lines (Strobel et al., 2016).

The internalization of *S. aureus* by endothelial cells induces the expression of cytokines, chemokines and adhesion molecules such as IL-6, IL-8, MCP-1, RANTES and ICAM-1 (Grundmeier et al., 2010; Strindhall et al., 2002; Tekstra et al., 1999) as well as genes associated with cell cycle, signaling and homeostasis (Grundmeier et al., 2010). These findings

suggest that in addition to inducing the inflammatory process, internalization also induces cellular events.

The prevention or activation of cell death may be a critical factor in the outcome of an infection and was well observed as a response to infection by several pathogens. It has been reported that pathogens are capable of modulating apoptosis and thus starting an infection in several types of host cells like osteoblasts, endothelial, and epithelial cells (Bayles et al., 1998; Fraunholz and Sinha, 2012; Haslinger-Loffler et al., 2005; Kubica et al., 2008; Lamkanfi and Dixit, 2014). Cell death of non-prophylactic phagocytes induces activation of caspase-2 due to the efflux of potassium caused by aerolysin or α-toxin (Imre et al., 2012). The induction of apoptosis in *S. aureus*-infected osteoblasts is associated with tumor necrosis factor-related ligation inducing ligand (TRAIL) release (Alexander and Hudson, 2001). TRAIL interacts with death receptor 4 (DR4), also known as TRAIL receptor 1 (TRAILR1) and death receptor 5 (DR5) (Young et al., 2011). Subsequently, TRAIL induces the activation of the intrinsic pathway of apoptosis through caspase- 9 and the extrinsic pathway through caspase-8, which leads to activation of caspase-3 and subsequently to apoptosis (Figure 2) (Alexander and Hudson, 2001; Claro et al., 2011; Josse et al., 2015, 2017).

8. Cytotoxicity induced by *S. aureus* extracellular vesicles

Secretion is an important component of bacterial pathogenesis once the delivery of molecules and virulence factors contribute to the process of pathogen infection. Growing interest has been given to the study of extracellular vesicles (EVs), nanoparticles (30-150 nm) of lipid bilayer that are secreted by any living cell and carry bioactive compounds such as proteins, metabolites and nucleic acids, able to modulate the metabolism and physiology of local or distant targets cells, participating in pathogenesis and immunomodulation (Brown et al., 2015). Various virulence factors have been identified being carried by EVs from different *S. aureus* strains, such as superantigens, toxins, coagulases, adhesins and other related proteins (Askarian et al., 2018; Gurung et al., 2011; Jeon et al., 2016; Lee et al., 2009; Tartaglia et al., 2018). Interestingly, cytotoxicity of EVs towards host cells varies according to EVs proteome and cell line type studied.

Since virulence factors vary from a strain to another, so can the cargo of EVs released, affecting cytotoxicity and host cell response. It was demonstrated that the presence of α-hemolysin in EVs is directly related to cell death, and EVs from α-hemolysin-negative strains have very low or no cytotoxic effect to different cell types (Hong et al., 2014; Thay et al., 2013).

As another example, EVs from M060 *S. aureus* strain containing exfoliative toxin A (ETA) were highly cytotoxic towards HEp-2 cells, contrary to EVs from three other *S. aureus* isolates that lack the same protein (Jeon et al., 2016). Moreover, both intact and disrupted M060 EVs show the same cytotoxic at low concentrations towards HaCaT cells, while disrupted ATCC 25923 *S. aureus* strain EVs are four times less cytotoxic than intact EVs (Kwon et al., 2019). On the other hand, exposure of HaCaT cells to EVs from ATCC 25923 induces a stronger immune response than that of M060 EVs (Kwon et al., 2019). In a study by Tartaglia et al (2018), EVs secreted by *S. aureus* N305 strain were not cytotoxic to PS and MAC-T cells but induced the expression of cytokines *in vitro*, and promoted tissue inflammation, deterioration, and local cytokine production, *in vivo* (Tartaglia et al., 2018). In some cases, EVs-associated molecules showed to be more efficient than extracts or soluble proteins to the induction of immune response and cell cytotoxicity (Hong et al., 2014; Kim et al., 2019). These findings suggest that EVs cargo and integrity can influence host cell response and that even non-cytotoxic EVs can promote immunomodulation.

Different cell types also influence host-EVs interaction and consequently cytotoxicity and host cell responses. EVs from *Staphylococcus aureus* subsp. *aureus* Rosenbach MSSA476 induced extensive cell death to human neutrophils and THP1 cells, while caused minimal cytotoxicity to keratinocytes (HaCaT) at the same concentrations (Askarian et al., 2018). This finding could be explained due to the difference between these cell types. Again in another study, *S. aureus* JE2 EVs showed to be less cytotoxic to airway epithelial cells (A549) than to erythrocytes and neutrophil-like HL60 cells (Wang et al., 2018). EVs can also induce production of cell adhesion molecules such as E-selectin, ICAM1 and VCAM1, and consequently, recruitment of monocytes (THP1) (Kim et al., 2019), contributing to the infiltration of immune cells. Cell response can also differ between cell types, for example, after exposure to 14458 *S. aureus* ATCC EVs, alveolar macrophages produced TNF-α and IL-6, while A549 cells produced only IL-6 (Kim et al., 2012). Table 1 shows some examples of strain-specific EVs cytotoxicity. Altogether, this data shows that EVs can interact with and modulate the immune response of host cells, suggesting that EVs can play an important role in staphylococcal pathogenesis.

9. Impact on the host cell cycle

We previously demonstrated that culture supernatant compounds of some *S. aureus* strains are able to induce a G2/M phase transition delay in HeLa cells (Alekseeva et al., 2013). Phenol-soluble modulin α (PSMα) peptides are responsible for this host cell cycle alteration, which also correlated with a decrease in the defensin genes expression suggesting a diminution of

antibacterial functions of epithelial cells. PSM production is strain-dependent and, by testing the supernatant of *S. aureus* human clinical isolates, we found that the degree of G2/M phase transition delay correlated with PSMα1 production (Deplanche et al., 2015). Similarly, the staphylococcal enterotoxin-like protein type O, an *S. aureus* superantigen, is also able to induce such phase transition delay. Aside from these PSM and SElO, also known as cyclomodulins (El-Aouar Filho et al., 2017), the "lipoprotein-like" proteins (lpl) in *S. aureus* contribute to internalization into non-professional antigen-presenting cells such as keratinocytes (Nguyen et al., 2016). Lpl1, a representative of the *lpl* cluster, also caused G2/M phase transition delay. As lpl genes are carried by strain-specific genomic islands (vSaα) (Baba et al., 2008), this Lpl associated phenotype is also strain-dependent. This mechanism might give an advantage to some strains in fostering an infection.

10. Impact on the innate immune response and on the inflammatory response

Some bacterial traits are associated with a higher inflammatory response (Chen et al., 2018). PBMC response in terms of IL-17 and IL-19 interleukins differs with the origin of the *S. aureus* strains. PBMC of a given individual will respond to interaction with the individual's endogenous strain by an increased production of IL-19 (an IL-17 suppressor), which may be regarded as a tolerogenic response. Contrarily, PBMC interaction with an exogenous strain (not carried by that individual) will induce a strong IL-17 response, which may facilitate *S. aureus* clearance. IL-17 is indeed essential for protection against extracellular pathogens at mucosal surfaces (Reiss-Mandel et al., 2018).

Non-proteinous surface components, like wall teichoic acid, may also play a role in the differential cell response. High WTA producer strains were shown to be more virulent in a mouse abscess model and induce stronger T cell proliferation and interferon gamma (IFNγ) production by human T cells (Wanner et al., 2017).

11. Impact on the epigenetic modifications in host cells

Epigenetic modifications as a consequence of host–pathogen interaction is of growing interest to understand and decipher how some opportunistic pathogens are rapidly cleared from the host body whereas others can evade the immune surveillance and cause recurrent and chronic infection. Epigenetic control mechanisms comprise DNA methylation, histone modification and the activity of non-coding RNAs. *S. aureus* induced epigenetic modifications in host cells is still poorly documented (Modak et al., 2014). Modak et al. (2014) investigated the inflammatory

response in mice mammary tissue and the epigenetic modifications induced by two strains of *S. aureus*, isolated from field samples (cow mastitis milk) when used in experimental intramammary infection. Their results showed strain specific hyperacetylation at histone H3K9 and H3K14 residues. Transcriptome analysis in mammary tissue in the infected mice revealed a set of upregulated genes that significantly correlated with the promoter specific, histone H3K14 acetylation. Small RNA sequencing revealed several differentially expressed miRNAs in the infected mice mammary tissue. One of 2 *S. aureus* strains activated the NF-κB signaling leading to severe inflammation and induction of immune surveillance, which could possibly lead to rapid clearance of *S. aureus* strain. The other strain repressed the inflammatory response, which might result in a long-term infection of the host mammary tissue (Modak et al., 2014).

12. Cell types and *S. aureus* invasion

The capacity for intracellular survival within professional phagocytes (monocytes, macrophages, and even neutrophils) is a critical factor facilitating the dissemination of *S. aureus* in the host (Garzoni and Kelley, 2009; Kubica et al., 2008). The interactions between *S. aureus* and phagocytes have been extensively studied in recent years and focused mainly on neutrophils and macrophages (Kubica et al., 2008; Lacoma et al., 2017; Nagl et al., 2002; Rigby and DeLeo, 2012; Spaan et al., 2013; Tranchemontagne et al., 2016), and recently on the mechanism of response of dendritic cells (DCs), which are cells that can influence immune responses promoting effector functions of other phagocytes (Schindler et al., 2012), in addition to activate the innate immune response (Chan et al., 2017). Bone marrow-derived dendritic cells (BMDCs) were able to kill *S. aureus*, however, certain strains of *S. aureus* were shown to have the ability to subvert DC death by autophagy (O'Keeffe et al., 2015).

It has been demonstrated that strains of *S. aureus* can subvert neutrophils in addition to being able to persist within macrophages derived from human monocytes suggesting that these phagocytic cells provide an intracellular niche that facilitates the dissemination of *S. aureus* in vivo, due to its mobility (Derby, 1961; Kubica et al., 2008).

This mechanism of *S. aureus* dissemination is also seen in traditional intracellular bacteria, such as *Mycobacterium tuberculosis* and *Listeria monocytogenes* (Drevets, 1999; Schlesinger, 1996). Besides, evidence exists that several strains of *S. aureus* can invade and persist within non-professional phagocytic cells such as endothelial, epithelial, osteoblasts, keratinocytes and fibroblasts (Ellington et al., 1999; Löffler et al., 2014; Sinha and Herrmann, 2005; Usui et al., 1992). A pioneering study demonstrated the ability of endothelial cells to encompass *S.*

aureus (Ogawa et al., 1985). Besides, *S. aureus* can adhere and invade cultured osteoblasts (Hudson et al., 1995). Primary endothelial cells and epithelial cells are known to absorb a large number of bacteria and can ingest *S. aureus*, as well as osteoblasts and fibroblasts cells (Löffler et al., 2014). These differences between cell types (primary cells and cell lines) regarding bacterial degradation and host inflammatory processes have been revised (Seidl et al., 2012; Tuchscherr et al., 2011).

A recent study demonstrated that most clinical isolates of *S. aureus* on endothelial cells have an invasive phenotype and the pro-inflammatory and cytotoxic effects depend on of invasion properties of the infectious strains (Strobel et al., 2016). This study showed the analysis of the uptake of *S. aureus* 6850, SH1000 and Cowan1 into different host cell types like epithelial, endothelial, fibroblasts and osteoblasts have differences in intracellular bacterial number after the infection process (Strobel et al., 2016). Besides, keratinocytes cells can ingest *S. aureus* but showed reduced levels if intracellular *S. aureus* in comparison with epithelial cells and endothelial cells.

S. aureus strains 6850 shown uptake different between primary cell and osteoblast cell line, with a low level of intracellular bacterium after infection in primary osteoblasts compared with an osteoblast cell line that presented a greater capacity of bacterial uptake (Strobel et al., 2016), suggesting differences in bacterial invasiveness. In addition, the intracellular persistence of *S. aureus* inside osteoblasts is also supported by the presence of SCVs. Clinical strains of *S. aureus* can persist within osteoblasts for several weeks. Persistence induces bacterial phenotypic diversity, including SCV phenotypes, accompanied by changes in virulence factor expression (Tuchscherr et al., 2019).

13. Conclusion

We have begun to appreciate many general aspects of the inflammatory process in immune cells and their expert contributions to homeostasis and disease. However, we are still far from predicting how information collected by recognizing a particular agent (e.g. *S. aureus*) will be decoded by the immune system. It has been shown in this review that *S. aureus* can exert various effects on eukaryotic host cells. This pathogen has developed many strategies for adhering, invading and persisting in intracellular localization for long periods. The host cell response is highly complex, as both the selection of bacterial strains and host cell types play an important role in different cell responses. Although much progress has been made in understanding the molecular biology of this pathogen, much is still unknown. There are many examples where both professional and nonprofessional phagocytes contribute to homeostasis, but an interesting question is: How do these two cell populations communicate to ensure that

inflammation is attenuated. The role in coupling innate and adaptive immunity via antigen presentation has unmasked several receptors, as well as signal transduction pathways and/or membrane trafficking routes that are only partially understood. Taken together, this shows that these speculative ideas are subject to experimental testing, which we hope will be the subject of further investigation.

Author Contributions

NB, YLL supervised the work and corrected the manuscript. ELL, BSRL, EG, FC, VA did the main part of the bibliographical survey. All the authors took part in the writing of the manuscript.

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Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figure legend

Figure 1: Staphylococcal mechanisms of adherence to and internalization into host cells. (A). The current model of some signaling events involved in the invasion of *S. aureus* into non-professional phagocytic cells. Fibronectin-binding proteins (FnBPs) expressed on the bacterial surface bind via host fibronectin in the extracellular matrix, which forms a bridge with receptors integrin α5β1 on the host cell membrane. The integrin-linked kinase (ILK) provides a link via α5β1 and the cytoskeleton and interacts through the cytoplasmic domain β1. The subsequent recruitment of paxillin (PAX) and focal adhesion kinase (FAK) lead to remodeling of the actin cytoskeleton. Other focal adhesion proteins such as vinculin (VLC) and tensin are also recruited to the site of bacterial uptake. **(B).** Different receptors involved in *S. aureus* internalization in non-professional phagocytes. The main mechanism of internalization involves the α5β1 integrin receptor, which is mediated by Fibronectin (Fn) and fibronectin-binding protein (FnBP). FnBP can interact with heat shock protein 60 (Hsp60) thus contributing to efficient *S. aureus* internalization by host cells. Extracellular adherence protein (Eap) also contributes to staphylococcal adherence and internalization if sufficiently expressed, but a host cell receptor has not been described yet. *S. aureus* autolysin (Atl) can bind to heat shock protein, Hsc70 or integrin α5β1 integrin via Fn. Wall teichoic acid (WTA) is required for nasal colonization by *S. aureus* and there is evidence that scavenger receptors are involved in WTA binding. Adapted by (Hirschhausen et al., 2010).

Figure 2: *Staphylococcus aureus* can also cause apoptosis of osteoblasts.
The induction of apoptosis of osteoblasts by the intrinsic and extrinsic caspase pathways. Both can lead to the release of intracellular *S. aureus* after the death of the osteoblast. Adapted by Josse et al, 2015.

Figure 1:

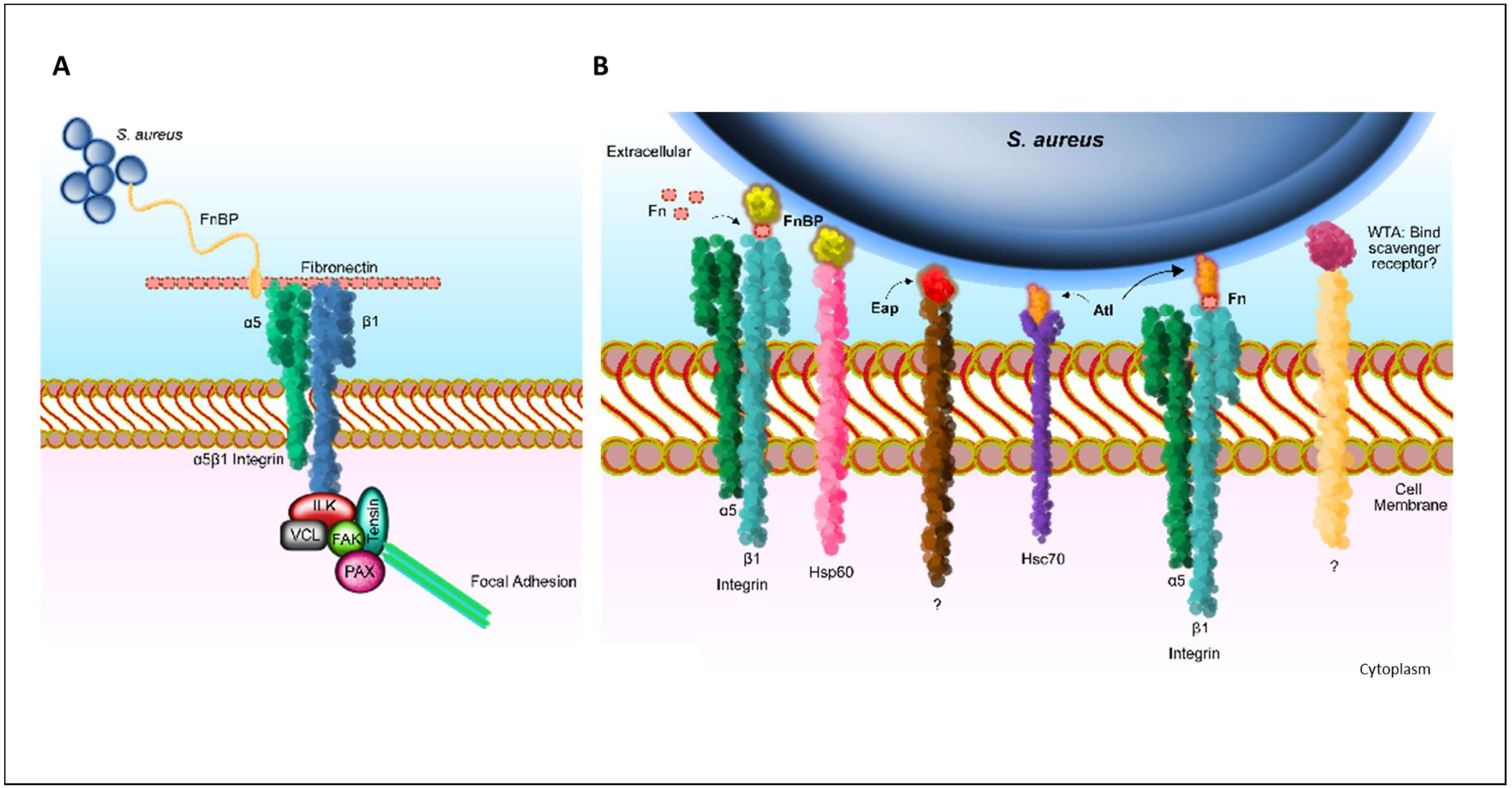
A
S. aureus
FnBP
Fibronectin
α5
β1
α5β1 Integrin
ILK
VCL
FAK
Tensin
PAX
Focal Adhesion
B
S. aureus
Extracellular
Fn
FnBP
Eap
Atl
Fn
WTA: Bind scavenger receptor?
α5
β1
Integrin
Hsp60
?
Hsc70
α5
β1
Integrin
?
Cell Membrane
Cytoplasm

Figure 2:

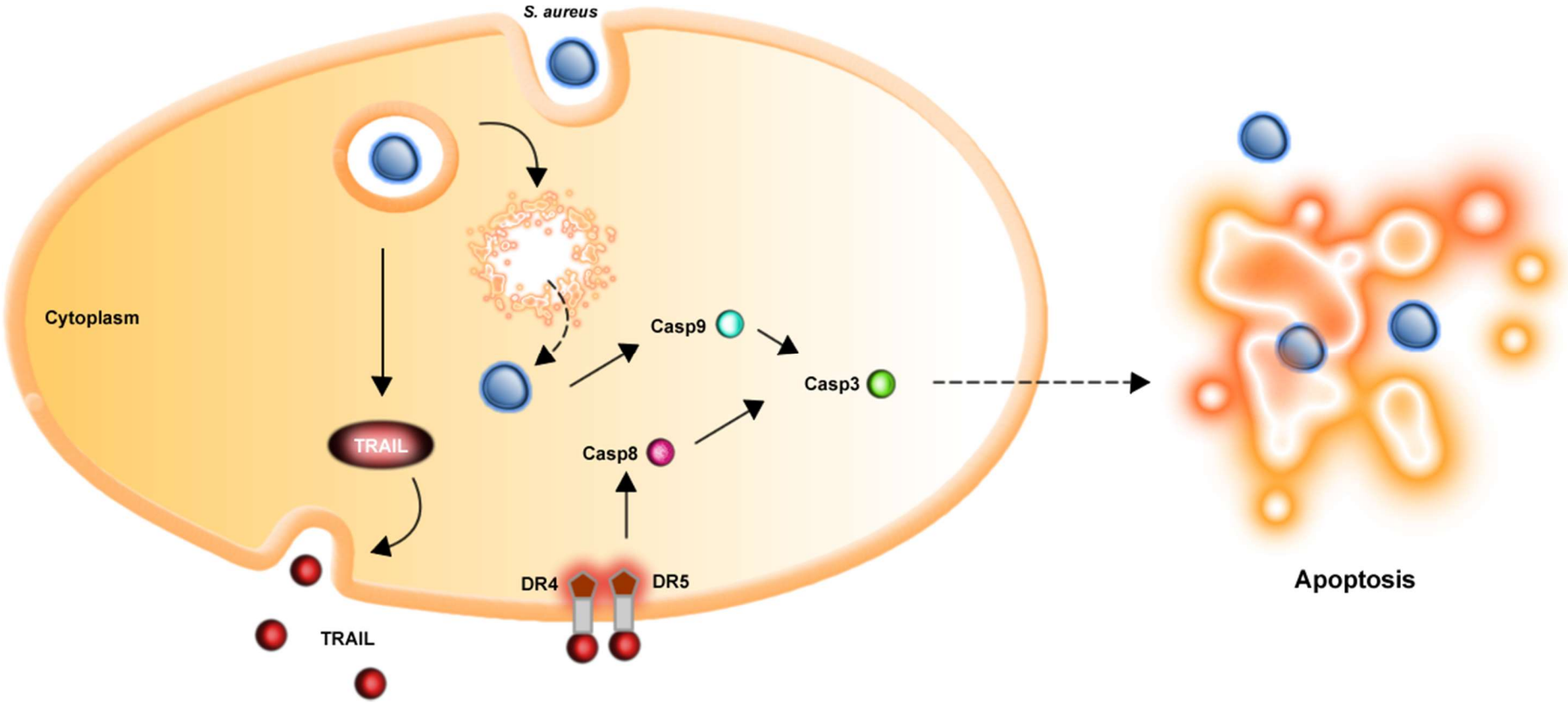
S. aureus
Cytoplasm
TRAIL
Casp9
Casp3
Casp8
DR4
DR5
TRAIL
Apoptosis

Table 1: Strain-specific EVs cytotoxicity

Strains	*In vitro*		*In vivo*		References
	Cell type	**Type of response**	**Model**	**Type of response**	
ATCC 14458	-	-	-	-	Lee et al, 2009
ATCC 14458	Airway ephitelial cell A549	EVs, internalization; production of TNF-α and IL-6 (dose-dependent)	Mouse airway exposure to Evs	Increase of inflammatory cells in bronchoalveolar lavage (BAL); increase of TNF-α, IL-12, and IL-6 (dose-dependent)	Kim et al, 2012
	Alveolar macrophages	EVs, internalization; production of IL-6		Repeated exposure (3 weeks): lung infiltration by inflammatory cells, especially neutrophils (dose-dependent.); increase in IgG1, INF-γ and IL-17	
	Peritoneal macrophages from TLR2-/- mice	Production of IL-6 and TNF-α absent in comparison with wt	TLR2-/- mouse airway exposure to EVs	Lower lung infiltration by inflammatory cells; absence of TNF-α and IL-6 production	
8325-4	rabbit erythrocytes, HeLa human cells	dose-dependent hemolysis; decrease in viable cells	-	-	Thay et al, 2013
DU1090 (8325-4 Δhla)	rabbit erythrocytes, HeLa human cells	low hemolitc avtivity; no cytotoxic effect			
ATCC 14458 (α-hemolysin positive)	HaCaT cells (immortalized human epidermal keratinocytes)	EVs internalized into the cytoplasm; α-hemolysin cytotoxic effect (dose-dependent); apoptotic death	Back skin of hairless mice	EVs induce skin barrier disruption via keratinocyte cell death; induces production of IL-1β and IL-6 and inhibit TNF-α production	Hong et al, 2014
			EVs administered epicutaneously into mouse skin	Epidermal thickening and higher infiltration by eosinophils	

continue…

Strains	*In vitro*		*In vivo*		References
	Cell type	**Type of response**	**Model**	**Type of response**	
M060 (SSSS)	Hep-2 epithelial cells and COS-7 fibroblasts	Cytotoxic to Hep-2 and COS-7 with <10µg/mL EVs (dose-dependent), and stronger in COS-7; internalization of ETA present in EVs into Hep-2 cells	-	-	Jeon et al, 2016
06ST1048	Hep-2 cells	cytotoxic 50 µg/mL			
01ST93	Hep-2 cells	non-cytotoxic 50 µg/mL			
03ST17	Hep-2 cells	non-cytotoxic 50 µg/mL			
subs. aureus Rosenbach MSSA476	Human blood (MSSA476 EVs)	Exogenous administred EVs (LB and BHI-EVs) promoted MSSA476 survival (dose-dependent); proteinase K treated-EVs do not confer protective effect	Murine intravenous infection model (MSSA476 EVs)	Increased S. aureus recovery from blood in mice infected with bacteria supplemented with EVs; no difference observed for kidney, liver and spleen	Askarian et al, 2018
	Neutrophils (MSSA476 EVs)	Exogenous Evs (LB and BHI-EVs) confered resistance to neutrophil killing; dramatic decrease of neutrophils viability; induce NET release			
	HaCaT	minimal cytoxicity			
	THP-1 cells (monocytes)	extensive induction of cell death			
N305	MAC-T	EVs were non-cytotoxic	Mouse mastitis model	After 24h: macroscopic signs of inflammation in glands exposed to 10 µg EVs (less extent with 1 µg); neutrophil recruitment to the alveoli of mamary glands (higher with 10 µg); induction of local levels of MCP-1, KC, MIP-2, BAFF (all dose-dependent) and RANTES	Tartaglia et al, 2018

continue…

Strains	*In vitro*		*In vivo*		References
	Cell type	**Type of response**	**Model**	**Type of response**	
JE2	Mutant strains for psmα, psmβ (psm=phenol-soluble modulins)	Reduction in EV production; reduced EV size	Lethal murine sepsis model: challenge with USA300 FPR3757	Immunization with EVs from JE2-Δagr-Δspa , but not JE2-Δagr, provided significant protection	Wang et al, 2018
	JE2 + sublethal concentration of penicillin (PenG)	Decreases peptidoglican (PGN) cross-linking (ref23) ↔ which consequently increases EVs production and size			
	Mutant strains for autolysins Sle1 and Atl	both mutants decreased EVs production, however only in Δsle1 strain the reduction was significative			
	A549 (human epithelial cells)	membrane integrity 50% with 5 ug/mL EVs	lethal murine sepsis model: challenge with USA300 LAC and USA500 NRS685 strains	immunization with JE2-Δagr-Δspa_eng-EVs provided significant protection against both isolates	
	HL60 (neutrophil like cells)	membrane integrity 0% with 5 ug/mL EVs			
	rabbit erythrocytes	membrane integrity 0% with 5 ug/mL EVs			
	human leucocytes	EVs from JE2-Δagr-Δspa were non toxic			
ATCC 6538	HDMECs (human dermal microvascular endothelia cells)	EVs non cytotoxic to HDMECs at conc. up to 1 µg/mL; EVs induces production of cell adhesion molecules like E-selectin, ICAM1 and VCAM1, and consequently recruitment of monocytes (THP-1); Among 42 analysed cytokines, only GRO a/b/g, GM-CSF, EGF and IL-6 were upregulated; EVs-induced HDMEC activation was dependent on both TLR4 and NF-κB signalling pathways; EVs trigger a more rapid and intense induction of cell adhesion molecules and inflammatory cytokines than S. aureus extracts	-	-	Kim et al, 2018
ATCC 25923	HaCaT cells	intact ATCC EVs toxic at ≥2,5 µg/mL, but not cytotoxic up to 10 µg/mL if disruped; both intact and disruped M060 EVs were cytotoxic at ≥1,25 µg/mL; both intact EVs induced expression of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) and chemokines (IL-8 and MCP-1) (dose-dependent), but expression was higher for ATCC 25923 EVs	-	-	Kwon et al, 2019
M060					

Chapter 4. Original article - Involvement of caspase-1 in inflammasomes activation and bacterial clearance in *S. aureus*-infected osteoblasts.

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Involvement of Caspase-1 in inflammasomes activation and bacterial clearance in *S. aureus*-infected osteoblasts.

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Running title: Inflammasome activation and bacterial clearance in osteoblasts

Abstract

Staphylococcus aureus, a versatile Gram-positive bacterium, is the main cause of bone and joint infections (BJI), which are prone to recurrence. The inflammasome is an immune signaling platform that assembles after pathogen recognition. It activates proteases, most notably caspase-1, which then proteolytically mature and promotes the secretion of pro-IL-1β and pro-IL-18, cytokines strongly implicated in the pathology of BJI. The role of the inflammasome in the infection of osteoblasts, which play a pivotal role in BJI, has not been investigated. We

show that *S. aureus*-infected osteoblast-like MG-63 but not caspase-1 knockout CASP1$^{-/-}$MG-63 cells, which were generated using CRISPR/Cas9 technology, activate the inflammasome as monitored by the release of mature IL-1β. The effect was strain-dependent and significantly smaller in a natural mutant of the Agr quorum-sensing system of *S. aureus* as well as isogenic mutants in the Agr-controlled genes encoding phenol-soluble modulins. Furthermore, we found that the lack of caspase-1 in CASP1$^{-/-}$MG-63 cells impair the defense functions of osteoblasts, as bacterial clearance was drastically decreased in CASP1$^{-/-}$ MG-63 compared to wild-type MG-63 cells. These data provide insight into the mechanism of *S. aureus* BJI and establish a crucial role of caspase-1 in bacterial clearance by osteoblasts.

Key words: Osteoblasts, *Staphylococcus aureus*, inflammasomes, caspase-1, IL-1β

1. Introduction

Staphylococcus aureus is a highly adaptive and versatile Gram-positive bacterium that has major importance for human and animal health (1). *S. aureus* causes diseases ranging from relatively minor skin infections to life-threatening infections such as bacteremia, pneumonia, meningitis, endocarditis, and sepsis (2). *S. aureus* is also the main cause of bone and joint infections (BJI), particularly in the presence of orthopedic devices (3). Despite adequate antimicrobial therapy, BJI is linked to a high percentage of relapse, often leading to the development of chronic disease demanding particularly challenging treatments (4). While research into treatment options has been focused mostly on antibacterial strategies, it is widely believed that the treatment of recurrent *S. aureus* infections would also benefit considerably from reinforcement of host defenses (5), and a deeper understanding of the immune response is crucial for the development of new anti-infective strategies.

The innate immune response plays a crucial role in the defense against pathogens and is initiated through pattern recognition receptors (PRRs), which recognize microbial pathogen-associated molecular patterns (PAMPs) and endogenous danger-associated molecular patterns (6). This leads to the activation of host defense pathways that results in the clearance of infections. The innate immune response against microbes involves a major inflammatory pathway known as the activation of inflammasomes, multi-protein signaling complexes that assemble after recognition of danger signals and/or pathogens (7). Inflammasomes consist of a family of cytosolic receptors called NLRs (nucleotide-binding domain and leucine-rich repeats containing receptors), PYHIN protein family and enzymatic components, most notably caspase-1 and less frequently caspase-11 (8). Caspase-1 is synthesized in cells as an inactive precursor of 45 kDa and forms two subunits of 20 and 10 kDa after inflammasome activation

(9). Most inflammasomes also use an adaptor molecule known as ASC (apoptosis-associated speck-like protein) (10).

After stimulation with pathogens or damage-associated molecular patterns, inflammasome assembly leads to the autocatalytic cleavage of caspase-1, processing of pro-IL-1β and pro-IL-18 into their mature active forms and their secretion from cells (11).

Furthermore, inflammasome activation may trigger pyroptosis, an inflammatory form of cell death. The role of different nuclear receptors, as well as the PYHIN proteins AIM2 and IFI16 in assembling inflammasomes and controlling the innate immune response against various microbes, has been intensively explored (12, 13). Special attention has been paid to the investigation of the role of inflammasomes during infection with intracellular pathogens, like *Salmonella typhimurium, Legionella pneumophila, Listeria monocytogenes* and other Gram-negative bacteria (14–17). Some studies were also performed investigating the role of inflammasomes in phagocytes and epithelial cells during infection with the Gram-positive pathogen *S. aureus* (18, 19). However, an involvement of inflammasomes and processed IL-1β, which plays a crucial role in bone homeostasis (20), in *S. aureus*-associated BJI has not yet been demonstrated.

In the present work, we discovered that in response to *S. aureus* infection, osteoblast-like wild-type (WT) MG-63 cells activate inflammasomes in contrast to caspase-1 knockout CASP1$^{-/-}$ MG-63 cells, which we generated using CRISPR/Cas9 technology. Inflammasome activation was monitored by caspase-1 activation and the release of mature soluble IL-1β. Mutant *S. aureus* strains were employed, demonstrating a key contribution of phenol-soluble modulins (PSMs) in triggering the release of soluble IL-1β, which is strongly implicated in the pathology of BJI (20, 21). We demonstrated that in addition to the key role of caspase-1 in the release of pro-inflammatory cytokines in infected non-phagocytic cells, caspase-1 is implicated in cell defense mechanisms like as a restriction of the intracellular replication of bacteria that is critical in the induction of both innate and adaptive immune processes.

2. Results

2.1. Caspase-1 activation and IL-1β release triggered by inflammasomes activators LPS and ATP in human osteoblasts and PMA-treated ThP1 cells

In order to monitor inflammasome activation in the human osteoblast-like MG-63 cells the detection of active caspase-1 by Western blot analysis was set up in samples containing cells and cell supernatants. As shown in Fig. 1A, exposing MG-63 cells to LPS+ATP led to the appearance of 20 kDa band corresponding to the active caspase-1 p20 fragment. The activation of caspase-1 was associated with the production of IL-1β, as the exposure of cells to LPS+ATP resulted in the production of mature IL-1β (Fig.1B). These results suggest that MG-63 cells form functional inflammasomes and that their activation can be estimated by IL-1β release.

To compare the ability of inflammasome activation by professional versus non-professional phagocytes, PMA-stimulated ThP1 cells differentiated into macrophagesor MG-63 osteoblast-like cells were exposed to inflammasome activators LPS+ATP and the level of IL-1β production was estimated 2 h and 6 h after the beginning of the treatment. PMA-treated control ThP1 cells produce ~ 7 pg/ml of IL-1β 2 h at the beginning of the treatment. Exposure of PMA-differentiated ThP1 cells to LPS+ATP resulted in a significant increase to ~ 30 pg/ml 2 h post treatment. After 6 h PMA-treated control ThP1 cells produce ~ 10 pg/ml of Il-1β, while the level of IL-1β produced by PMA-treated ThP1 cells after an exposure to LPS+ATP was ~ 650 pg/ml. Supernatants of ThP-1+LPS+ATP (6 h) were diluted 1:5, the result presented in Fig.1C is multiplied by the dilution factor. In contrast, IL-1β was not detected 2 h post-treatment, while only 4 pg/ml of IL-1β was detected in MG-63 cells 6 h after the exposure to LPS+ATP.

2.2. Deletion of caspase-1 gene in human osteoblasts MG-63 using the CRISPR/Cas9 gene editing system.

It has been shown that stimulation is required for IL-1β transcription and protein expression by MG-63 cells [22]. Since MG-63 cells are commonly used as a model to study BJI [23] we used MG-63 cells for CRISPR/Cas9-mediated CASP1 deletion to study the involvement of inflammasomes in *S. aureus* infection of osteoblasts. There are 6 isoforms of caspase-1 encoded by the CASP1 gene, located on chromosome 11 [24]. Caspase-1 alpha and beta are the main transcripts in human cells. To generate CASP1 deletion MG-63 cells we used a previously validated sgRNA ATTGACTCCGTTATTCCGAA [25] (Fig.2A).

Based on sequences analysis, the sgRNA is predicted to target four Caspase-1 isoforms including alpha and beta. Following CRISPR lentiviral infection and puromycine selection, individual clones, which were identified as a result of limiting dilution of puromycin-treated cells, were tested by Western blotting for the lack of the 45-kDa band corresponding to pro-caspase-1. Finally, a deletion clone (CASP1$^{-/-}$ MG-63, G2 clone) showing CRISPR-induced genomic alteration (Fig. 2B) was selected for further analysis. Western blot analysis of WT MG-63 vs CASP1$^{-/-}$ MG-63 cells confirmed the lack of pro-caspase-1 in CASP1$^{-/-}$ MG-63 cells (Fig. 3A).

2.3. Inflammasomes involvement in caspase-1 dependent IL-1β release by *S. aureus* infected MG-63 osteoblasts

Activation of the NLRP3 inflammasome involves the oligomerization of the adaptor protein ASC (apoptosis-associated speck-like protein containing A CARD) into speck-like aggregates [26]. This structure recruits and activates pro-caspase-1, which promotes the maturation of IL 1β and IL-18.

To ensure that the deletion of CASP1 does not impair upstream events of inflammasome formation, the level of NLRP3 protein expression and the formation of ASC specks was analyzed in WT MG-63 and CASP1$^{-/-}$ MG-63 cells. NLRP3 protein expression was estimated by FACS. As shown in Fig. 3B, equal amounts of NLRP3 was observed in WT and CASP1$^{-/-}$ MG-63 cells. The exposure of both types of cells to *S. aureus* SA113 resulted in similarly increased levels of fluorescence. Mean fluorescence intensity (MFI) increased from 8000 to 13500 in WT MG-63 cells after *S. aureus* infection, similarly to CASP1$^{-/-}$ MG-63 cells, which showed an MFI increase from 7900 to 14000 (Fig. 3B).

Taking into account that both types of cells contain NLRP3 (Fig. 3B), while pro-caspase-1 is present only in WT MG-63 cells (Fig. 3A) we next determined whether an exposure of both cell lines to *S. aureus* SA113 leads to the formation of ASC specks, since their formation does not require the presence of pro-caspase-1. Consequently, we analyzed immunofluorescence staining for the adaptor ASC in WT and CASP1$^{-/-}$MG-63 cells. As shown in Fig. 3C, *S. aureus* infection triggers assembly of the ASC specks in the cytosols of WT as well as CASP1–/– MG-63 cells (green).

Previously it was reported that the transcripts of IL-1β and IL-18 were observed after an exposure of mice osteoblasts to *S. aureus*, however the secretion of IL-1β and IL-18 was not detected despite the presence of active caspase-1 [27].

To understand whether *S. aureus* activates inflammasomes in human osteoblast-like MG-63 cells, we measured IL-1β production in WT and CASP1$^{-/-}$MG-63 cells. The presence of NLRP3 and the lack of pro-caspase-1 in CASP1$^{-/-}$MG-63 cells allows these cells to be used as a control for the estimation of NLRP3 inflammasome activity in MG-63 cells by the measurement of IL-1β, which is processed due to caspase-1. The assessment of IL-1β in WT and CASP1$^{-/-}$ MG-63 cells exposed to inflammasomes activators LPS+ATP (Fig. 3D) as well as to *S. aureus* LAC (Fig. 3E) revealed the lack of IL-1β production in CASP1$^{-/-}$MG-63 in contrast to WT MG-63 cells.

To clarify the possible role of human osteoblasts in the production of IL-1β, we analyzed the kinetics (from 2 to 11 days) of IL-1 β production by WT and CASP1$^{-/-}$MG-63 cells exposed to *S. aureus* SA113. As demonstrated in Fig. 4A, the level of IL-1β increased from day 2 to 11. In contrast, IL-1β was not detected in supernatants from infected CASP1$^{-/-}$MG-63 cells during the tested period.

To verify whether IL-1β release by infected osteoblasts was strain-dependent, we analyzed the kinetics of IL-1 β release by MG-63 exposed to different *S. aureus* strains: LAC (USA300), MW2 (USA 400) and SA113. As shown in Fig. 4B, all strains induce IL-1β release; however, MW2 and LAC induced significantly higher levels of IL-1β than SA113. IL-1β was not detected in the supernatants of MG-63 cells exposed to killed bacteria of any of the three strains, suggesting that factors associated with viable bacteria are involved in inflammasome activation.

2.4. Pivotal role of *S. aureus* PSM toxins in stimulation of IL-1β release by infected osteoblasts.

The quorum-sensing system in *S. aureus* known as the accessory gene regulator (Agr) regulates the expression of many virulence factors. Agr regulates main *S. aureus* toxins, among which regulation of PSMs (PSMα 1 to 4, PSMβ 1 and 2, and δ-toxin sometimes called PSMɣ) is exceptionally direct and strict [28], [29]. It has previously been shown that PSMs stimulate the production of inflammatory cytokines by infected keratinocytes [30]. Furthermore, our results on strain dependence of IL-1β stimulation in osteoblasts suggested an involvement of Agr, as the low levels of IL-1β in cells infected with strain SA113 as opposed to those infected with strains MW2 and LAC correlate with levels of Agr functionality in those strains. SA113 is functionally Agr-defective, while MW2 and LAC show high Agr activity. These considerations prompted us to investigate the role of PSMs in IL-1β production by infected osteoblasts.

First, we analyzed LAC (USA300) wild-type and its isogenic mutant LACΔ*psmαβhld* (*S. aureus* strain lacking PSMα, PSMβ and δ-toxin) for their ability to stimulate the release of IL-1β. As shown in Fig. 5A, the level of IL-1β was significantly decreased in the supernatants of WT MG-63 cells exposed to LAC Δ*psmαβhld* compared to WT MG-63 cells exposed to LAC wild-type on days 5 and 9 post-infection. To further investigate which PSM were involved in the simulation of IL-1β release, we used the PSM deletion strain LACΔ*psmαβhld* and complemented strains, expressing either the four PSMα peptides (LACΔ*psm*αβ*hld*-pTXΔα1-4), the two PSMβ peptides (LACΔ*psm*αβ*hld*-pTXΔβ1-2), or the δ-toxin (LACΔ*psm*αβ*hld*-pTXΔ*hld*) and monitored IL-1β levels in cell exposed to those strains up to 9 days post-infection. As shown in Fig. 5B, there was a significant decrease in the IL-1β released by cells exposed to the LACΔ*psmαβhld* strain compared to LAC. Exposure to complemented mutants demonstrated that the release was partially restored when the strains were complemented with PSMα (LACΔ*psm*αβ*hld*-pTXΔα1-4), PSMβ (LACΔ*psm*αβ*hld* pTXΔβ1-2) or δ-toxin (LACΔ*psm*αβ*hld*-pTXΔ*hld*). However, the difference was statistically significant only when strains were complemented with PSMβ or, at earlier time points, δ-toxin (LACΔ*psm*αβ*hld*-pTXΔ*hld*) (Fig. 5).

2.5. *S. aureus* clearance by osteoblasts depends on caspase-1

It was reported that caspase-1 is essential for the restriction of intracellular pathogen replication in professional phagocytes [14], however the role of caspase-1 in bacterial clearance in non-professional phagocytes, which include osteoblasts, has not been investigated. Therefore, we analyzed whether the lack of caspase-1 in *CASP1*$^{-/-}$MG-63 cells is correlated with a failure to control the intracellular replication of *S. aureus* bacteria. The quantity of intracellular bacteria during *S. aureus* infection of WT and *CASP1*$^{-/-}$MG-63 cells was determined after gentamicin treatment of infected cells as described in Material and Methods. According to CFU determination bacterial internalization was not impaired in *CASP1*$^{-/-}$MG-63 cells, as we observed equivalent levels of internalized bacteria in WT MG-63 cells at 2 h post-infection at ~ 10^2 CFU/10^5 host cells. Six and 24 hours post-infection significantly higher numbers of viable bacteria were recovered from *CASP1*$^{-/-}$MG-63 cells compared to WT MG-63 cells (Fig. 6A). These results suggest that the lack of caspase-1 impairs the ability of osteoblasts to restrict *S. aureus* growth. These findings were further confirmed using confocal microscopy. As shown in Fig. 6B, 6 h post-infection a considerably higher number of intracellular *S. aureus* bacteria (red) was observed in *CASP1*$^{-/-}$ MG-63 cells than WT MG-63 cells, while assembly of the ASC specks was observed in both cell types (green).

3. Discussion

Despite antibiotic therapy associated with complex surgical procedures, *Staphylococcus aureus* BJI is particularly difficult to treat because bacteria employ multiple approaches such as the formation of biofilm and small-colony variants as well as the invasion and intra-osteoblastic persistence, leading to chronicity and relapse [31]–[33]. We aimed to investigate the contributions of internalized *S. aureus* on alteration of host defense events such as inflammasomes activation and bacterial clearance in infected osteoblasts.

Activation of inflammasomes during infection have been extensively characterized in professional phagocytes [34]–[37]. The implication of inflammasomes in response of *S. aureus* infected osteoblasts, which play a pivotal role in BJI, was not demonstrated. Bone homeostasis is kept by an equilibrium between bone resorption and bone formation [20]. Osteoblasts synthesize various bone matrix proteins that are involved in bone formation, as well as regulate the maturation of osteoclasts responsible for bone resorption. IL-1β, a proinflammatory cytokine, plays a pivotal role in bone formation and bone resorption [21]. IL-1β is mainly expressed by monocytes, macrophages, and dendritic cells [38]–[41], and is processed into a soluble mature form by the inflammasomes compound, active caspase-1. In the present work we focused on the monitoring of soluble IL-1β produced by infected osteoblasts.

We demonstrated that human osteoblast-like MG-63 cells release a soluble IL-1β starting from 6 h post treatment with inflammasomes activator LPS+ATP, matching the of active caspase-1 20 kDa subunit (Fig. 1) that suggests inflammasomes activation in MG-63 cells. The production of IL-1β by MG-63 cells started later and IL-1β level was much lower than IL-1β production by ThP1 cells whose level of IL-1β production corresponds to the findings of other researchers [42]. The discrepancy between levels of the soluble IL-1β released by MG-63 and ThP1 is likely related to the different origin of both cells' types: osteoblast-like MG-63 cells and PMA-treated ThP1 differentiated into macrophages [43].

Taking into account the pivotal role of caspase-1 in the maturation of IL-1β we generated the CASP1$^{-/-}$MG-63 cell line using CRISPR/CAS9 gene editing for the investigation of inflammasomes involvement in *S. aureus* infected osteoblasts. The equal NLRP3 protein expression together with the similar formation of ASC specks in both WT MG-63 and CASP1$^{-/-}$MG-63 cells, as well as the absence of active caspase-1 and IL-1β release following the stimulation with inflammasomes activators in CASP1$^{-/-}$MG-63 cells, validate caspase-1 knock out in osteoblastic cells. Therefore, we generated CASP1$^{-/-}$MG-63 cell line that is a powerful tool that allows the function of inflammasomes to be investigated. We observed that an exposure of MG-63 cells to *S. aureus* results in the progressive increase of IL-1β from several

hours to several days in contrast to the findings of Marriott et al. describing the increase of IL-1β RNA expression along with the absence of IL-1β protein synthesis in 24 h infected mice osteoblasts. The discrepancy between our and Marriott et al. results are likely related to the different origin of *S. aureus* strains used in both laboratories, because *S. aureus*-induced IL-1β release is strongly strain-dependent as we have demonstrated in the present work. Moreover, a low level of IL-1β is produced at the beginning of the infection, thus the monitoring of IL-1β depends from the sensitivity of the detection method. Rising IL-1β production in *S. aureus*-infected MG-63 cells in contrast to its lack in infected CASP1$^{-/-}$MG-63 cells demonstrated caspase-1 dependent mechanisms of IL-1β production by human osteoblasts. Our data corroborate observations of others that demonstrated caspase-1 depending mechanism of IL-1 beta processing and its release by mice macrophages during infection [44].

The absence of IL-1β production by cells exposed to heat-killed bacteria indicates that factors associated with viable bacteria are involved in the activation of NLRP3 inflammasome. Employment of deletion and complemented PSMs mutants demonstrated a pivotal role of *S. aureus* toxins PSMs in inflammasomes related IL-1β production by infected osteoblastic cells. We would like to highlight that IL-1β production by infected osteoblasts appears to be specifically dependent on PSM betas among PSMs. This is noteworthy, as we don't know any specific phenotypes attributable to those PSMs so far. In addition to PSMs the action of other factors cannot be excluded since complemented mutants did not restore entirely the level of secreted IL-1β.

The role of caspase-1 in the production of inflammatory cytokines has been extensively investigated [39], [45], while much less attention has been paid to its impact on other immune processes. Nevertheless, recent investigations point out that additionally to the processing of IL-1β and IL-18 caspase-1 regulates unconventional protein secretion [46], activates lipid metabolic pathway [47], restricts pathogens replication in professional phagocytes [48], [49] and induces pyroptosis, proinflammatory cell death [50]. Our investigation of host defense event during *S. aureus* infection of non-professional phagocytes, such as the control of intracellular bacterial proliferation, reveals a drastic increase in the proliferation of internalized bacteria in osteoblastic CASP1$^{-/-}$MG-63 cells. This finding is in keeping with the previous reports demonstrating the correlation of the lack of caspase-1 activation with a failure to restrict the replication of *S. aureus* inside of phagocytic cells [51], [52]. Indeed, it has been demonstrated that during *S. aureus* infection of professional phagocytes NLRP3 inflammasomes and caspase-1 regulate phagosomes acidification that is essential for the activation of the hydrolytic enzymes required for killing of internalized bacteria [51], [53]. Moreover, it was suggested that early activation of NLRP3 [51], [53] inflammasomes is

triggered by events, such as production of ROS that are associated with the phagocytic process in macrophages [51]. Recently, we demonstrated that *S. aureus* induce ROS in WT MG-63 cells [54]. We hypothesize that *S. aureus*-induced ROS play the similar role in inflammasome activation in non-phagocytic cells.

Collectively, our results demonstrate that human osteoblast-like MG-63 cells play an important role in the immune response against *S. aureus* infection through inflammasomes activation and processing of IL-1β, the master cytokine of inflammation [55]. The outcome of the infection depends on the balance between the host immune response and the action of main *S. aureus* virulence factors, PSMs, whose production may differ among the *S. aureus* strains, as we already observed in our previous work [56]. Besides our observations of inflammasomes activation, we demonstrated that CASP1$^{-/-}$MG-63 cells were unable to restrict the intracellular replication of *S. aureus.* It points out that the active caspase-1 prevent exacerbated intracellular replication of *S. aureus* in non-professional phagocytes in addition to professional phagocytes, suggesting the universal pivotal role of caspase-1 in pathogens clearance independently from the type of cells. Our results raise the fascinating possibility, that pathogens, which inhibit caspase-1 activation, do so not only to govern the generation of inflammatory cytokines but also to impede the bacterial replication in infected osteoblasts that are critical for the defense response during BJI. These findings deserve further investigation to elucidate the ability of pathogens to inhibit inflammasomes activation and disturb the function of phagocytic vesicles in osteoblasts in order to evade host cells defense response. Current findings redefine our understanding of the role of osteoblasts in BJI, suggesting that osteoblasts are not passive bystanders, but active players in host defenses against *S. aureus* infection.

4. Materials and Methods:

4.3. Maintenance of eukaryotic cells lines

The human osteoblast-like MG-63 (LGC Standards, Teddington, UK) cells and human monocyte ThP1 cells (ATCC TIB-202) were cultured in DMEM and RPMI 1640 respectively, supplemented with 10% fetal calf serum (Gibco), 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C with 5% CO2 as described [54]. Trypsin/EDTA (Sigma) was used for cell subculturing to release adherent cells. In order to differentiate ThP1 cells into macrophages, cells were treated with 20 nM phorbol 12-myristate 13-acetate (PMA) for 48 h [57].

4.4. Deletion of the caspase-1 gene in human osteoblast-like MG-63 cells using the CRISPR/Cas9 gene editing system.

The caspase-1 gene in MG-63 cells was deleted using CRISPR/Cas9 technology (58). The sgRNA sequence (ATTGACTCCGTTATTCCGAA), which as previously published targets the *CASP1* gene (25), was cloned into the lentiCRISPR v2 (Addgene #52961) (59). Deletion was achieved *via* lentiviral infection of MG-63 cells. Lentiviral production has been performed as recommended (http://tronolab.epfl.ch) and previously described (60). After antibiotic selection [puromycine (3 days, 1µg/mL)], cells were seeded in 96-well plates at 0.3 cells/well for single-cell clonal expansion. Single-cell clones were tested by Western blot analysis for the presence of the 45-kDa band corresponding to pro-caspase-1. Clones that did not present 45-kDa bands were recloned in 96-well plates (10 and 30 cells per plate). Afterward, single-cell clones were transferred in 6-wells plate and grown to 80% of confluence. Again, the clones that lacked 45-kDa bands (Western Blot) were selected and grown in 25-ml flasks. CRISPR-induced genomic modifications were verified by nucleotide sequence analysis, and the lack of caspase-1 was confirmed by Western blot analysis.

4.5. *S. aureus* strains description

The methicillin-resistant strains *S. aureus* MW2 (USA400), LAC (USA300) wild type, the isogenic mutant LAC Δ*psmαβhld*, which lacks the psmα and psmβ operons and in which translation of the *hld* gene is abolished by mutation of the start codon, LAC (pTXΔ16) which carries the control plasmid, the deletion mutant LACΔ*psmαβhld* (pTXΔ16) and the complemented strains expressing either the four PSMα peptides (LACΔpsmαβhld-pTXΔα1-4), the two PSMβ peptides (LACΔ*psmαβhld*-pTXΔβ1-2), or the δ-toxin (LACΔ*psmαβhld*-pTXΔhld) were obtained from the Laboratory of Bacteriology, NIH, USA (61, 62). The pTXΔ16 plasmids were derived from plasmid pTX15 with the deletion of the *xylR* repressor gene. The tetracycline-resistant strains harboring plasmid pTXΔ16 were grown in BHI containing 12.5 µg/ml of tetracycline. *S. aureus* SA113 and SA113 (pctuf-mCherry) strain which carries plasmid-encoded mCherry (red fluorescence) fused with the propeptide of lipase for fluorescence enhancement were obtained from the Laboratory of Microbial Genetics, University of Tübingen (63). Aliquots from overnight cultures on BHI broth were diluted (1:50) in DMEM. The growth curves of mutants were similar to that of the wild type. Strains were grown at 37°C under anaerobic conditions until cultures reached an optical density of 0.6 at 600 nm, corresponding to 10^8 CFU/ml (Colony Forming Units). CFU was determined after plating a bacterial suspension on BHI agar followed by overnight incubation as described (64).

4.6. Cell culture infection

$2.5x10^5$ of either MG-63, CASP1$^{-/-}$MG-63, or ThP1 cells were seeded in the wells of 12-well plates. ThP1 were treated with PMA as described above before treatment. Cells then were exposed to *S. aureus* strains at MOI 50:1. An MOI value of 50:1 was selected in order to prevent the induction of host cell death. Bacterial concentrations were estimated spectrophotometrically and were confirmed by the determination of CFU. Extracellular bacteria were removed 2 h post-infection by incubating cells in cDMEM with 20 µg/ml lysostaphin and 100 µg/ml gentamicin for 2 h, which eliminates extracellular bacteria without altering intracellular bacteria (56, 64), followed by incubation in cDMEM containing 25 µg/ml of gentamicin. After the indicated periods adherent and floating, cells were collected, centrifuged, and prepared either for Western blot analysis or for analysis by cytofluorometry. For the determination of the number of internalized bacteria, we used the method we have previously described (64, 65). Briefly, to determine the number of internalized bacteria after 2 h of infection, infected cells were lysed with 0.05% Triton X-100 in PBS, cell lysates were plated on BHI agar at different dilutions, and CFU was determined after overnight incubation as described (64, 65).

4.7. Western blot analysis

Pro-caspase-1 and cleaved caspase-1 were detected by Western blot analysis as described (66). Briefly, $2.5x10^5$ of cells were grown in 12-well plates for 12 h. Pro-caspase-1 was detected in non-stimulated and stimulated cells, while active caspase-1 was detected solely in stimulated cells. Wild type (WT) MG-63 or CASP1$^{-/-}$ MG-63 cells were primed for 3 h with LPS (1µg/ml) (Sigma) and selected samples were stimulated with ATP (5 mM) for 15 min or after the exposure to *S. aureus* strains. Six hours after the beginning of the treatment cells together with cell-culture supernatants were collected and diluted in RIPA lysis buffer supplemented with complete protease inhibitor cocktail, P8340 (Sigma Aldrich®). Protein samples were prepared in 5x polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer. Proteins were separated on 12% SDS-PAGE gels and transferred onto 0.2 µM PVDF membranes. The membranes were washed three times in PBS-T (Tris-buffered saline containing 0.5% Tween 20) and blocked in 10% skim milk in PSB-T for 1 h at room temperature before being incubated with primary antibody overnight at 4°C. Caspase-1 was detected with anti-human caspase-1 antibody (1:1,000 dilution, mAb p/20, AdipoGen). Membranes were washed four times in PBS-T and incubated with HRP-conjugated secondary anti-mouse IgG-HRP antibody 1:10,000 dilution, Cell Signaling Ozyme) for 1 h at room temperature, washed

another four times, and incubated with Western chemiluminescent HRP substrate (ECL kit) (GE Healthcare). The image was processed using a GBOX imaging system (Syngene, Ozyme, Poitiers, France). To assess the quantity of loaded protein, membranes were re-probed with rabbit anti-tubuline (Cell Signaling Ozyme France) (1:1000) and bands were visualized using the ECL kit and a GBOX imaging system.

4.8. Flow cytometry analysis

For an estimation of NLRP3 protein expression in WT MG-63 vs CASP1$^{-/-}$ MG-63 cells, cells were exposed to S. aureus LAC for 2 h and were fixed in 4% paraformaldehyde/PBS followed by permeabilization in 0.1% Triton/0.5% BSA/PBS. After centrifugation, cells were resuspended in 200 µl of 0.5% BSA/PBS, 5µl of Alexa Fluor 488 rat anti-human NLRP3 antibody (RDsystems, France, IC7578G) per 106 cells was added, and the samples were incubated for 45 min. NLRP3 expression was analyzed with an Accuri C6 flow cytometer. Data were collected from 20,000 cells, and analyzed with CFlow software (Becton Dickinson) as described [67].

4.9. Confocal microscopy

WT MG-63 or CASP1$^{-/-}$MG-63 cells were grown on coverslips and exposed to *S. aureus* SA113 (MOI 50:1) for 2 h. Six and 24 h post-infection, cells were fixed with 4% paraformaldehyde/PBS for 20 min, followed by permeabilization in 0.1% triton/PBS and incubation with 20% goat serum (Sigma) as described (54). Rabbit anti-PYCARD antibody (Coger France) diluted 1:50 in 1% BSA/PBS was applied overnight at 4°C, followed by incubation with Alexa Fluor 488 labeled goat anti-rabbit antibody (Cell Signaling Ozyme) (1:1000) for 2 h. Coverslips were mounted with ProLong antifade containing DAPI (Vectashield, Biovalley). Confocal laser scanning microscopy (CLSM) observation of specimens was performed using a ZEISS LSM 880 inverted confocal microscope (Carl Zeiss AG, Oberkochen, Germany) equipped with immersion objective 63× (Plan Apochromat objective, oil immersion, NA 1.4) driven by Zen software.

4.10. IL-1β quantification by ELISA

Undiluted cell culture supernatants were subjected to human IL-1β detection by sandwich-ELISA (Thermofischer Life Technologies) according to the manufacturer's instructions. Briefly,

wells of 96-wells plates were coated with capture anti-human IL-1β antibody and were incubated overnight at 4°C. After washing the wells were incubated in the blocking solution. Then tested samples were added to the appropriate wells. A standard curve was measured with 2-fold serial dilutions of the maximal standard concentration of 150 pg/ml. Recombinant human IL-1β was used for generating the standard curve and calibrating samples. After 2 h of incubation, biotin-conjugated anti-human IL-1β antibody was added to the wells followed by incubation with avidin-HRP for 30 min. After the addition of the tetramethylbenzidine substrate solution for 15 min, the reaction was stopped with stop solution and absorbance was read at 450 nm. The sensitivity of IL-1β detection was 2 pg/ml.

4.11. Statistical analysis

At least three independent assays were performed per experiment. The differences among the groups were assessed by analysis of variance (ANOVA). P-values < 0.05 were considered to be significant. Tukey's Honestly Significant Difference test was applied for comparison of means between the groups. The values are expressed as mean ± standard deviation (±SD).

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Conflict of Interest

The authors declare no competing financial and/or non-financial interests, all authors concur with the submission.

Figure legend

Figure 1. Caspase-1 activation and IL-1β release triggered in human osteoblasts

A. MG-63 cells were incubated in 12-well plates. Afterwards, cells were primed with LPS (1 µg/ml) and stimulated with ATP (5 mM). Six hours after the beginning of the treatment cells together with cell-culture supernatants were diluted in RIPA lysis buffer. Protein samples were prepared in 5 x SDS-PAGE sample loading buffer. Detection of 45-kDa pro-caspase-1 in MG-63 cells by Western blot analysis was performed using anti-caspase-1 antibody (AdipoGen) as described in Material and Methods. The protein load was verified with anti-tubulin antibody. Three independent assays were performed.

B, C. MG-63 or ThP1 cells were incubated in 12-well plates. Afterwards, cells were primed for 2 h with LPS (1 µg/ml) and stimulated with ATP (5 mM) for 15 min. ThP1 were treated with PMA before LPS+ATP treatment as described above. Two or six hours after the beginning of the treatment cell supernatants were collected, centrifuged, and the level of IL-1β was determined by a commercial sandwich-ELISA (Thermofischer Life Technologies) as described in Material and Methods. Supernatants of ThP-1+LPS+ATP were diluted 1:5. The values are presented as concentrations in pg/ml. Three independent assays were performed. The differences among the groups were assessed by analysis of variance (ANOVA). Comparisons with P-values < 0.05 (*) were considered to be significant. *, $P < 0.05$; **, $P < 0.01$. Tukey's Honestly Significant Difference test was applied for comparison of means between the groups. The values are expressed as means ± standard deviation (±SD).

Figure 2. Deletion of caspase-1 gene in human osteoblasts MG-63 using the CRISPR/Cas9 gene editing system

A. Scheme showing the caspase-1 deletion targeting exon 2 of the CASP1 gene (Sequence ID NG 029124.2).

B. Trace file from Sanger sequencing of the targeted deletion in the exon 2 of the CASP1 gene (WT MG-63 and $CASP1^{-/-}$ MG-63 clone).

Figure 3. NLRP3 protein expression, speck-like aggregates formation and IL-1β secretion by WT MG-63 vs CASP1$^{-/-}$MG-63 cells exposed to *S. aureus*

A. WT MG-63 or *CASP1*$^{-/-}$ MG-63 cells were incubated in 12-well plates. Cells were then detached by trypsin treatment and cell pellets were obtained by centrifugation. Protein samples were prepared in 5 x SDS-PAGE sample loading buffer. Detection of 45-kDa pro-caspase-1 in WT MG-63 and *CASP1*$^{-/-}$ MG-63 cells by Western blot analysis using anti-caspase-1 antibody (AdipoGen) was performed as described in Material and Methods. Three independent assays were performed.

B. WT MG-63 or *CASP1*$^{-/-}$ MG-63 cells were exposed to *S. aureus* LAC (USA300) for 2 h. Following fixation and permeabilization of cells, NLRP3 expression at the protein level was determined by FACS using Alexa Fluor 488 anti-NLRP3 antibody. NLRP3 expression was analyzed with an Accuri C6 flow cytometer. Data were collected from 20,000 cells, and analyzed with CFlow software (Becton Dickinson). Cells are analyzed using FSC-A x SSC-A plot. The major density of events is captured by the gate. The events that represent debris, cell fragments and pyknotic cells are eliminated. Values shown on the right side of the graph refer to the respective mean fluorescence intensities (MFIs). Three independent assays were performed.

C. WT MG-63 or CASP1$^{-/-}$ MG-63 cells were exposed to a fluorescent derivative of *S. aureus* SA113, which carries plasmid-encoded mCherry (red fluorescence), at MOI 1:50 for 2 h followed by antibiotic treatment as described in Materials and Methods. Six hours post-infection cells were immunostained with rabbit anti PYCARD antibody (Coger France), followed by incubation with Alexa Fluor 488 labeled goat anti-rabbit antibody (Cell Signaling Ozyme) at a dilution of 1:50 for 2 h at room temperature (green staining, red arrow). Nuclear DNA was labeled with DAPI (blue staining). Samples were viewed with a Zeiss laser-scanning microscope equipped with a 63 × plan Apo-NA 1.4 immersion objective driven by Zen software. Scale bar: 1 µm.

D, E. WT MG-63 or CASP1$^{-/-}$ MG-63 cells were exposed to LPS+ATP (D) or *S. aureus* strain SA113 at MOI 1:50 for 2 h (E) followed by antibiotic treatment as described in Material and Methods. Six hours post-infection or 6 h after the beginning of LPS+ATP treatment cell supernatants were collected, centrifuged and the level of IL-1β was determined by a commercial sandwich-ELISA (by sandwich-ELISA (Thermofischer Life Technologies) as described in Materials and Methods. The values are presented as concentrations in pg/ml. Three independent assays were performed. The differences among the groups were assessed by analysis of variance (ANOVA). P-values < 0.05 were considered to be significant. Tukey's Honestly Significant Difference test was applied for comparison of means between the groups. The values are expressed as means ± standard deviation (±SD).

Figure 4. *S. aureus* strain-dependent release of IL-1β by infected MG-63 cells

A. WT MG-63 or CASP1$^{-/-}$ MG-63 cells were grown in 6-well plates, then were exposed to *S. aureus* strain SA113 at MOI 1:50 for 2 h followed by antibiotic treatment as described in Materials and Methods. After various times post-infection (6 h, 2 days, 5 days, 7, days and 11 days) cell supernatants were collected, centrifuged and the level of IL-1β was determined by a sandwich-ELISA (Thermofischer Life Technologies) as described in Material and Methods. The values are presented as concentrations in pg/ml. Three independent assays were performed. The differences among the groups were assessed by analysis of variance (ANOVA). P-values < 0.05 (*) were considered to be significant. Tukey's Honestly Significant Difference test was applied for comparison of means between the groups. The values are expressed as means ± standard deviation (±SD).

B. WT MG-63 cells were exposed to *S. aureus* strains LAC (USA300, red color), MW2 (USA 400, green color), or SA113 (yellow color) at MOI 1:50 for 2 h followed by antibiotic treatment as described in Material and Methods. After various times post-infection (6 h, 2 days, 5 days, 7, days and 11 days) cell supernatants were collected, centrifuged and the level of IL-1β was determined by a commercial sandwich-ELISA (Invitrogen, France) as described in Material and Methods. The values are presented as concentrations in pg/ml. Three independent assays were performed. The differences among the groups were assessed by analysis of variance (ANOVA). P-values < 0.05 (*) were considered to be significant. Tukey's Honestly Significant Difference test was applied for comparison of means between the groups. The values are expressed as means ± standard deviation (±SD).

Figure 5. *S. aureus* phenol-soluble modulins stimulate IL-1β release from infected osteoblasts

A. WT MG-63 cells were exposed to wild type LAC (USA300) and its isogenic mutant LAC Δ*psmαβhld* at MOI 1:50 for 2 h followed by antibiotic treatment as described in Material and Methods. After various time post-infection (2 days and 5 days) cell supernatants were collected, centrifuged and the level of IL-1β was determined by a sandwich-ELISA (Thermofischer Life Technologies) as described in Material and Methods. The IL-1β values are presented as concentrations in pg/ml. Three independent assays were performed. The differences among the groups were assessed by analysis of variance (ANOVA). P-values < 0.05 (*) were considered to be significant. Tukey's Honestly Significant Difference test was applied for comparison of means between the groups. The values are expressed as means ± standard deviation (±SD).

B. MG-63 cells were exposed to USA300 LAC (pTXΔ16), which carries the control plasmid, the deletion mutant LACΔ*psmαβhld* (pTXΔ16) and the complemented strains expressing the

four PSMα peptides (LACΔ*psmαβhld*-pTXΔα1-4), the two PSMβ peptides (LACΔ*psmαβhld*-pTXΔβ1-2), or the δ-toxin (LACΔ*psmαβhld*-pTXΔhld) at MOI 1:50 for 2 h followed by antibiotic treatment as described in Material and Methods. After various times post-infection (2 days, 5 days, 7 days and 9 days) cell supernatants were collected, centrifuged and the level of IL-1β was determined by a sandwich-ELISA (Thermofischer Life Technologies) as described in Material and Methods. The values are presented as concentrations in pg/ml. Three independent assays were performed. The differences among the groups were assessed by analysis of variance (ANOVA). P-values< 0.05 (*) were considered to be significant. Tukey's Honestly Significant Difference test was applied for comparison of means between the groups. The values are expressed as means ± standard deviation (±SD).

Figure 6. Involvement of caspase-1 in bacterial clearance

A. WT MG-63 or CASP1$^{-/-}$ MG-63 cells were grown in 12-well plates overnight, then were exposed to *S. aureus* MW2 strain at MOI 1:50 for 2 h followed by antibiotic treatment as described. Two hours, 6 h and 24 h post-infection cells were lysed with 0.05% Triton X-100 in PBS, cell lysates were plated on BHI agar, and CFU was determined after overnight incubation. CFU values were normalized to 10^5 host cells. Experiments were performed in triplicate. The data are presented as means ± SD. Tukey's Honestly Significant Difference test was applied for comparison of means between the groups. **, P < 0.01, for the comparison of the number of internalized bacteria in CASP1$^{-/-}$MG-63 cells with those in WT MG-63 cells.

B. WT MG-63 or CASP1$^{-/-}$ MG-63 cells were grown on the slides of 12-well plates overnight, then cells were exposed to a fluorescent derivative of strain *S. aureus* SA113 fluorescent, which carries plasmid-encoded mCherry (red fluorescence), at MOI 1:50 for 2 h followed by antibiotic treatment as described. Six hours post-infection cells were immune-stained with rabbit anti PYCARD antibody (Coger France), followed by incubation with Alexa Fluor 488 labeled goat anti-rabbit antibody (Cell Signaling Ozyme) at a dilution of 1:50 for 2 h at room temperature (green staining). Nuclear DNA was labeled with DAPI (blue staining). Samples were viewed with a Zeiss laser-scanning microscope equipped with a 63× plan Apo-NA 1.4 immersion objective driven by Zen software. Scale bar: 3 μm.

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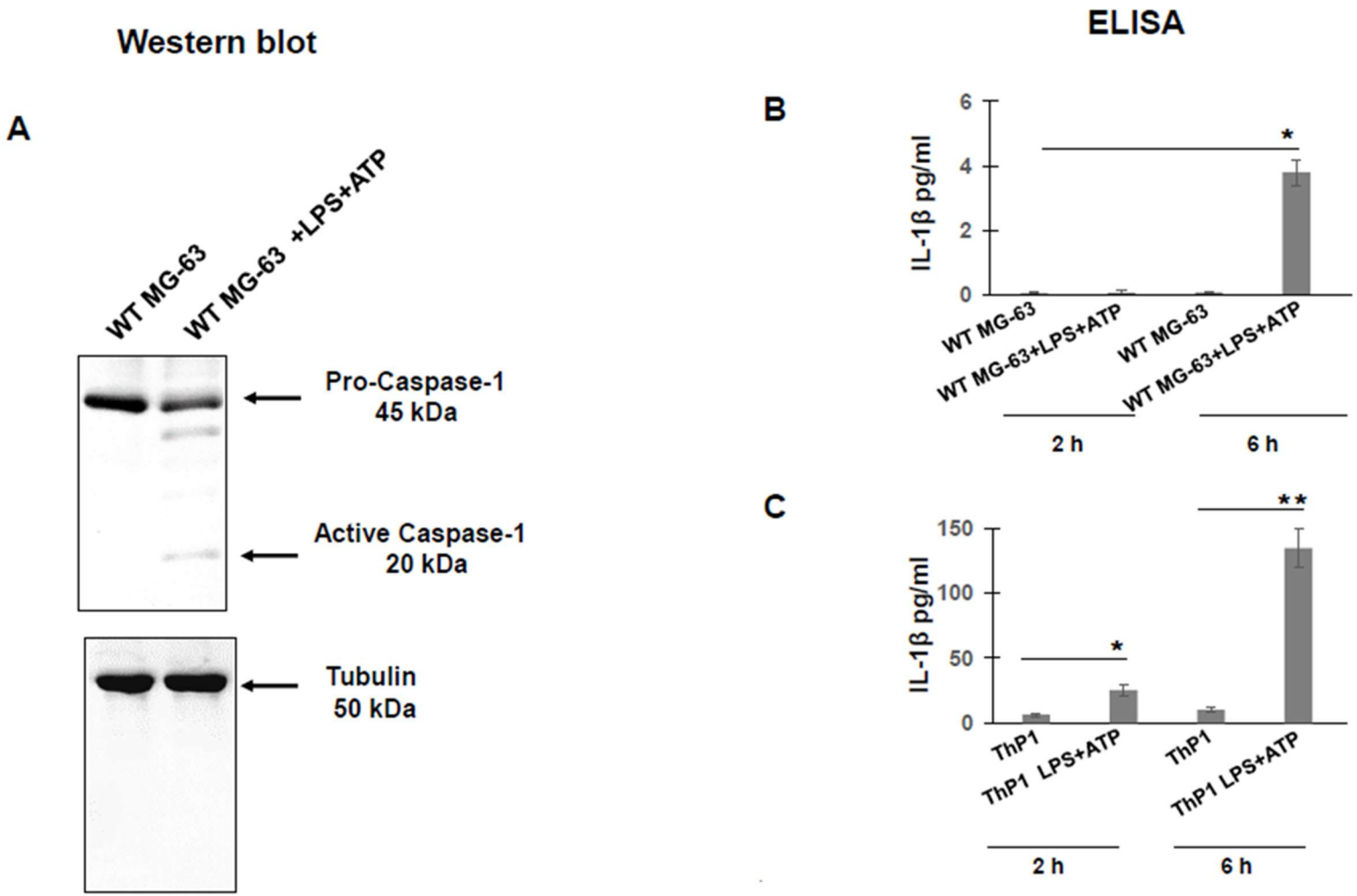
Figure 1:


Western blot
A
WT MG-63
WT MG-63 +LPS+ATP
Pro-Caspase-1
45 kDa
Active Caspase-1
20 kDa
Tubulin
50 kDa
ELISA
B
IL-1β pg/ml
6
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WT MG-63
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WT MG-63
WT MG-63+LPS+ATP
2 h
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150
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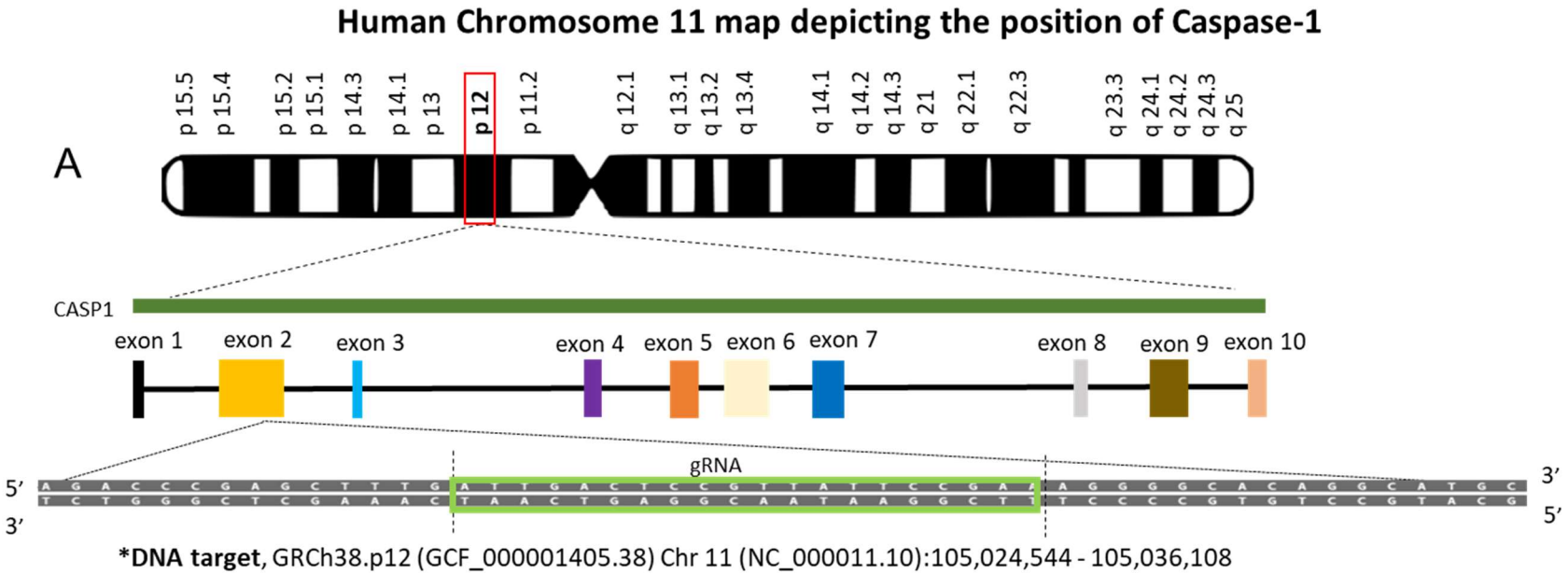
6 h

Figure 2:


Human Chromosome 11 map depicting the position of Caspase-1
A
p 15.5 p 15.4 p 15.2 p 15.1 p 14.3 p 14.1 p 13 p 12 p 11.2 q 12.1 q 13.1 q 13.2 q 13.4 q 14.1 q 14.2 q 14.3 q 21 q 22.1 q 22.3 q 23.3 q 24.1 q 24.2 q 24.3 q 25
CASP1
exon 1 exon 2 exon 3 exon 4 exon 5 exon 6 exon 7 exon 8 exon 9 exon 10
gRNA
5′
3′
3′
5′
*DNA target, GRCh38.p12 (GCF_000001405.38) Chr 11 (NC_000011.10):105,024,544 - 105,036,108

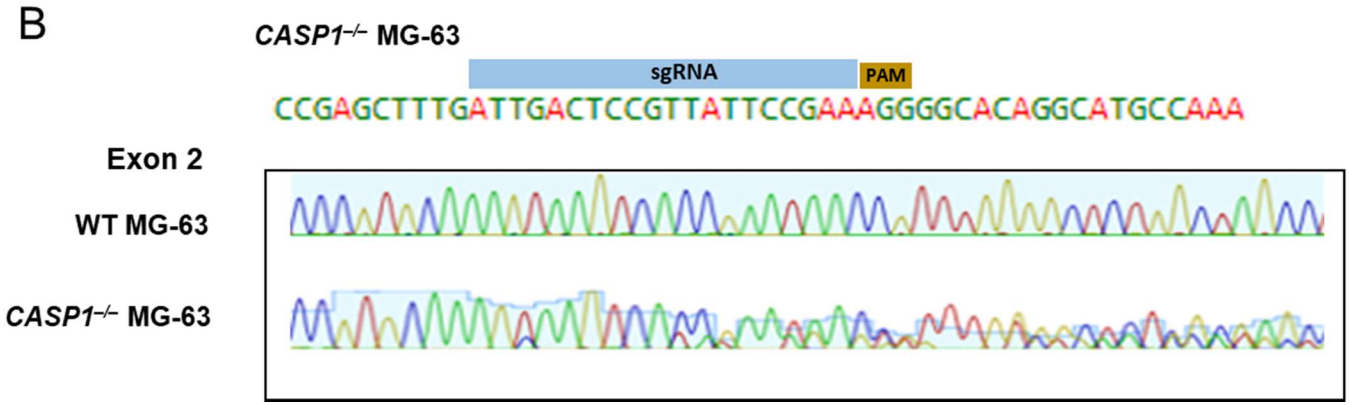

B
CASP1−/− MG-63
sgRNA
PAM
CCGAGCTTTGATTGACTCCGTTATTCCGAAAGGGGCACAGGCATGCCAAA
Exon 2
WT MG-63
CASP1−/− MG-63

Figure 3:

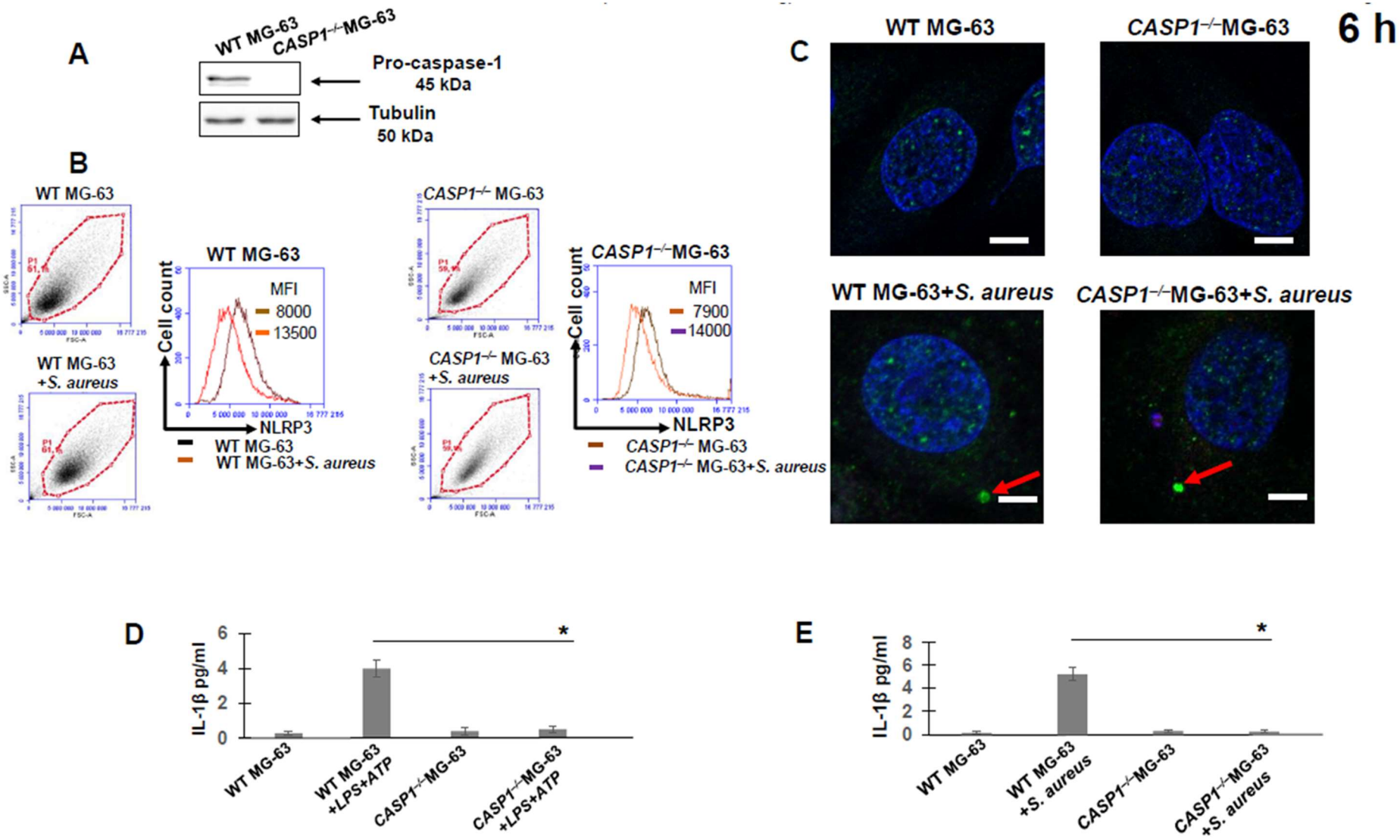

A
WT MG-63
CASP1−/−MG-63
Pro-caspase-1
45 kDa
Tubulin
50 kDa
B
WT MG-63
WT MG-63
+S. aureus
WT MG-63
MFI
8000
13500
Cell count
NLRP3
WT MG-63
WT MG-63+S. aureus
CASP1−/− MG-63
CASP1−/− MG-63
+S. aureus
CASP1−/−MG-63
MFI
7900
14000
Cell count
NLRP3
CASP1−/− MG-63
CASP1−/− MG-63+S. aureus
C
WT MG-63
CASP1−/−MG-63
6 h
WT MG-63+S. aureus
CASP1−/−MG-63+S. aureus
D
IL-1β pg/ml
WT MG-63
WT MG-63 +LPS+ATP
CASP1−/−MG-63
CASP1−/−MG-63 +LPS+ATP
E
IL-1β pg/ml
WT MG-63
WT MG-63 +S. aureus
CASP1−/−MG-63
CASP1−/−MG-63 +S. aureus

Figure 4:

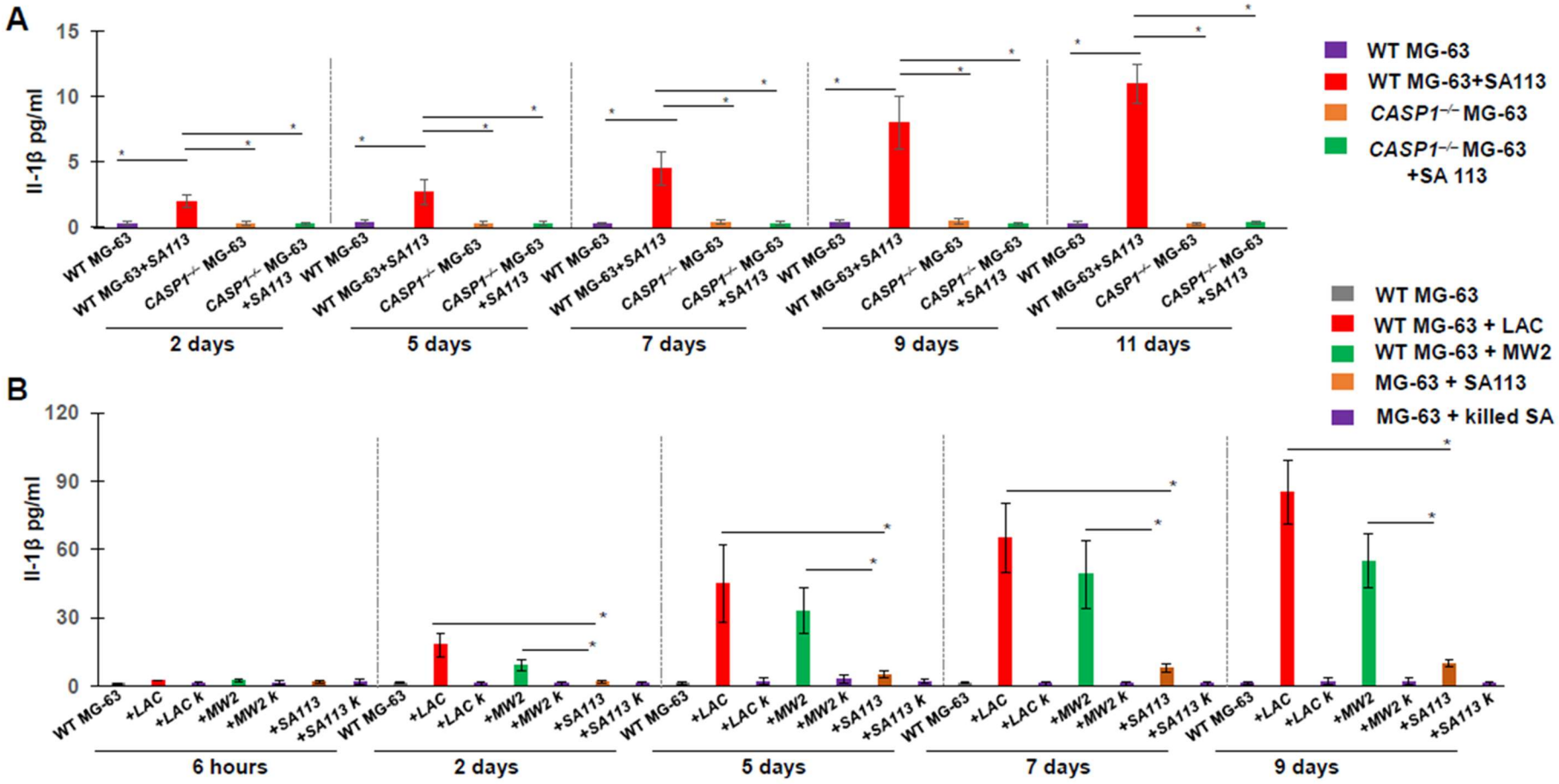

A
Il-1β pg/ml
15
10
5
0
WT MG-63
WT MG-63+SA113
CASP1-/- MG-63
CASP1-/- MG-63 +SA113
2 days
5 days
7 days
9 days
11 days
WT MG-63
WT MG-63+SA113
CASP1-/- MG-63
CASP1-/- MG-63 +SA113
B
Il-1β pg/ml
120
90
60
30
0
WT MG-63
+LAC
+LAC k
+MW2
+MW2 k
+SA113
+SA113 k
6 hours
2 days
5 days
7 days
9 days
WT MG-63
WT MG-63 + LAC
WT MG-63 + MW2
MG-63 + SA113
MG-63 + killed SA

Figure 5:

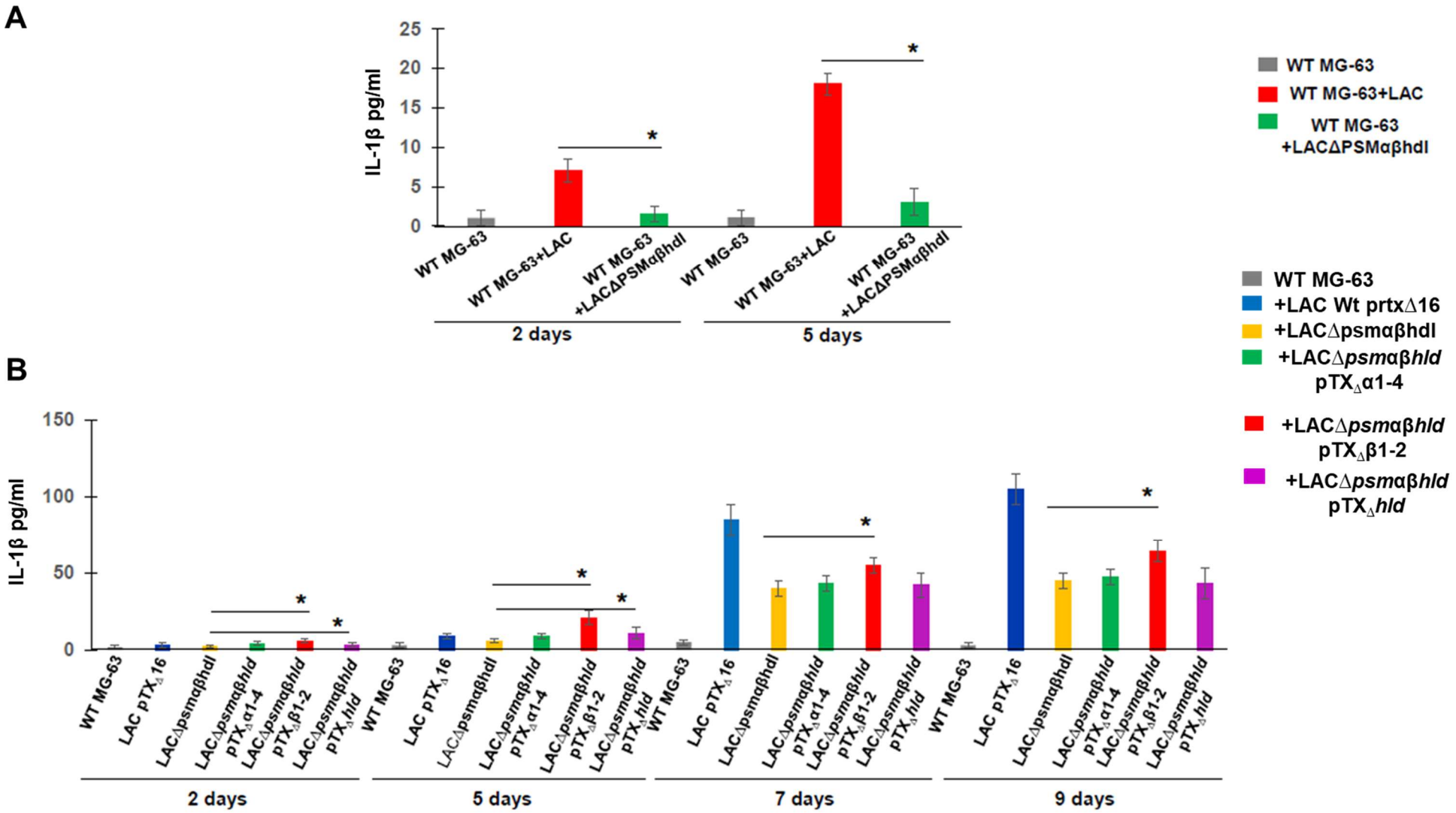
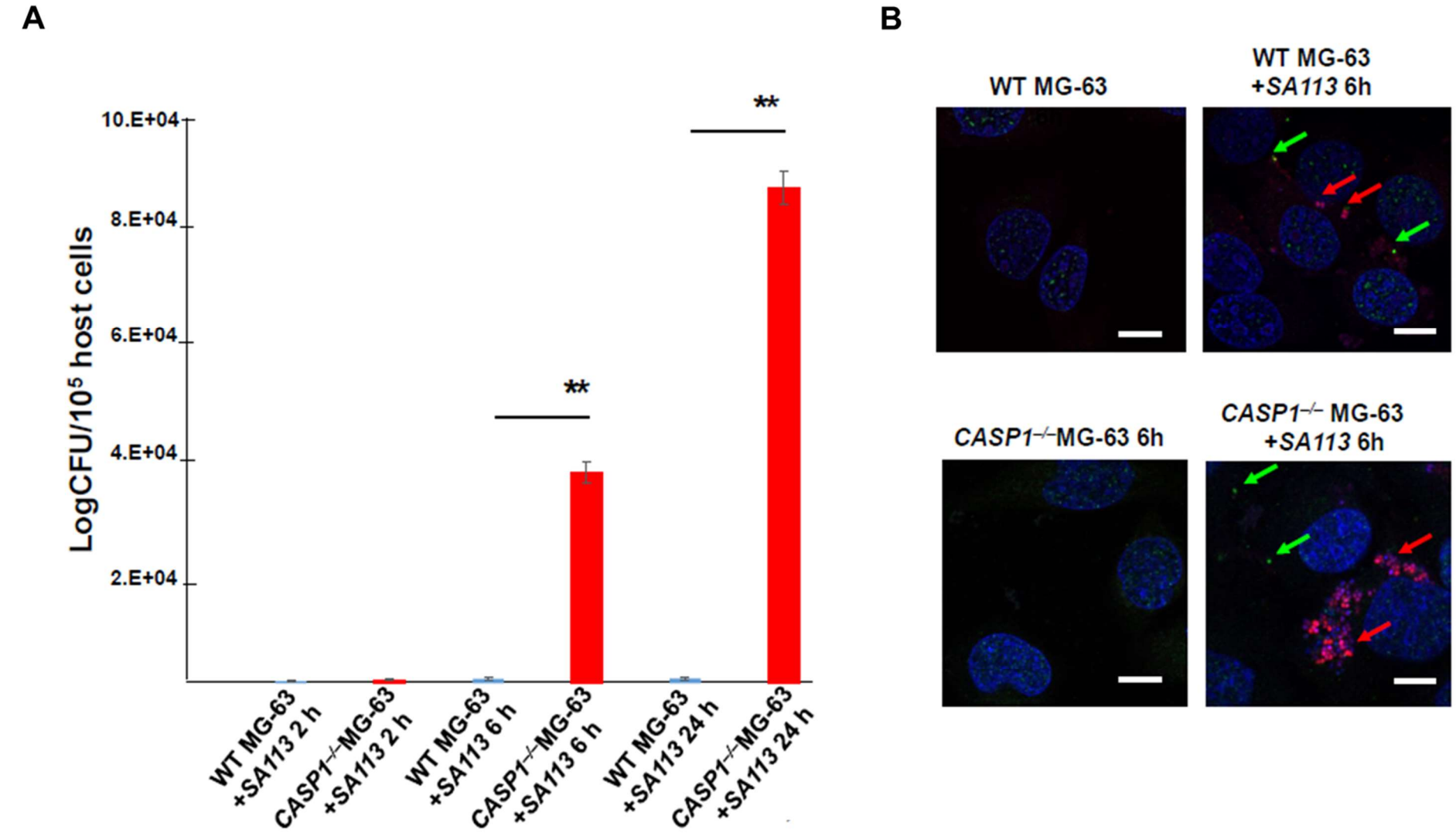

A
IL-1β pg/ml
25
20
15
10
5
0
*
*
WT MG-63
WT MG-63+LAC
WT MG-63 +LACΔPSMαβhdl
WT MG-63
WT MG-63+LAC
WT MG-63 +LACΔPSMαβhdl
2 days
5 days
WT MG-63
WT MG-63+LAC
WT MG-63 +LACΔPSMαβhdl
B
IL-1β pg/ml
150
100
50
0
WT MG-63
+LAC Wt prtxΔ16
+LACΔpsmαβhdl
+LACΔpsmαβhld pTXΔα1-4
+LACΔpsmαβhld pTXΔβ1-2
+LACΔpsmαβhld pTXΔhld
WT MG-63
LAC pTXΔ16
LACΔpsmαβhdl
LACΔpsmαβhdl pTXΔα1-4
LACΔpsmαβhdl pTXΔβ1-2
LACΔpsmαβhdl pTXΔhld
2 days
5 days
7 days
9 days

Figure 6:


A
B
LogCFU/10⁵ host cells
10.E+04
8.E+04
6.E+04
4.E+04
2.E+04
**
**
WT MG-63 +SA113 2 h
CASP1⁻/⁻MG-63 +SA113 2 h
WT MG-63 +SA113 6 h
CASP1⁻/⁻MG-63 +SA113 6 h
WT MG-63 +SA113 24 h
CASP1⁻/⁻MG-63 +SA113 24 h
WT MG-63
WT MG-63 +SA113 6h
CASP1⁻/⁻MG-63 6h
CASP1⁻/⁻ MG-63 +SA113 6h

Chapter 5. Results and general discussion

Crucial difficulties to fight *S. aureus* infections links to the capacity of bacterium to cause chronic infections where it can survive in an intracellular niche and can avoid detection by either the innate or acquired immune system. An understanding of the impact of internalized *S. aureus* on the alteration of host defense events will indicate new pathways for the development of antibacterial treatment.

The mechanisms of activation of inflammasome and secretion of IL-1β by human osteoblastic cells and the ability of these non-phagocytic cells to induce the activation of caspase-1 is essential for the understanding of the mechanisms of chronic infections caused by *S. aureus*.

Caspases consist of a family of proteases classically divided into two groups according to their function: inflammatory or apoptotic (CHANG; YANG, 2000; CHEN; KANG; FU, 2018; DENES; LOPEZ-CASTEJON; BROUGH, 2012). Caspases are produced in their inactive form and activated through inflammasome (DENES; LOPEZ-CASTEJON; BROUGH, 2012; LATZ, 2010). Activation of caspase-1 has been associated with the response of cells of the phagocytic system such as (monocytes, dendritic cells, and macrophages) against diff erent bacterial pathogens (BROZ; MONACK, 2011; KUBICA et al., 2008; LAMKANFI, 2011). This process occurs through the release of several antigens (PAMPs) into the intracellular medium that binds to cytosolic standard recognition receptors culminating in the activation of caspase-1 that leads to cleavage and activation of pro-inflammatory cytokines IL-1β and IL-18 (SCHRODER; TSCHOPP, 2010).

We initially evaluated whether *S. aureus* was able to activate signaling pathways that culminated in the endogenous activation of caspase-1 in human osteoblastic (MG-63) cells (non-professional phagocytes). Classically, the detection of mature subunits of caspase-1 (p20 and/or p10) extracellularly serve as markers for the activation of inflammasome *in vitro* (SHAMAA et al., 2015). Thus, based on observations provide by Shamaa and collaborators (2015) and in others studies (MARTINON et al., 2006; MARTINON; BURNS; TSCHOPP, 2002; SCHRODER; TSCHOPP, 2010), reporting that mature caspase-1 is released into the supernatant after stimulation through signals such as PAMPs or DAMPs, we used the solution of trichloroacetic acid (TCA) 100 % to precipitate proteins from the supernatant since it is well reported that TCA is a very effective protein precipitating agent (NOVÁK; HAVLÍČEK, 2013) we investigated the activation of caspase-1.

The evaluation of the activation of the inflammasome was made based on the activation of caspase-1 subunit p/10 and measured by western blotting. Cells were exposed to *S. aureus* strains at MOI 50:1. A maximal MOI value of 50:1 was selected to prevent the induction of a host cell death. Bacterial concentrations were estimated spectrophotometrically and were confirmed by determination of colony-forming unit (CFU). CFU was determined after overnight incubation as described previously (ALEKSEEVA et al., 2013). Our results demonstrated that MG-63 cells in culture medium with Fetal Calf Serum (FCS) infected by *S. aureus* strains USA 300 and MW2 (USA 400), as well as with the combination of LPS and nigericin, which are known activators of inflammasome (LATZ; XIAO; STUTZ, 2013a; MAN; KANNEGANTI, 2015) were able to activate caspase-1.

Interestingly, as observed in **fig. 11**, the band of 10-kDa was also identified in the negative control (MG-63-without activators). Therefore, in the first instance, we consider that the band present in the negative control was some protein from the FCS. In fact, the use of the complete medium (FCS) may lead to the binding of the caspase-1 to albumin and/or other serum components causing the effective concentration of caspase-1 in western blotting analysis to be reduced. Besides, cells infected with *S. aureus* strains were not well labeled with Anti-caspase-1 (Polyclonal Anti-human caspase-1 antibody, Acris-Origene, dilution of 1:250) used to recognize the p10 subunit of active caspase-1, showing a non-specificity interaction. Based on this observation, detection criteria were modified, such as: use of serum-free medium to avoid the cross-reaction of our antibodies with FCS proteins and the use of another primary monoclonal antibody to the caspase-1 subunit p/20 (Anti-human caspase-1 antibody, mAb p/20, dilution 1:1000), which has high affinity for active 20-kDa caspase-1.

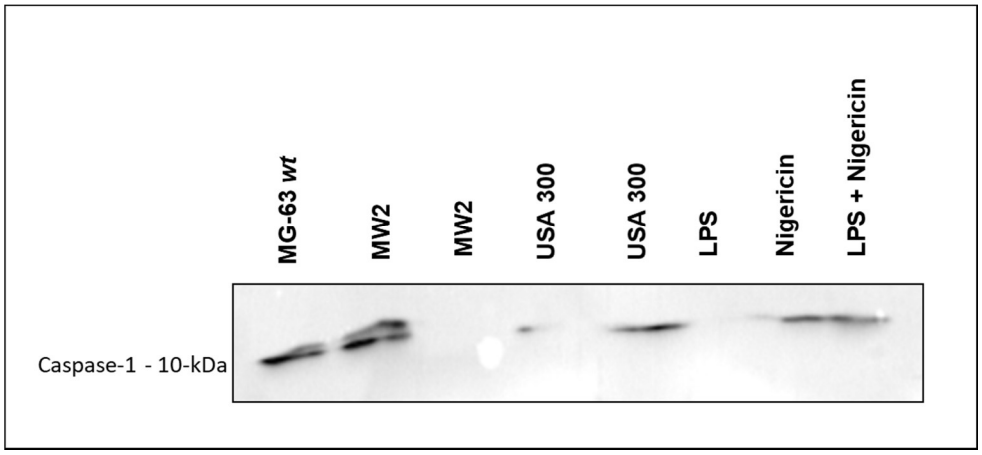


Figure 11: Detection of active caspase-1 in MG-63 cell supernatants by Western blot.

The supernatant proteins were precipitated with trichloroacetic acid (TCA) 100%. Polyclonal Anti-human caspase-1 antibody, Acris-Origene, dilution of 1:250.

In addition, human monocytic cell line (ThP1) (BOSSHART; HEINZELMANN, 2016), extensively described as a cellular model able to activate caspase-1 and produce large quantities of IL-1β notably, after differentiation by phorbol 12-myristate 13-acetate (PMA) otherwise known as 12-O-Tetradecanoylphorbol-13-acetate (TPA) was investigated in our newest protocol for identification of mature caspase-1 by Western blot (AKITA et al., 1997; FRANCHI et al., 2009; GAIDT et al., 2016; KIM et al., 2016; LI; GUNTER; FUKAGAWA, 2012; SCHUMANN et al., 1998; SHAMAA et al., 2015; STARR et al., 2018). This differentiation has been described for several years in the literature and is accompanied by a change in the morphology and cell adhesion (TSUCHIYA et al., 1982).

Initially, different protocols were used for sample preparation and active caspase-1 detection (**Table 3**) tested with different incubation times and using inducers of caspase-1 activation such as used anteriorly (LPS, Nigericin) and ATP (LOPEZ-CASTEJON; BROUGH, 2011; PICCINI et al., 2008) as positive control and the activation of caspase-1 was measured by western blotting. Surprisingly, we observed that in contrast to (KAHLENBERG; DUBYAK, 2004; LALIBERTE; EGGLER; GABEL, 1999; NIU et al., 2017; SHAMAA et al., 2015) literature findings, caspase-1 activation did not occur in response to the stimuli used. Indeed, studies reported discrepancies in the expression of caspase-1 in human monocytes (ThP1) ranging from different activators to different activation times, as well as conflicting evidence regarding the expression of active caspase-1 in this cell line (BAKER et al., 2015). These results suggest a degree of variability among these ThP1 cells differentiated with (PMA) grown in separate facilities.

It is well characterized in the literature that inactive pro-caspase-1 is converted into an active enzyme via dimerization, followed by an autocatalytic reaction that generates an active molecule composed of two large and two small subunits. As well as, that the autocatalysis of pro-caspase-1 to active caspase-1 is tightly controlled by the caspase-1 inflammasome (BROZ et al., 2012; LAMKANFI, 2011; RATHINAM; VANAJA; FITZGERALD, 2012; SCHRODER; TSCHOPP, 2010). To evaluate the activation of the inflammasome by activation of caspase-1, we performed assays to detect pro-caspase-1 in the cytosol of the ThP1 differentiated and MG-63 cells. In **fig. 12**, the MG-63 cells and PMA-differentiated ThP1 cells with the combination of LPS and nigericin demonstrated the presence of 45-kDa pro-caspase-1 in the cytosol by Western blot.

Stimuli	Time	Method to purify proteins	Antibiody
LPS - 1 µg/ml	3 h	trichloroacetic acid (TCA) 100%	Polyclonal Anti-human caspase-1 antibody, Acris-Origene, p/10, dilution 1:250.
Nigericin 5 mg/ml	1 h		
LPS - 1 µg/ml	3 h	trichloroacetic acid (TCA) 100%	Monoclonal antibody (Anti-human caspase-1 antibody, mAb p/20, dilution 1:1000)
Nigericin 5 mg/ml	1 h		
	3h		
	6h		
LPS - 1 µg/ml	3 h	Chloroform/Methanol Precipitation	Monoclonal antibody (Anti-human caspase-1 antibody, mAb p/20, dilution 1:1000)
ATP 5mM	1h		
	6h		
	16h		
LPS - 1 µg/ml	3 h	Two-dimensional electrophoresis (2DE)	Monoclonal antibody (Anti-human caspase-1 antibody, mAb p/20, dilution 1:1000)
ATP 5mM	1h		
	6h		
	16h		
LPS - 1 µg/ml	30 min	Culture supernatants plus cell lysates (Sup + CL)	Monoclonal antibody (Anti-human caspase-1 antibody, mAb p/20, dilution 1:1000)
ATP 5mM	15 min		

Table 3: Different protocols used to detect active caspase-1 in ThP1 cells differentiated with (PMA) by western blotting.

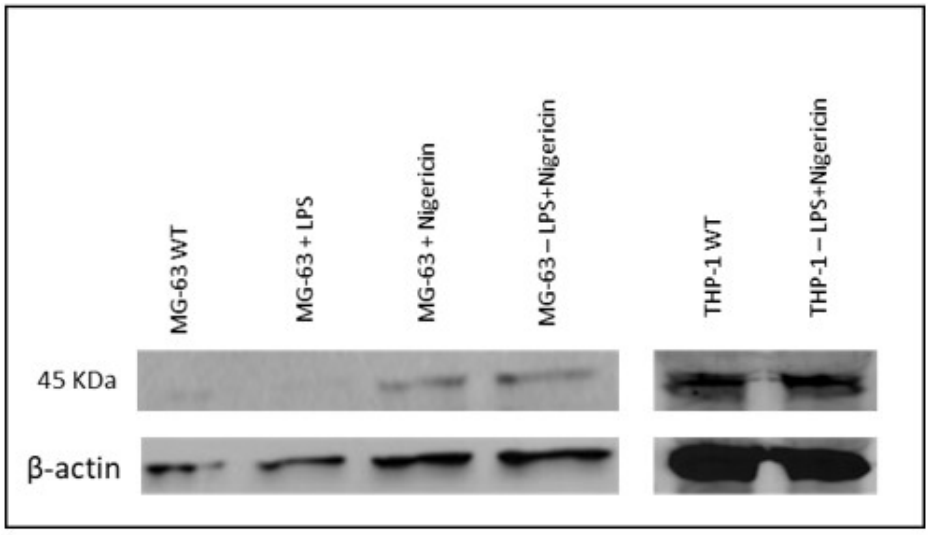


Figure 12: Detection of pro-caspase-1 in lysates of MG-63 and ThP1 cells by western blotting.

Negative controls: WT MG-63 and PMA-differentiated ThP1 cells and positive controls: 1µg/ml of LPS and 5 mg/ml of nigericin were used. Pro-caspase-1 (45-kDa) is present in the cytosol as an inactive zymogen.

5.1. Caspase-1 activation and IL-1β release triggered by inflammasome activators LPS and ATP in human osteoblasts and PMA-treated ThP1 cells

Several mechanisms are associated with the activation of inflammasome and secretion of IL-1β. Previous studies have extensively demonstrated the importance of caspase-1 cleavage for its activation (MARTINON; BURNS; TSCHOPP, 2002). Caspase-1 is present in the cytoplasm of phagocytic cells as a 45-kDa inactive zymogen, which needs to be cleaved to acquire its biological function (BROZ et al., 2012; RATHINAM; VANAJA; FITZGERALD, 2012; VANAJA; RATHINAM; FITZGERALD, 2015). It is known that activation of some inflammasomes, such as NLRP3, is characterized by two signals: the first signal is an exogenous derived microbial (PAMPs), which triggers the production of cytokine precursors e.g., pro-IL-1β; and a second signal, represented by the interaction of DAMPs with their PRRs. There is accumulated evidence indicating the relevance of this second sign of activation, particularly extracellular ATP in the regulation of the NLRP3 inflammasome through purinergic receptors (P2X), which are important mediators of inflammation and apoptosis (GOMBAULT; BARON; COUILLIN, 2013; LOPEZ-CASTEJON; BROUGH, 2011; MCCALL et al., 2008; PICCINI et al., 2008; SAVIO et al., 2018).

Therefore, we investigate whether activation caspase-1 and secretion of IL-1β both occur in MG-63 cells, non-professional phagocytes and professional phagocytes, human monocytes. For this purpose, human osteoblastic cells (MG-63) and ThP1 cells were used. Differentiation of ThP1 monocytes into macrophages was obtained by the treatment with 200 nM phorbol 12-myristate 13-acetate (PMA). This differentiation is following by a change in morphology and cell adhesion, described in the literature for several years (TSUCHIYA et al., 1982). The cells were stimulated as follows: *i)* primates with 1 µg/ml LPS per 3 h for MG-63 cells and 30 min for ThP1 cells, as the first sign of inflammasome activation, and stimulated with 5 mM of ATP for 15 min as the second signal of activation of inflammasome and *ii)* stimulated with only 1 µg/ml of LPS. After different times post-infection (2h, 6h, 2 days, 5 days, 7 days and 9 days) cell supernatants were collected and the level of IL-1β was determined by commercial sandwich-ELISA (Invitrogen, France) and the activation of caspase-1 in MG-63 cells was measured by Western blot.

As shown in **fig. 13**, differentiation of ThP1 cells by PMA, induced IL-1β secretion significantly 24 h post-treatment, corroborating with the findings of Park et al. (2007), which demonstrate that PMA-differentiated cells are able to induce IL-1β production, showing that PMA plays an important role in the production of detectable levels of IL-1β (PARK et al., 2007). Besides, there was an elevation of this secretion when we primed the monocytes with LPS when we

compared the times of 4h and 24h after the treatment (**Fig. 13**). It is well established in the literature that stimulation with LPS activates the TLR4 pathway and induces inflammation (GUIJARRO-MUÑOZ et al., 2014; SOUZA et al., 2017; WU; CHEN; CHEN, 2009; ZHANG et al., 2019). However, there is controversy over the use of TLR ligands to activate the secretion of caspase-1 and IL-1β. It was suggested by Martinon and Tschopp (2004) that stimulation by LPS alone would be ineffective as an activator of the inflammasome and successful secretion of IL-1β (MARTINON; TSCHOPP, 2004). Our results showed that In PMA-differentiated ThP1 cells, LPS-treatment provides the "first signal" to induce a pro-inflammatory response that culminates with the secretion of IL-1β in the supernatants. Further, according to the literature, PMA treatment induces the characteristic expression of CD14, a TLR4 co-receptor, which suggests that, without PMA treatment, ThP1 cells are not able to respond to the LPS stimulus, and can not then induce the production of IL-1β, demonstrating that induction can occur through the LPS-dependent signaling pathway and thus TLR4/CD14 (KOHRO et al., 2004; SCHWENDE et al., 1996).

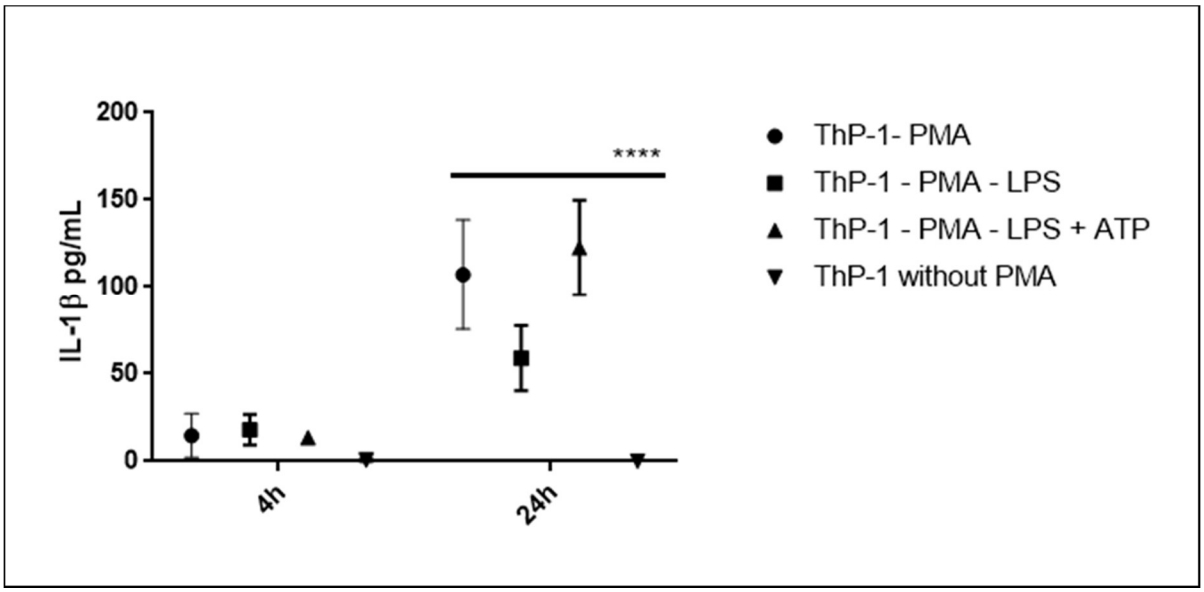


Figure 13: Influence of PMA on IL-1β production in culture supernatants of THP1 cells.

IL-1β production in ThP1 supernatants was measured between 4 and 24 hours after treatment with 200 nM PMA and stimulated with LPS (1 µg/ml) for 30 min and 5 mM ATP for 15 min. Tukey's Honestly Significant Difference test was applied for comparison of means between the groups. The values are expressed as mean ± standard deviation (±SD). Experiments are performed in triplicate and are representative of data obtained in three independent experiments. The asterisk represents a statistically significant difference: **** $p < 0.0001$ by two-way ANOVA.

The results showed that there was a potentiation of IL-1β secretion in ThP1 cells that received ATP as the second signal (**Fig. 13**). These data corroborate with findings from the literature that subsequent stimulation with inflammatory inducers such as ATP as a second signal for activation of the inflammasome induces caspase-1 activation and IL-1β maturation and secretion (HE; HARA; NÚÑEZ, 2016; JO et al., 2016). It is well known that ATP acts via the purinergic pathway to activate inflammasomes such as NLRP3 (MARIATHASAN et al., 2006). ATP also involves another intermediate signal, which is the generation of reactive oxygen species (ROS) (ABAIS et al., 2015; MARTÍNEZ-REYES; CUEZVA, 2014; ZHENG et al., 2014). It has been shown that both ATP and ROS have different activation pathways, however, both lead to caspase-1 activation and IL-1β production through the purinergic pathway (COUILLIN; GOMBAULT; BARON, 2013; CRUZ et al., 2007; LATZ; XIAO; STUTZ, 2013a). Indeed, ATP causes the activation of the NLRP3 inflammasome by the production of ROS, which will then act on another inflammasome and give rise to the production of IL-1β. ROS would also cause the production of endogenous ATP, which would be excreted via through pannexin hemichannels (mainly Panx1) or P2X7R and which, in turn, would act in the purinergic channels to activate the NLRP3 inflammasome (CHEN et al., 2017b; DAHL, 2015; DAHL; QIU; WANG, 2013; MUÑOZ-PLANILLO et al., 2013; SHOJI et al., 2014).

In order to monitor inflammasome activation in the human osteoblast-like MG-63 cells the detection of active caspase-1 by Western blot analysis was set up in samples containing cells and cell supernatants. As shown in **Fig. 14-A**, exposing MG-63 cells to LPS+ATP led to the appearance of 20 kDa band corresponding to the active caspase-1 p20 fragment. The p20 subunit of mature caspase-1 was investigated in culture supernatants plus cell lysates (Sup+CL) by Western blot. In fact, in the absence of the activation of the inflammasome, caspase-1 is detected in its precursor form, pro-caspase-1, in cell lysates. Upon activation, pro-caspase-1 is cleaved proteolytically in its p20 and p10 active forms which are detectable in cell lysates early in the activation of the inflammasome, and then most of these active forms are secreted and detected in the supernatant (BOUCHER et al., 2018; BROZ; MONACK, 2011; MARTINON et al., 2006; MARTINON; BURNS; TSCHOPP, 2002; TSUCHIYA et al., 2019; WANG et al., 2017). The activation of caspase-1 was associated with the production of IL-1β, as the exposure of cells to LPS+ATP resulted in the production of mature IL-1β (**Fig.14-B**). These results suggest that MG-63 cells form functional inflammasomes and that their activation can be estimated by IL-1β release.

To compare the ability of inflammasome activation by professional versus non-professional phagocytes, PMA-stimulated ThP1 cells differentiated into macrophagesor MG-63 osteoblast-

like cells were exposed to inflammasome activators LPS+ATP and the level of IL-1β production was estimated 2 h and 6 h after the beginning of the treatment. PMA-treated control ThP1 cells produce ~ 7 pg/ml of IL-1β 2 h at the beginning of the treatment. Exposure of PMA-differentiated ThP1 cells to LPS+ATP resulted in a significant increase to ~ 30 pg/ml 2 h post treatment. After 6 h PMA-treated control ThP1 cells produce ~ 10 pg/ml of Il-1β, while the level of IL-1β produced by PMA-treated ThP1 cells after an exposure to LPS+ATP was ~ 650 pg/ml. Supernatants of ThP-1+LPS+ATP (6 h) were diluted 1:5, the result presented in **Fig.14-C** is multiplied by the dilution factor. In contrast, IL-1β was not detected 2 h post-treatment, while only 4 pg/ml of IL-1β was detected in MG-63 cells 6 h after the exposure to LPS+ATP.

Thus, we demonstrated that human osteoblast-like MG-63 cells release a soluble IL-1β starting from 6 h post-treatment with inflammasomes activator LPS+ATP, matching the appearance of active caspase-1 20-kDa subunit (**Fig. 14**) that suggests inflammasomes activation in MG-63 cells. The production of IL-1β by MG-63 cells started later and IL-1β level was much lower than IL-1β production by ThP1 cells whose level of IL-1β production corresponds to the findings of other researchers (GRAHAMES et al., 1999). The discrepancy between levels of the soluble IL-1β released by MG-63 and ThP1 is likely related to the different origin of both cells' types: osteoblast-like MG-63 cells and PMA-treated ThP1 differentiated into macrophages (SPANO; BARNI; SCIOLA, 2013).

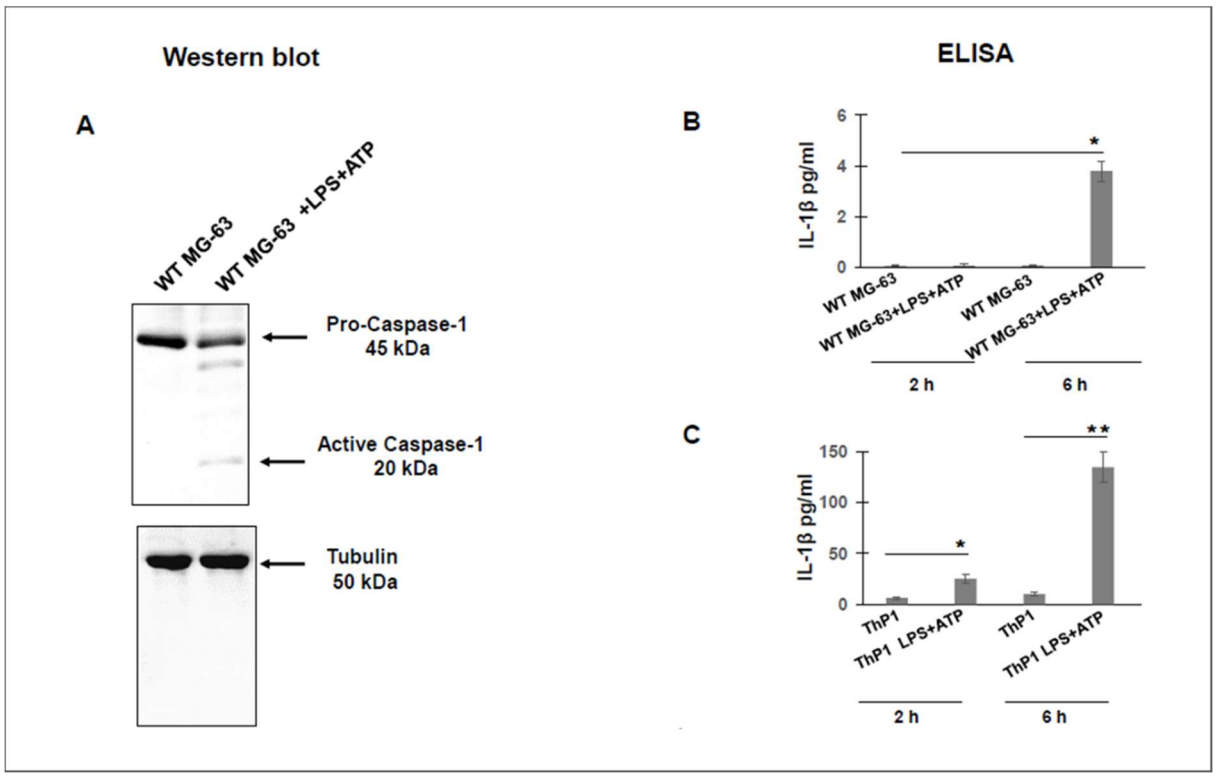


Figure 14: Caspase-1 activation and IL-1β release triggered in human osteoblasts.

A. MG-63 cells were incubated in 12-well plates. Afterwards, cells were primed with LPS (1 µg/ml) and stimulated with ATP (5 mM). Six hours after the beginning of the treatment cells together with cell-culture supernatants were diluted in RIPA lysis buffer. Protein samples were prepared in 5 x SDS-PAGE sample loading buffer. Detection of 45-kDa pro-caspase-1 in MG-63 cells by Western blot analysis was performed using anti-caspase-1 antibody (AdipoGen) as described in Material and Methods. The protein load was verified with anti-tubulin antibody. Three independent assays were performed.
B, C. MG-63 or ThP1 cells were incubated in 12-well plates. Afterwards, cells were primed for 2 h with LPS (1 µg/ml) and stimulated with ATP (5 mM) for 15 min. ThP1 were treated with PMA before LPS+ATP treatment as described above. Two or six hours after the beginning of the treatment cell supernatants were collected, centrifuged, and the level of IL-1β was determined by a commercial sandwich-ELISA (Thermofischer Life Technologies) as described in Material and Methods. Supernatants of ThP-1+LPS+ATP were diluted 1:5. The values are presented as concentrations in pg/ml. Three independent assays were performed. The differences among the groups were assessed by analysis of variance (ANOVA). Comparisons with P-values < 0.05 (*) were considered to be significant. *, $P < 0.05$; **, $P < 0.01$. Tukey's Honestly Significant Difference test was applied for comparison of means between the groups. The values are expressed as means ± standard deviation (±SD).

5.2. Deletion of caspase-1 gene in human osteoblasts MG-63 using the CRISPR/Cas9 gene editing system

NLRP3 is one of the most studied and best-characterized inflammasomes and has been associated with several diseases and conditions characterized by chronic inflammation, including gout, cancer, type 2 diabetes, and rheumatoid arthritis, as well as periodontal diseases, among others (SCHRODER; TSCHOPP, 2010). Thus, in the first part of this study, we were interested to set up an experimental model for the investigation of the involvement of the NLRP3 inflammasome in the response of *S. aureus*-infected MG-63 cells.

It has been shown that stimulation is required for IL-1β transcription and protein expression by MG-63 cells (KOH et al., 1997). Since MG-63 cells are usually used as a model to study bone and joint infections (BJI) (DEPLANCHE et al., 2019), we used MG-63 cells for CRISPR/Cas9-mediated caspase-1 knockout to study the involvement of inflammasomes in *S. aureus* infection of osteoblasts cells. Indeed, the study of the response of WT MG-63 *vs* CAS1$^{-/-}$MG-63 human cell line, non-professional phagocytes, allowed us to estimate the role of inflammasomes and its component caspase-1 in staphylococcal infection of osteoblasts.

To develop this model, we aimed to achieve the generation of a cell line deficient for the caspase-1 gene that will help to investigate the involvement of the inflammasome activation

during infection of MG-63 cells. The selection of caspase-1 as a potential candidate for the gene editing is linked to the pivotal role of this protease in the processing and maturation of the pro-inflammatory cytokine IL-1β by canonical inflammasome pathway.

The caspase-1 gene in MG-63 cells was deleted using CRISPR/Cas9 technology (MALI et al., 2013). The knockout was achieved via lentiviral infection of MG-63 cells. Lentiviral productions have been performed as recommended (http://tronolab.epfl.ch) and previously described (GILOT et al., 2017), more detail in the main article materials and methods session. There are 6 isoforms of caspase-1 encoded by the CASP1 gene, located in chromosome 11. Caspase-1 *alpha* and *beta* are the main transcripts in human cells. To generate a caspase-1 knockout in MG-63 cells we used a previously validated sgRNA-ATTGACTCCGTTATTCCGAA (SCHMID-BURGK et al., 2016). Based on sequences analysis, the sgRNA is predicted to target four caspase-1 isoforms including *alpha* and *beta*.

Four major groups of DNA bind ing proteins used in gene editing are known: meganucleases from microbial mobile genetic elements, nucleases based on eukaryotic transcription factors such as zinc fingers (ZFN), transcription activator-like effectors (TALENs), from the *Xanthomonas* bacterium and the Cas9 endonuclease of the adaptive immune system of some bacteria such as *S. pyogenes* (ADLI, 2018; BOCH, 2011; HSU; LANDER; ZHANG, 2014; JINEK et al., 2012; KIM; CHA; CHANDRASEGARAN, 1996; LEDFORD, 2015; RAMIREZ et al., 2008).

The great difference between Cas9 and the rest is the fact that recognition between RNA and DNA is based on the complementarity of bases. The Cas9 nuclease is directed by a small RNA sequence that recognizes the target DNA by complementarity making it accessible to the action of Cas9, which has innumerable advantages compared to the previous nucleases, e.g. in CRISPR/Cas9 technology, several target genetic sites can be targeted simultaneously using multiple RNA sequences guide (gRNA) and all redirecting of the Cas9 nuclease depends on two RNA sequences, which is much easier and simpler (ADLI, 2018; CONG et al., 2013; LIU et al., 2018; MAGGIO; GONÇALVES, 2015; MALI et al., 2013).

Thus, after obtaining the genetically modified lineage, using the CRISPR/Cas9 technique, we used the single cell clonal expansion method to select the monoclones, which did not express the caspase-1 gene. For this, first, we needed to select several unique clones to obtain a true knockout cell lineage. This process is important after engineering mutations, especially with something like CRISPR/Cas9 that can generate heterogeneous mutants. Thus, based on the literature showing the use of multiple approaches that were developed for the isolation of

monoclonal cell lines as Fluorescence-activated cell sorting (FACS), Magnetic-activated cell sorting (MACS), Laser capture microdissection (LCM), Manual cell picking and Microfluidic (CITRI et al., 2011; DATTA et al., 2015; GROSS et al., 2015; LECAULT et al., 2012; WELZEL; SEITZ; SCHUSTER, 2015). According to Hu and colleagues (2016), in the single cell clonal expansion method, the probability of obtaining a single cell in an aliquot is statistical in nature, and although limiting dilution provides the most versatile approach, the experimental procedure requires optimization to increase the chance of successful isolation of the single clone (GROSS et al., 2015; HU et al., 2016).

Therefore, after establishing the method for the isolation of the monoclones MG-63 cells were transduced and selected with 0.2 µg/ml of puromycin for 3 days. Individual cells from a stable pool of cells were then expanded for another 3 weeks under a selection of puromycine. Western blotting with anti-caspase-1 antibody was performed with cell lysates to detect the 45-kDa precursor form of caspase-1 (pro-caspase-1). And WT MG-63 cells expressing pro-caspase-1 were included as a control. Thus, clones exhibiting an absence of 45-kDa bands were selected as potential knockout cells for the caspase-1 gene. Our results showed that of the 142 monoclones selected by the single cell clones method (data not are shown), only three were selected as possible candidates for the Casp1 knockout gene (**Fig. 15**).

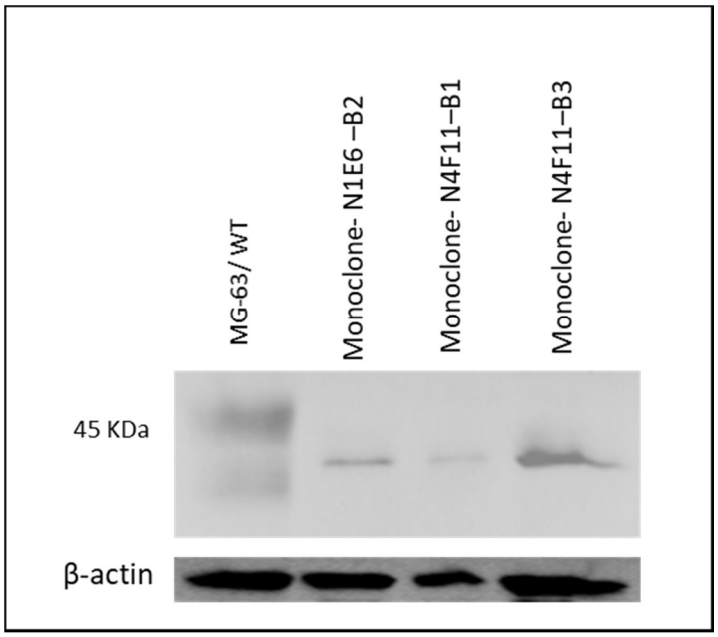


Figure 15: Detection of pro-caspase-1 in lysates of MG-63-CRISPR/cas9 cells by Western blot.

Three monoclones were then selected as possible candidates for CASP1 gene knockout: N13E6-B2, N4F11-B1 and N4F11-B3. Negative control: WT MG-63 cells. Monoclonal antibody anti-human caspase-1, mAb p/20 dilution 1:1000, Adipogene.

Interestingly, as shown in **fig. 15**, there was the expression of a weak band that could still correspond to pro-caspase-1 of 45-kDa in the cellular extract. We assume that in our experimental model the development of wild type cells occurred which could seriously confuse the data obtained. In fact, the presence of wild cells can overcome the growth of a mutant clone over time. We decided to perform an additional selection of monoclones using a higher concentration of puromycin, so the selection was made with 1µg/mL of puromycin for 3 days. Individual cells from a stable pool of cells were then expanded for another 3 weeks under a selection of puromycine. The detection of the 45-kDa band corresponding to pro-caspase-1 was performed by Western blotting of cell extracts with anti-caspase 1 antibody. Wild type MG-63 (WT MG-63) cells expressing pro-caspase-1 were included as a control. Our data demonstrated that of the total of 35 monoclones tested only 3 did not present the band of 45-kDa in cellular extracts corresponding to pro-caspase-1 (**Fig. 16** and **Fig. 17**). Thus, in order to ensure the generation of Cas1$^{-/-}$ MG-63 cells we performed a second cloning by limited dilution of these 3 monoclones. Monoclonal cell colonies were tested by western blotting (**Fig. 18**). As shown in **fig. 18**, our results demonstrated the obtaining of monoclones with the deletion of the 45-kDa band from the reference to pro-caspase-1 and, therefore, we selected to be subjected to DNA sequencing the monoclones: N2E11-G2 (G2), N2E11-B9 (B9) (**Fig. 18**) and N5G2-C3 (C3) (data not shown).

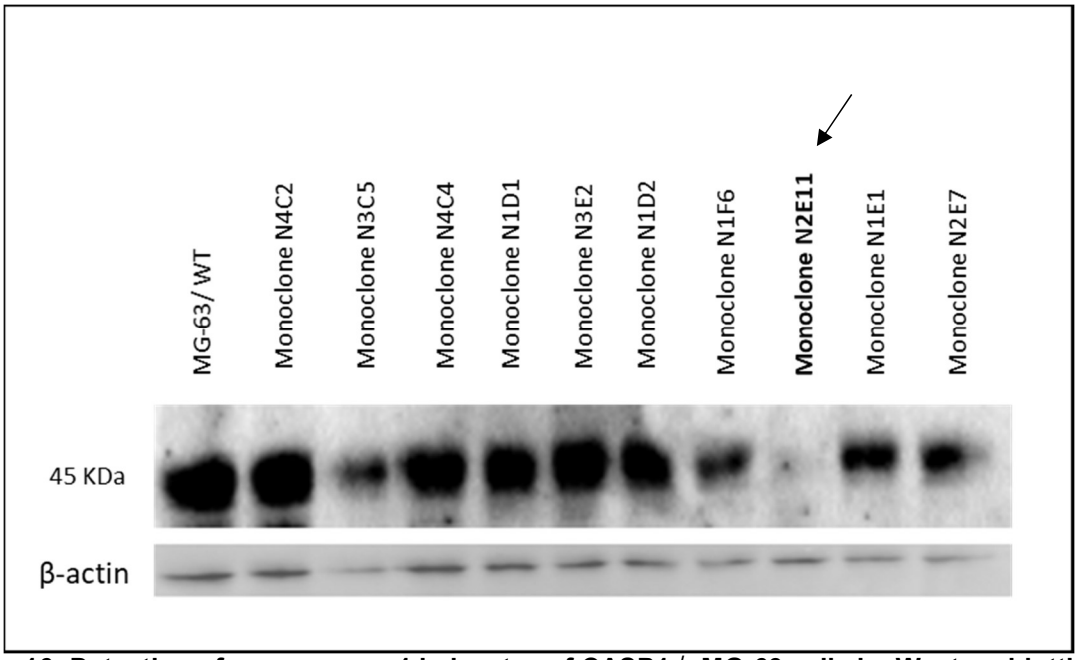


Figure 16: Detection of pro-caspase-1 in lysates of CASP1$^{-/-}$ MG-63 cells by Western blotting.

Negative control: WT MG-63 cells. Absence of the 45-kDa subunit band of the caspase-1 gene for the monoclone N2E11, in bold. Anti-human caspase-1 antibody, mAb p / 20 1: 1000, Adipogene.

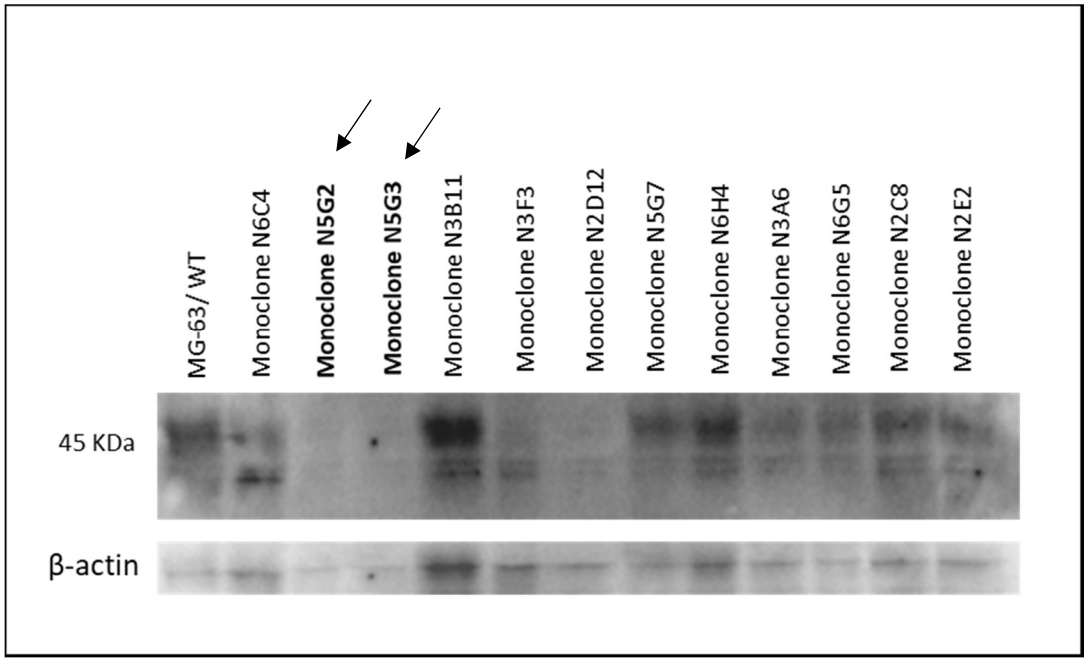


Figure 17: Detection of pro-caspase-1 in lysates of CASP1$^{-/-}$ MG-63 cells by Western blotting.

Negative control: WT MG-63 cells. Absence of the 45-kDa subunit band of the caspase-1 gene for the monoclone: N5G2 and N5G3, in bold. Anti-human caspase-1 antibody, mAb p / 20 1: 1000, Adipogene.

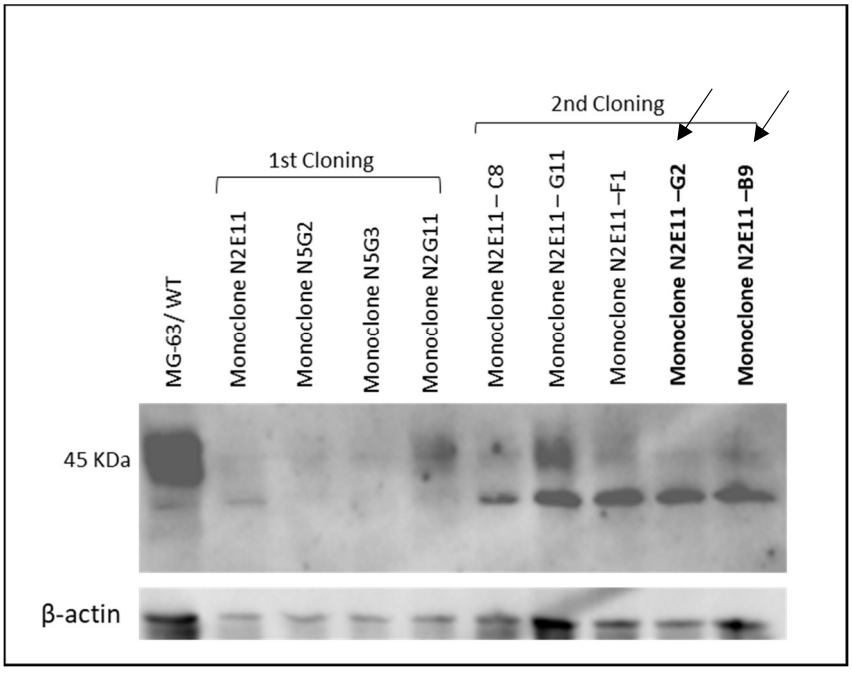


Figure 18: Detection of pro-caspase-1 in lysates of CASP1$^{-/-}$ MG-63 cells by Western blot.

Negative control: WT MG-63 cells. Selection of monoclones: N2E11-G2 (monoclone G2) and N2E11–B9 (monoclone B9), in bold, for sequencing. Anti-caspase-1 human antibody, mAb p/20 1:1000, Adipogene.

Thus, we evaluated the caspase-1 gene region of the selected monoclones G2, B9, and C3 (see Fig. 18), and WT MG-63 cells as a negative control by DNA sequencing analysis (Sanger sequencing method) (HEATHER; CHAIN, 2016). The monoclone G2 (CASP1$^{-/-}$ MG-63 cells) (**Fig. 19-B**), B9 and C3 (data not shown), which were identified as a result of limiting dilution of puromycin-treated cells, were tested by Western blotting for the lack of the 45-kDa band corresponding to pro-caspase-1. Finally, a deletion clone (CASP1$^{-/-}$ MG-63, G2 clone) showing CRISPR-induced genomic alteration (**Fig. 19-B**) was selected for further analysis. Western blot analysis of WT MG-63 *vs* CASP1$^{-/-}$ MG-63 cells confirmed the lack of pro-caspase-1 in CASP1$^{-/-}$ MG-63 (G2 clone) cells (**Fig. 20-B**).

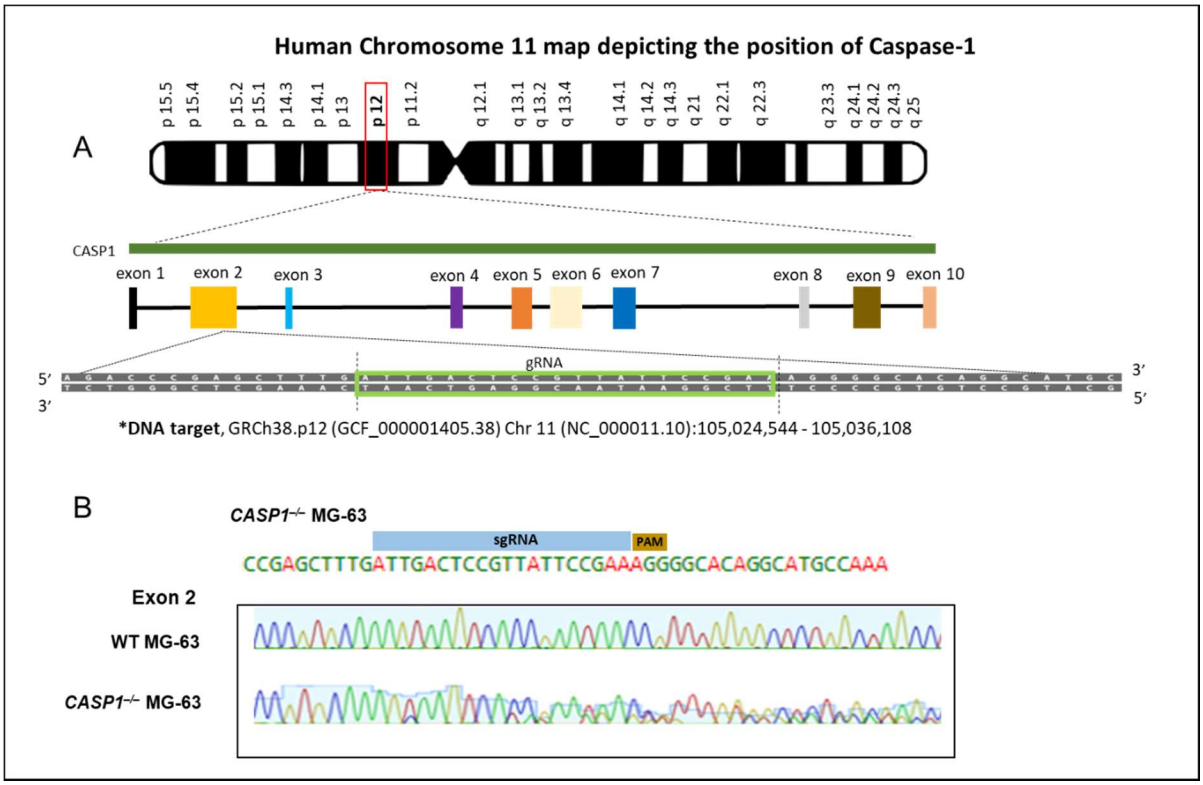


Figura 19: Deletion of caspase-1 gene in human osteoblasts MG-63 using the CRISPR/Cas9 gene editing system

A. Scheme showing the caspase-1 deletion targeting exon 2 of the CASP1 gene (Sequence ID NG 029124.2).

B. Trace file from Sanger sequencing of the targeted deletion in the exon 2 of the CASP1 gene (WT MG-63 and CASP1–/– MG-63 clone).

5.3. Inflammasome involvement in caspase-1 dependent IL-1β release by *S. aureus* infected osteoblasts

Activation of the NLRP3 inflammasome is formed with the junction of an NLR receptor, the adapter protein ASC (Apoptosis-associated speck-like protein containing a CARD) and pro-caspase-1 succeeding by caspase-1 activation that leads to the maturation of IL-1β and IL-18 (MARTINON; BURNS; TSCHOPP, 2002). Upon inflammasome activation, ASC is rapidly depleted from its steady-state homogeneous cellular distribution and self associates to form a single punctum inside the cell of about 1µm in diameter, called a “speck” detectable by immunofluorescence as a single perinuclear focus (FERNANDES-ALNEMRI et al., 2007; LATZ; XIAO; STUTZ, 2013a; LU et al., 2014; MASUMOTO et al., 1999; VOJTECH et al., 2012). The polymerized ASC recruits and dimerizes caspase-1 within the specks, allowing caspase-1 activation by autoproteolysis of the enzyme (FERNANDES-ALNEMRI et al., 2007). Recent studies have shown that ASC “speck” is a platform for caspase-1 activity (FRANKLIN; LATZ; SCHMIDT, 2018). Thus, it is necessary that the expression of all these upstream components occurs so that the subsequent activation of the inflammasome (MARTINON; BURNS; TSCHOPP, 2002).

Thus, to ensure that the deletion of CASP1 does not impair upstream events of inflammasome formation, the level of NLRP3 protein expression and the formation of ASC specks was analyzed in WT MG-63 and CASP1$^{-/-}$ MG-63 cells. NLRP3 protein expression was estimated by FACS. As shown in **Fig. 20-B**, equal amounts of NLRP3 was observed in WT and CASP1$^{-/-}$ MG-63 cells. The exposure of both types of cells to *S. aureus* SA113 resulted in similarly increased levels of fluorescence. Mean fluorescence intensity (MFI) increased from 8000 to 13500 in WT MG-63 cells after *S. aureus* infection, similarly to CASP1$^{-/-}$ MG-63 cells, which showed an MFI increase from 7900 to 14000 (**Fig. 20-B**).

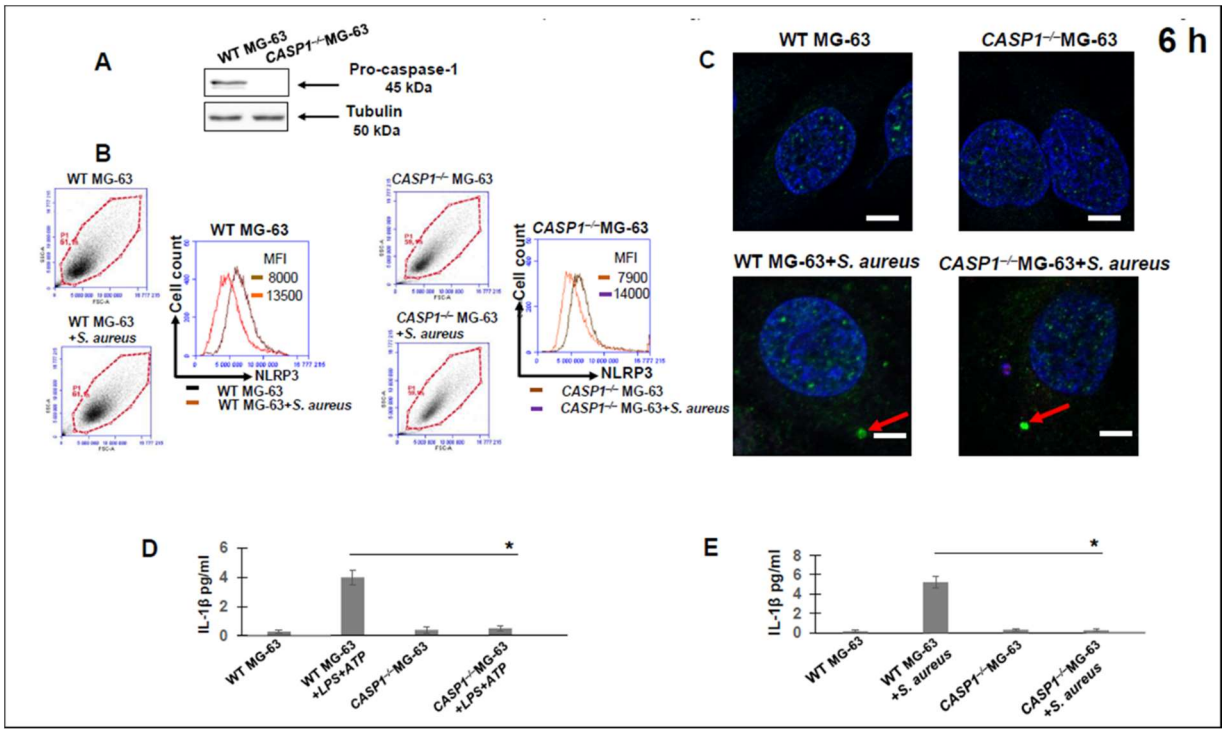


Figure 20: NLRP3 protein expression, speck-like aggregate formation and IL-1β secretion by WT MG-63 vs CASP1$^{-/-}$ MG-63 cells exposed to *S. aureus*.

A. WT MG-63 or *CASP1*$^{-/-}$ MG-63 cells were incubated in 12-well plates. Cells were then detached by trypsin treatment and cell pellets were obtained by centrifugation. Protein samples were prepared in 5 x SDS-PAGE sample loading buffer. Detection of 45-kDa pro-caspase-1 in WT MG-63 and *CASP1*$^{-/-}$ MG-63 cells by Western blot analysis using anti-caspase-1 antibody (AdipoGen) was performed as described in Material and Methods. Three independent assays were performed.

B. WT MG-63 or *CASP1*$^{-/-}$ MG-63 cells were exposed to *S. aureus* LAC (USA300) for 2 h. Following fixation and permeabilization of cells, NLRP3 expression at the protein level was determined by FACS using Alexa Fluor 488 anti-NLRP3 antibody. NLRP3 expression was analyzed with an Accuri C6 flow cytometer. Data were collected from 20,000 cells, and analyzed with CFlow software (Becton Dickinson). Cells are analyzed using FSC-A x SSC-A plot. The major density of events is captured by the gate. The events that represent debris, cell fragments and pyknotic cells are eliminated. Values shown on the right side of the graph refer to the respective mean fluorescence intensities (MFIs). Three independent assays were performed.

C. WT MG-63 or CASP1$^{-/-}$ MG-63 cells were exposed to a fluorescent derivative of *S. aureus* SA113, which carries plasmid-encoded mCherry (red fluorescence), at MOI 1:50 for 2 h followed by antibiotic treatment as described in Materials and Methods. Six hours post-infection cells were immunostained with rabbit anti PYCARD antibody (Coger France), followed by incubation with Alexa Fluor 488 labeled goat anti-rabbit antibody (Cell Signaling Ozyme) at a dilution of 1:50 for 2 h at room temperature (green staining, red arrow). Nuclear DNA was labeled with DAPI (blue staining). Samples were viewed with a Zeiss laser-scanning microscope equipped with a 63 × plan Apo-NA 1.4 immersion objective driven by Zen software. Scale bar: 1 µm.

D, E. WT MG-63 or CASP1$^{-/-}$ MG-63 cells were exposed to LPS+ATP (D) or *S. aureus* strain SA113 at MOI 1:50 for 2 h (E) followed by antibiotic treatment as described in Material and Methods. Six hours post-infection or 6 h after the beginning of LPS+ATP treatment cell supernatants were collected, centrifuged and the level of IL-1β was determined by a commercial sandwich-ELISA (by sandwich-ELISA (Thermofischer Life Technologies) as described in Materials and Methods. The values are presented as concentrations in pg/ml. Three independent assays were performed. The differences among the groups were assessed by analysis of variance (ANOVA). P-values < 0.05 were considered to be significant. Tukey's Honestly Significant Difference test was applied for comparison of means between the groups. The values are expressed as means ± standard deviation (±SD).

Taking into account that both types of cells contain NLRP3 (**Fig. 20-B**), while pro-caspase-1 is present only in WT MG-63 cells (**Fig. 3-A**) we next determined whether an exposure of both cell lines to *S. aureus* SA113 leads to the formation of ASC specks, since their formation does not require the presence of pro-caspase-1. Consequently, we analyzed immunofluorescence staining for the adaptor ASC in WT and CASP1$^{-/-}$MG-63 cells. As shown in Fig. 20-C, *S. aureus* infection triggers assembly of the ASC specks in the cytosols of WT as well as CASP1–/– MG-63 cells (green). These results corroborate with the findings of (GUEY et al., 2014), that shows that in WT cells (macrophages and dendritic cells) treated with lethal factor (LT), are able to formed ASC specks and also that in the absence of caspase-1, ASC specks are still formed (GUEY et al., 2014). Moreover, it has been shown that ASC specks can accumulate in the microenvironment of cells dying by pyroptosis. Interestingly, these aggregates of ASC specks can then be phagocytosed by surrounding immune cells and, by their "prion" activity, spread the inflammation by activating caspase-1 in these cells (FERNANDES-ALNEMRI et al., 2007; FRANKLIN; LATZ; SCHMIDT, 2018).

Previously it was reported that the transcripts of IL-1β and IL-18 were observed after an exposure of mice osteoblasts to *S. aureus*, in contrast, secretion of IL-1β and IL-18 was not detected despite the presence of active caspase-1 (MARRIOTT; HUGHES; BOST, 2002).

To understand whether *S. aureus* activates inflammasomes in human osteoblast-like MG-63 cells, we measured IL-1β production in WT and CASP1$^{-/-}$MG-63 cells. The presence of NLRP3 and the lack of pro-caspase-1 in CASP1$^{-/-}$MG-63 cells allows these cells to be used as a control for the estimation of NLRP3 inflammasome activity in MG-63 cells by the measurement of IL-1β, which is processed due to caspase-1. The assessment of IL-1β in WT and CASP1$^{-/-}$ MG-63 cells exposed to inflammasomes activators LPS+ATP (**Fig. 20-D**) as well as to *S. aureus* LAC (**Fig. 20-E**) revealed the lack of IL-1β production in CASP1$^{-/-}$MG-63 in contrast to WT MG-63 cells.

To clarify the possible role of human osteoblasts in the production of IL-1β, we analyzed the kinetics (from 2 to 11 days) of IL-1 β production by WT and CASP1$^{-/-}$MG-63 cells exposed to *S. aureus* SA113. As demonstrated in **Fig. 21-A**, the level of IL-1β increased from day 2 to 11. In contrast, IL-1β was not detected in supernatants from infected CASP1$^{-/-}$MG-63 cells during the tested period.

To verify whether IL-1β release by infected osteoblasts was strain-dependent, we analyzed the kinetics of IL-1 β release by MG-63 exposed to different *S. aureus* strains: LAC (USA300), MW2 (USA 400) and SA113. As shown in **Fig. 21-B**, all strains induce IL-1β release; however, MW2 and LAC induced significantly higher levels of IL-1β than SA113. IL-1β was not detected in the supernatants of MG-63 cells exposed to killed bacteria of any of the three strains, suggesting that factors associated with viable bacteria are involved in inflammasome activation.

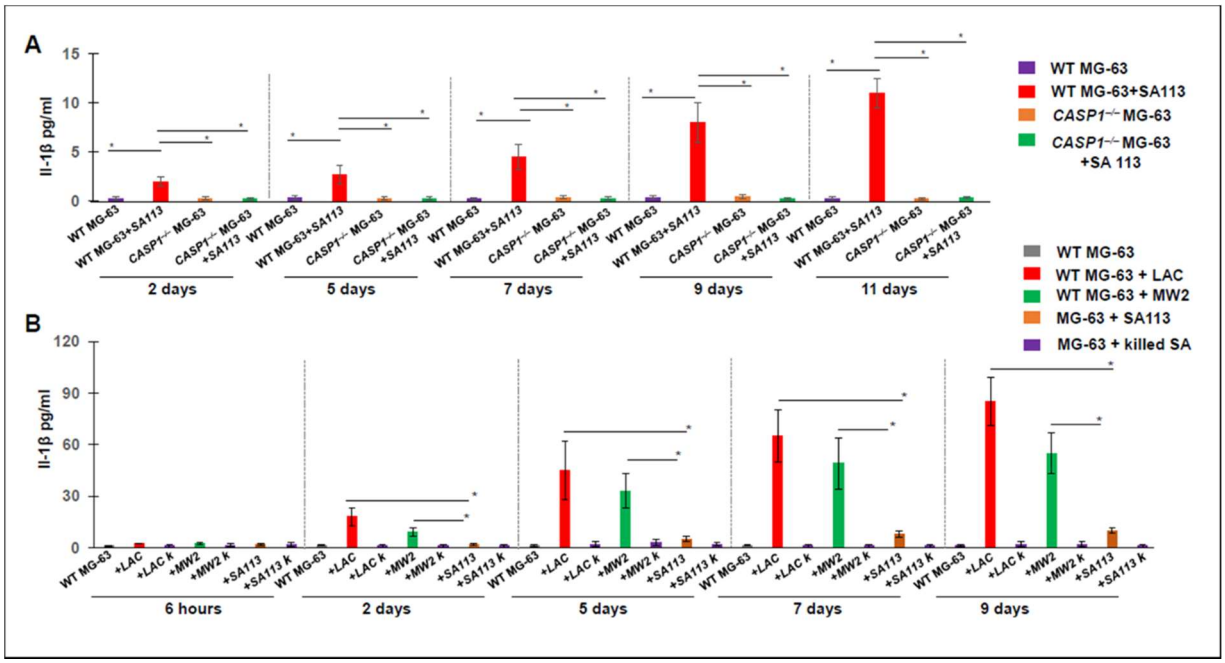


Figure 21: *S. aureus* strain-dependent release of IL-1β by infected MG-63 cells.

A. WT MG-63 or CASP1$^{-/-}$ MG-63 cells were grown in 6-well plates, then were exposed to *S. aureus* strain SA113 at MOI 1:50 for 2 h followed by antibiotic treatment as described in Materials and Methods. After various times post-infection (6 h, 2 days, 5 days, 7, days and 11 days) cell supernatants were collected, centrifuged and the level of IL-1β was determined by a sandwich-ELISA (Thermofischer Life Technologies) as described in Material and Methods. The values are presented as concentrations in pg/ml. Three independent assays were performed. The differences among the groups were assessed by analysis of variance (ANOVA). P-values < 0.05 (*) were considered to be significant. Tukey's

Honestly Significant Difference test was applied for comparison of means between the groups. The values are expressed as means ± standard deviation (±SD).

B. WT MG-63 cells were exposed to *S. aureus* strains LAC (USA300, red color), MW2 (USA 400, green color), or SA113 (yellow color) at MOI 1:50 for 2 h followed by antibiotic treatment as described in Material and Methods. After various times post-infection (6 h, 2 days, 5 days, 7, days and 11 days) cell supernatants were collected, centrifuged and the level of IL-1β was determined by a commercial sandwich-ELISA (Invitrogen, France) as described in Material and Methods. The values are presented as concentrations in pg/ml. Three independent assays were performed. The differences among the groups were assessed by analysis of variance (ANOVA). P-values < 0.05 (*) were considered to be significant. Tukey's Honestly Significant Difference test was applied for comparison of means between the groups. The values are expressed as means ± standard deviation (±SD).

Besides, the lack of production of IL-1β in the other clones proposed also was evaluated. For this, WT MG-63 cells, (CASP1$^{-/-}$ MG-63 clone B9) and (CASP1$^{-/-}$ MG-63 clone C3) were infected with *S. aureus* SA113 at MOI 50:1. Cultured cells were monitored during (9 days post-infection), and the supernatant was used to quantify IL-1β levels by ELISA. Our results show that there was no secretion of IL-1β in the (CASP1$^{-/-}$ MG-63 clone B9) and (CASP1$^{-/-}$ MG-63 clone C3), after the stimulation with *S. aureus* SA113, when compared to WT MG-63 infected with the strain SA113, that presented high levels of IL-1β (**Fig. 22**).

Thus, the equal NLRP3 protein expression together with the similar formation of ASC specks in both WT MG-63 and CASP1$^{-/-}$MG-63 cells, as well as the absence of active caspase-1 and IL-1β release following the stimulation with inflammasomes activators in CASP1$^{-/-}$MG-63 cells, validate caspase-1 knockout in osteoblastic cells. Therefore, we generated CASP1$^{-/-}$MG-63 cell line that is a powerful tool that allows the function of inflammasomes to be investigated. We result show that *S. aureus*-induced IL-1β release is strongly strain-dependent. Rising IL-1β production in *S. aureus*-infected MG-63 cells in contrast to its lack in infected CASP1$^{-/-}$MG-63 cells demonstrated caspase-1 dependent mechanisms of IL-1β production by human osteoblasts. Our data corroborate observations of others that demonstrated caspase-1 depending mechanism of IL-1β processing and its release by mice macrophages during infection (RAUPACH et al., 2006). Besides, the absence of IL-1β production by cells exposed to heat-killed bacteria indicates that factors associated with viable bacteria are involved in the activation of the NLRP3 inflammasome.

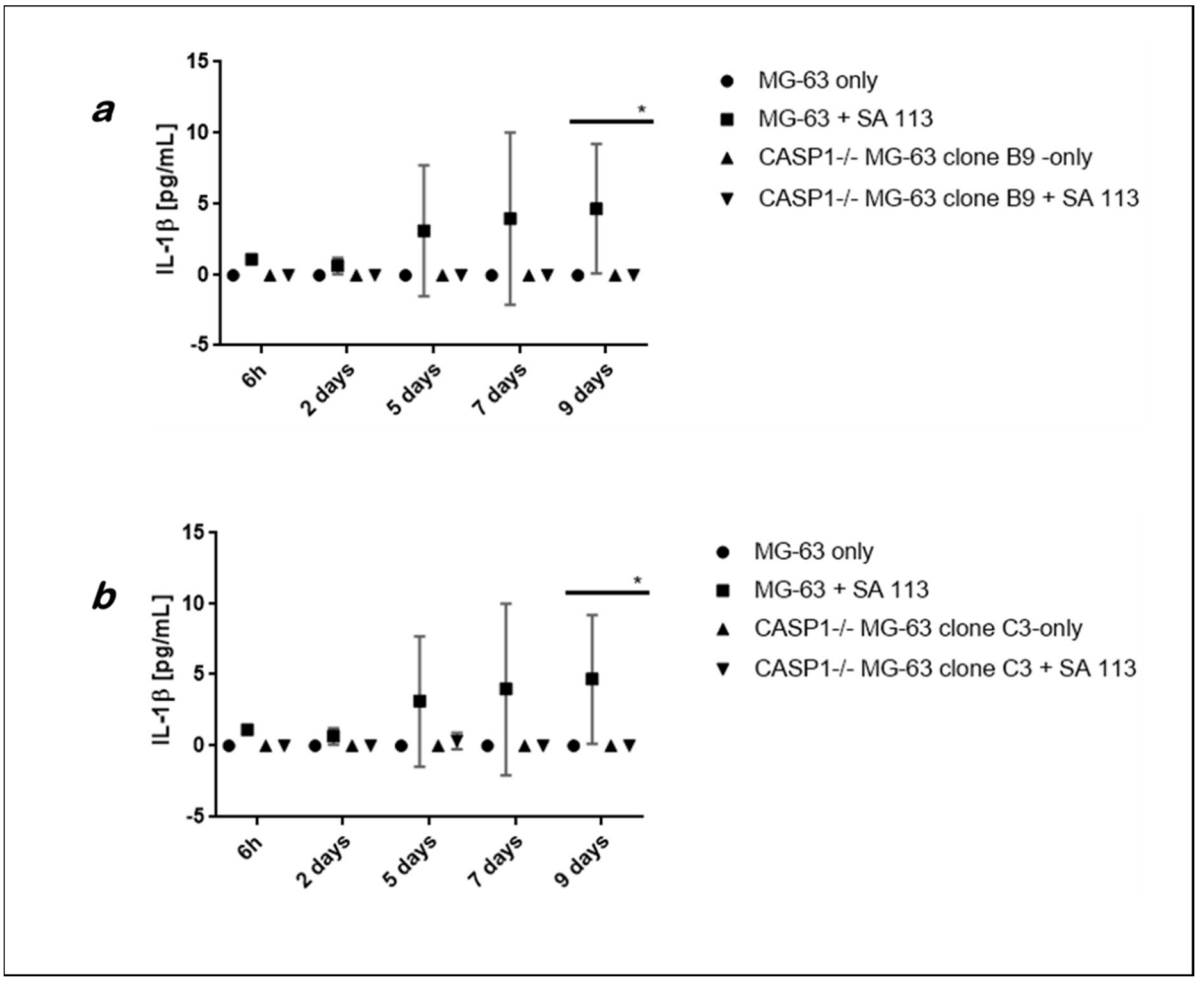


Figure 22: Caspase-1 is required for secretion of IL-1β in *S. aureus*-infected MG-63 cells.

WT MG-63 cells were infected or not with *S. aureus* SA113 at MOI 50:1 and compared with (CASP1$^{-/-}$ MG-63 clone B9) and (CASP1$^{-/-}$ MG-63 clone C3) infected or not with *S. aureus* SA113 at MOI 50: 1. IL-1β production was measured in the cell-free supernatant by ELISA. Data show monitoring for a period of 6 h to 9 days post-infection. Tukey's Honestly Significant Difference test was applied for comparison of means between the groups. The values are expressed as mean ± standard deviation (±SD). Experiments are performed in triplicate and are representative of data obtained in three independent experiments. Asterisk represent statistically significant difference: *p < 0.05 by two-way ANOVA.

5.4. Pivotal role of *S. aureus* PSM toxins in stimulation of IL-1β release by infected osteoblasts

Pathogens have developed strategies to avoid and subvert the defense functions of infected cells for extended periods (RATHINAM; FITZGERALD, 2016; RATHINAM; VANAJA; FITZGERALD, 2012). Certain bacteria such as *Yersinia pseudotuberculosis, Yersinia*

enterocolitica, *Salmonellae* species, *Pseudomonas aeruginosa,* among others, use strategies that manipulate inflammasome activation, in most cases, interfere in the production or recognition of bacterial ligands that trigger inflammasomes (BRODSKY et al., 2010).

The quorum-sensing system in *S. aureus* known as the accessory gene regulator (Agr) regulates the expression of many virulence factors. Agr regulates main *S. aureus* toxins, among which regulation of Phenol-soluble modulins (PSMs) reviewed (PESCHEL; OTTO, 2013) contain five α-peptides (δ-toxin and PSMα1-4) and two β-peptides (PSMβ1-2), which all share an amphipathic α-helical structure, thereby acting as biological detergents. Thus, PSMs are regarded as a new class of Staphylococcal leukocidins (KRETSCHMER et al., 2010; NAKAMURA et al., 2013; PESCHEL; OTTO, 2013; RICHARDSON et al., 2019). PSMs first attract innate immune cells like neutrophils, macrophages, and dendritic cells (DCs) by binding to the formyl peptide receptor 2 (FPR2) (RICHARDSON et al., 2019; SCHREINER et al., 2013; WANG et al., 2017). Was been reported that PSMs can activate inflammasome-like signaling and trigger IL-1β and IL-18 secretion (MELEHANI; DUNCAN, 2016). In addition, was demonstrated that PSMs stimulate the production of inflammatory cytokines by infected keratinocytes (SYED et al., 2015), thus it is essential that this role is unraveled in our experimental model.

Furthermore, our results on strain dependence of IL-1β stimulation in osteoblasts suggested an involvement of Agr, as the low levels of IL-1β in cells infected with strain SA113 as opposed to those infected with strains MW2 and LAC correlate with levels of Agr functionality in those strains. SA113 is functionally Agr-defective, while MW2 and LAC show high Agr activity. These considerations prompted us to investigate the role of PSMs in IL-1β production by infected osteoblasts.

First, we analyzed LAC (USA300) wild-type and its isogenic mutant LACΔ*psmαβhld* (*S. aureus* strain lacking PSMα, PSMβ and δ-toxin) for their ability to stimulate the release of IL-1β. As shown in **Fig. 23-A**, the level of IL-1β was significantly decreased in the supernatants of WT MG-63 cells exposed to LAC Δ*psmαβhld* compared to WT MG-63 cells exposed to LAC wild-type on days 5 and 9 post-infection. To further investigate which PSM were involved in the simulation of IL-1β release, we used the PSM deletion strain LACΔ*psmαβhld* and complemented strains, expressing either the four PSMα peptides (LACΔ*psm*α*β*-*hld*-pTXΔα1-4), the two PSMβ peptides (LACΔ*psm*α*β*-*hld*-pTXΔβ1-2), or the δ-toxin (LACΔ*psm*α*β*-*hld*-pTXΔ*hld*) and monitored IL-1β levels in cell exposed to those strains up to 9 days post-infection. As shown in **Fig. 23-B**, there was a significant decrease in the IL-1β released by cells exposed to the LACΔ*psmαβhld* strain compared to LAC. Exposure to complemented

mutants demonstrated that the release was partially restored when the strains were complemented with PSMα (LACΔ*psm*αβ*hld*-pTXΔα1-4), PSMβ (LACΔ*psm*αβ*hld* pTXΔβ1-2) or δ-toxin (LACΔ*psm*αβ*hld*-pTXΔ*hld*). However, the difference was statistically significant only when strains were complemented with PSMβ or, at earlier time points, δ-toxin (LACΔ*psm*αβ*hld*-pTXΔ*hld*) **(Fig. 23**).

Employment of deletion and complemented PSMs mutants demonstrated a pivotal role of *S. aureus* toxins PSMs in inflammasomes related IL-1β production by infected osteoblastic cells. We would like to highlight that IL-1β production by infected osteoblasts appears to be specifically dependent on PSM betas among PSMs. This is noteworthy, as we don't know any specific phenotypes attributable to those PSMs so far. In addition to PSMs the action of other factors cannot be excluded since complemented mutants did not restore entirely the level of secreted IL-1β.

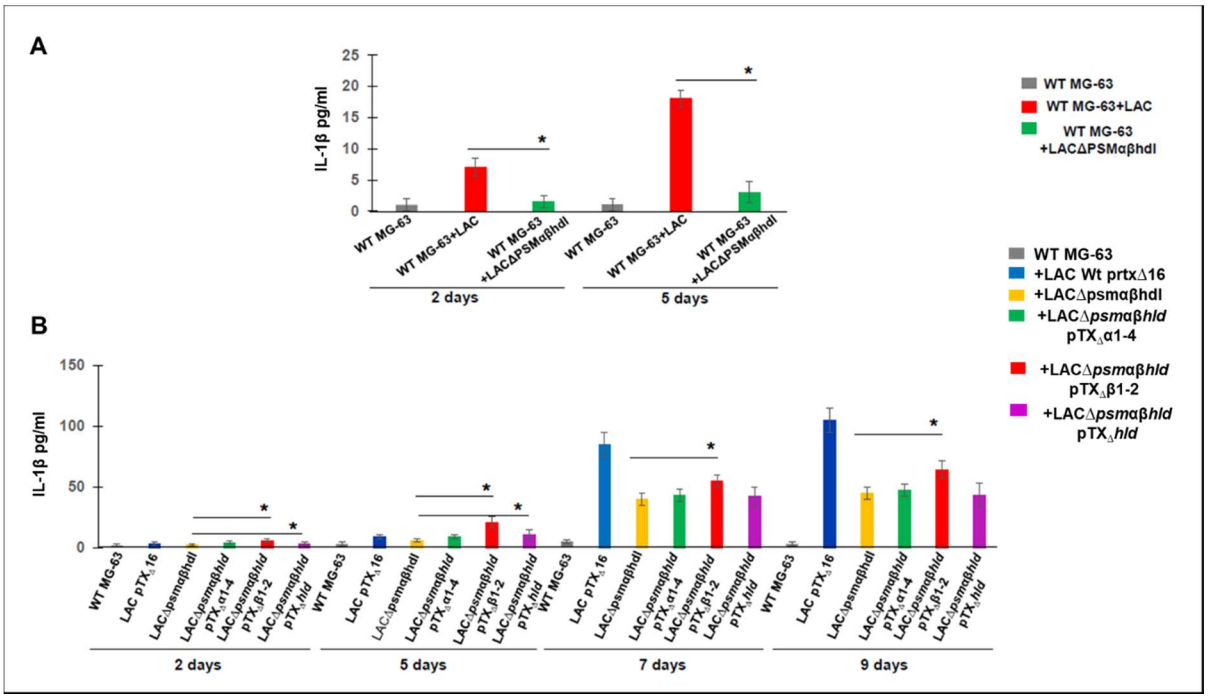


Figure 23: *S. aureus* phenol-soluble modulins stimulate IL-1β release from infected osteoblasts.

A. WT MG-63 cells were exposed to wild type LAC (USA300) and its isogenic mutant LAC Δ*psmαβhld* at MOI 1:50 for 2 h followed by antibiotic treatment as described in Material and Methods. After various time post-infection (2 days and 5 days) cell supernatants were collected, centrifuged and the level of IL-1β was determined by a sandwich-ELISA (Thermofischer Life Technologies) as described in Material and Methods. The IL-1β values are presented as concentrations in pg/ml. Three independent assays were performed. The differences among the groups were assessed by analysis of variance (ANOVA). P-values < 0.05 (*) were considered to be significant. Tukey's Honestly Significant Difference test was

applied for comparison of means between the groups. The values are expressed as means ± standard deviation (±SD).

B. MG-63 cells were exposed to USA300 LAC (pTXΔ16), which carries the control plasmid, the deletion mutant LACΔ*psmαβhld* (pTXΔ16) and the complemented strains expressing the four PSMα peptides (LACΔ*psmαβhld*-pTXΔα1-4), the two PSMβ peptides (LACΔ*psmαβhld*-pTXΔβ1-2), or the δ-toxin (LACΔ*psmαβhld*-pTXΔhld) at MOI 1:50 for 2 h followed by antibiotic treatment as described in Material and Methods. After various times post-infection (2 days, 5 days, 7 days and 9 days) cell supernatants were collected, centrifuged and the level of IL-1β was determined by a sandwich-ELISA (Thermofischer Life Technologies) as described in Material and Methods. The values are presented as concentrations in pg/ml. Three independent assays were performed. The differences among the groups were assessed by analysis of variance (ANOVA). P-values< 0.05 (*) were considered to be significant. Tukey's Honestly Significant Difference test was applied for comparison of means between the groups. The values are expressed as means ± standard deviation (±SD).

In addition, we evaluated the secretion of IL-1β in osteoblast cells exposed to *S. aureus* strains obtained from patients with osteomyelitis. We analyzed MG-63 cells infected by six *S. aureus* human clinical isolates. The level of IL-1β was measured by ELISA. Three pairs of *S. aureus* strains used were obtained from patients with staphylococcal BJI provided by CNF Staphylococcus (National Reference Center for Staphylococci) of Lyon. Three couples of isolates were selected from patients P1, P2, P3 who were diagnosed with initial acute (i) and recurrent (r) staphylococcal BJI: isolates from the same patient were named 45i and 46r (P1), 47i and 48r (P2), 51i and 53r (P3) for initial and recurrent BJI correspondently. (Supplementary Material). Previous studies conducted by our group have demonstrated that the eukaryotic cell cycle delay induced by *S. aureus* human clinical isolates is associated with the production of PSMα1 that reveals a new mechanism to promote infection (DEPLANCHE et al., 2015).

The analysis of the kinetics of IL-1β release by WT MG-63 cells exposed to either six clinical isolates or *S. aureus* USA400 strain (MW2) demonstrated an increase of the level of IL-1β in the supernatants from the 2nd day post-infection up to 9 days post-infection. Furthermore, we found different levels of IL-1β production induced by clinical strains collected from three patients. When comparing its acute (i) and recurrent (r) forms, we find that IL-1β production induced by the strains (45i and 46r) was similar (**Fig. 24-A**). The IL-1β production induced by the acute strain (47i) was significantly higher then induced by the recurrent strain (48r) (**Fig. 24-B**). A comparison of strains (51i) and (52r) showed that strain (52r) induced higher IL-1β levels compared to strain (51i) (**Fig. 24-C**). These results demonstrated that clinical strains of *S. aureus* obtained from patients with staphylococcal BJI induce IL-1β secretion in infected

MG-63 cells and suggest that *S. aureus* virulence factors may be involved in inflammasome activation and IL-1β secretion.

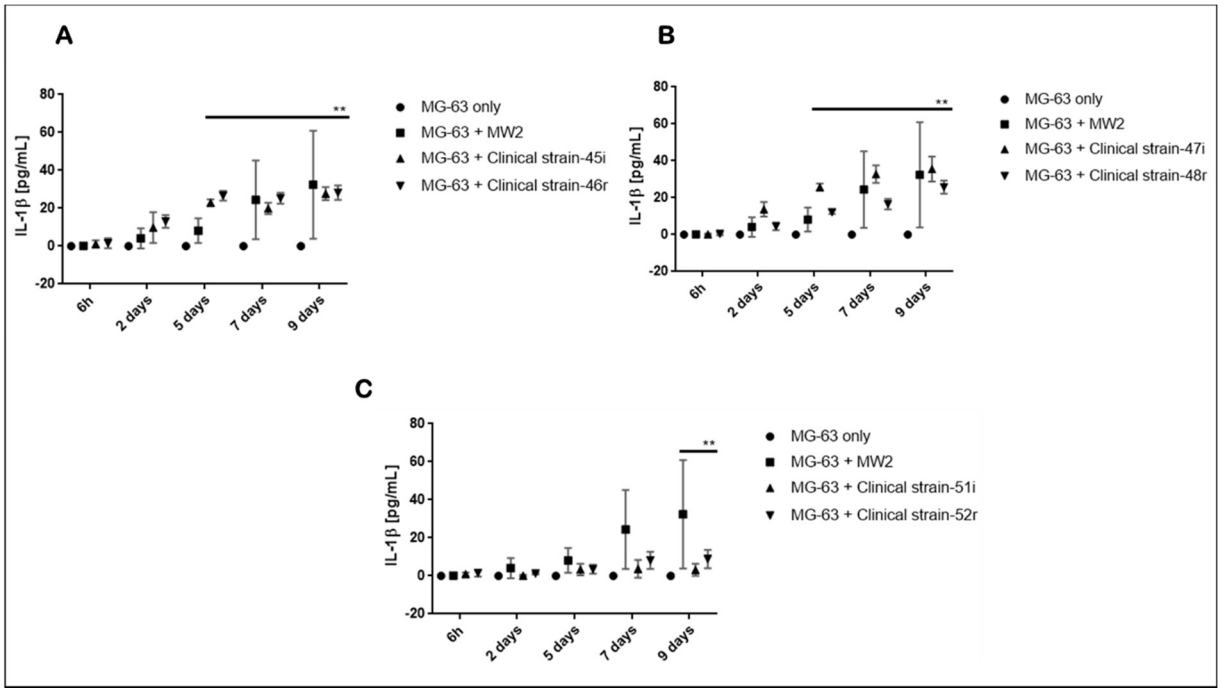


Figure 24: kinetics of IL-1β production between 6 h and 9 days post-infection in MG-63 cells by *S. aureus* clinical strains.

A. *S. aureus* clinical strain (45i) *vs S. aureus* clinical strain (46r). **B.** *S. aureus* clinical strain (47i) *vs S. aureus* clinical strain 48r. **C.** *S. aureus* clinical strain (51i) *vs S. aureus* clinical strain (52r). IL-1β production was measured by ELISA in supernatants of WT MG-63 cells. Tukey's Honestly Significant Difference test was applied for comparison of means between the groups. The values are expressed as mean ± standard deviation (±SD). Experiments are performed in triplicate and are representative of data obtained in three independent experiments. Significance is denoted as thus: **p < 0.01 by two-way ANOVA.

5.5. *S. aureus* clearance by osteoblasts depends on caspase-1

Several studies have demonstrated the importance of caspase-1 activation in the control of intracellular replication of several pathogens, such as *Salmonella* (BRENNAN; COOKSON, 2000), *Shigella* (CHEN; KANIGA; GALÁN, 1996) and *Legionella* (ZAMBONI et al., 2006). Cell culture models have demonstrated that *S. aureus* invades osteoblasts of different species, such as mice, birds, and humans (ELLINGTON et al., 1999; JEVON et al., 1999; REILLY et al., 2000). By electron microscopy, Reilly et al. (2000) were able to observe the presence of *S. aureus* within osteoblasts and osteocytes of *S. aureus*-infected chicken embryos, thus

demonstrating that bacterial invasion of these cells can occur *in vivo* (REILLY et al., 2000). Bosse et al. (2005) revealed in the interior of the osteoblasts and osteocytes removed from a patient suffering from a chronic infection of the fibula, the presence of gram-positive cocci intracellular bacteria presented a suggestive morphology by transmission electron microscopy (BOSSE; GRUBER; RAMP, 2005). In this context, the hypothesis that the facultative intracellular character of *S. aureus* provides the bacterium with a protective niche, protected from the immune system and most antibiotics, was gradually accepted by most authors; is also considered a plausible explanatory phenomenon of the chronic character of certain bone infections (ELLINGTON et al., 2003, 2006).

Previous studies reported that the professional phagocytes lacking caspase-1 are permissive to intracellular pathogen replication (KHWEEK; AMER, 2010; SIMON; HILBI, 2015). Sokolovska et al. (2013) demonstrated that activation of caspase-1 is required for the processing and killing of intracellular *S. aureus* by professional phagocytes (SOKOLOVSKA et al., 2013). According to our knowledge, the role of caspase-1 in bacterial clearance in non-professional phagocytes, which include osteoblasts, has not been investigated.

Consequently, we analyzed whether the lack of caspase-1 in *CASP1*$^{-/-}$MG-63 cells is correlated with a failure to control the intracellular replication of *S. aureus* bacteria. The quantity of intracellular bacteria during *S. aureus* infection of WT and *CASP1*$^{-/-}$MG-63 cells was determined after gentamicin treatment of infected cells as described in Material and Methods. According to CFU determination bacterial internalization was not impaired in *CASP1*$^{-/-}$MG-63 cells, as we observed equivalent levels of internalized bacteria in WT MG-63 cells at 2 h post-infection at ~ 10^2 CFU/10^5 host cells. Six and 24 hours post-infection significantly higher numbers of viable bacteria were recovered from *CASP1*$^{-/-}$MG-63 cells compared to WT MG-63 cells (**Fig. 25-A**). These results suggest that the lack of caspase-1 impairs the ability of osteoblasts to restrict *S. aureus* growth. These findings were further confirmed using confocal microscopy. As shown in **Fig. 25-B**, 6 h post-infection a considerably higher number of intracellular *S. aureus* bacteria (red) was observed in *CASP1*$^{-/-}$ MG-63 cells than WT MG-63 cells, while assembly of the ASC specks was observed in both cell types (green). These results corroborate with the findings of Amer et al. (2006), which demonstrate that caspase-1 activation is critical for the clearance of the bacteria independently of IL-1β activity (AMER et al., 2006).

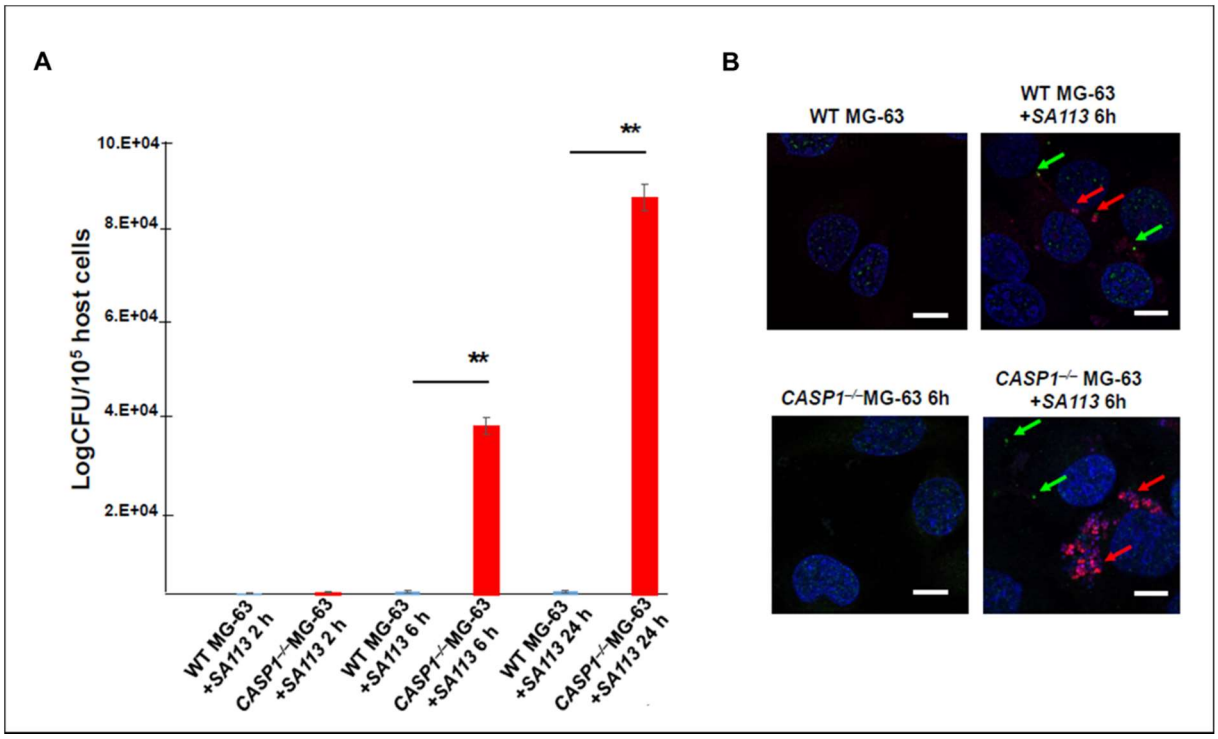


Figure 25: Involvement of caspase-1 in bacterial clearance.

A. WT MG-63 or CASP1$^{-/-}$ MG-63 cells were grown in 12-well plates overnight, then were exposed to *S. aureus* MW2 strain at MOI 1:50 for 2 h followed by antibiotic treatment as described. Two hours, 6 h and 24 h post-infection cells were lysed with 0.05% Triton X-100 in PBS, cell lysates were plated on BHI agar, and CFU was determined after overnight incubation. CFU values were normalized to 10^5 host cells. Experiments were performed in triplicate. The data are presented as means ± SD. Tukey's Honestly Significant Difference test was applied for comparison of means between the groups. **, $P < 0.01$, for the comparison of the number of internalized bacteria in CASP1$^{-/-}$MG-63 cells with those in WT MG-63 cells.

B. WT MG-63 or CASP1$^{-/-}$ MG-63 cells were grown on the slides of 12-well plates overnight, then cells were exposed to a fluorescent derivative of strain *S. aureus* SA113 fluorescent, which carries plasmid-encoded mCherry (red fluorescence), at MOI 1:50 for 2 h followed by antibiotic treatment as described. Six hours post-infection cells were immune-stained with rabbit anti PYCARD antibody (Coger France), followed by incubation with Alexa Fluor 488 labeled goat anti-rabbit antibody (Cell Signaling Ozyme) at a dilution of 1:50 for 2 h at room temperature (green staining). Nuclear DNA was labeled with DAPI (blue staining). Samples were viewed with a Zeiss laser-scanning microscope equipped with a 63× plan Apo-NA 1.4 immersion objective driven by Zen software. Scale bar: 3 µm.

Chapter 6. General conclusion and perspectives of the work performed during PhD project

General conclusion and perspective

Staphylococcus aureus is a bacterium that is widely studied due to its great impact on human and animal health as well as its role in the food industry. The purpose of this thesis was to investigate the contributions of internalized *S. aureus* on the alteration of host defense events such as inflammasomes activation and bacterial clearance in infected osteoblasts-like MG-63 cells that are widely used for the study of bone and joint infections (BJI).

We demonstrated that human osteoblast-like MG-63 cells treated with inflammasomes activators, form inflammasomes resulting in the activation of IL-1β-converting enzyme (ICE; caspase-1) and secretion of Pro-inflammatory cytokine Interleukin-1β (IL-1β). The production of IL-1β by osteoblastic MG-63 cells started later and its level was much lower then IL-1β production by differentiated ThP1 cells that are professional phagocytes, macrophages. This discrepancy is likely related to different origins in both types of cells.

Due to the ability of the inflammasomes compound in the activation of caspase-1 to process IL-1β and IL-18 we used CRISPR/Cas9 gene editing to generate osteoblast-like MG-63 deficient for *caspase-1* gene that are a powerful tool, which allows the study of the function of inflammasomes to be investigated. We observed a progressive increase of IL-1β levels in infected MG-63 cells from several hours to several days in contrast to the lack of IL-1β production in infected CASP1$^{-/-}$ MG-63 cells demonstrated caspase-1-dependent mechanisms and IL-1β production. Besides, the effect was strongly *S. aureus* strains dependent. The similar results were obtained with freshly isolated clinical *S. aureus* isolates: this emphasizes the biological significance of our findings. The absence of IL-1β production by cells exposed to heat-killed bacteria indicates that factors associated with viable bacteria are involved in the activation of the NLRP3 inflammasome. Employment of deletion and complemented PSMs mutants demonstrated a pivotal role of *S. aureus* toxins PSMs (especially PSM-β and PSM-γ) in inflammasomes related to IL-1β production by infected osteoblasts cells. This is noteworthy, as we don't know any specific phenotypes attributable to those PSMs so far. However, the presence of other factors can not be excluded since complemented mutants did not restore entirely the level of secreted IL-1β.

The investigation of another host cell defense event, such as the control of intracellular bacterial proliferation, reveals a drastic increase in the proliferation of internalized bacteria in osteoblast-like CASP1$^{-/-}$MG-63 cells. This finding, in addition to the previous reports demonstrating the correlation of the lack of caspase-1 activation with a failure to restrict the

replication of *S. aureus* inside of phagocytic cells points out that the active caspase-1 prevent exacerbated intracellular replication of *S. aureus* in professional as well as in non-professional phagocytes.

Collectively, our results demonstrated that human osteoblast-like MG-63 cells play an important role in the immune response against *S. aureus* infection. Infected osteoblasts are not passive bystanders but active players in defense against *S. aureus* infection. The outcome of the infection depends on the balance of the immune response of the host and the action of multiple *S. aureus* virulence factors among which PSMs play a pivotal role and whose production may differ among the *S. aureus*. Besides our observations of inflammasome activation (through measurement of IL-1β production), we demonstrated that caspase-1 deficient osteoblast-like cells were unable to restrict the intracellular replication of *S. aureus* suggesting the universal pivotal role of caspase-1 in pathogens clearance independently from the type of cells. These findings deserve further investigation to improve our understanding of *S. aureus*-host interactions and to uncover the underlying mechanisms.

And the next steps

The developed model is a powerful tool for tracking inflammation *in vitro*. Although this model is based on a single cell line, it opens new perspectives for the generation of similar models of non-phagocytic cells that allow the infectious process associated with *S. aureus* to be explored.

Furthermore, we consider other action mechanisms for the formation of the NLRP3 inflammasome in *S. aureus*-infected MG-63 cells. It is known that process like lysosomal membrane disturbance, ionic imbalance (K^+ efflux) and induction of oxidative stress, signals critical for the activation of the NLRP3 inflammasomes, are processes depend on an active participation of the bacterium (HALLE et al., 2008; JIN; FU, 2019; LATZ, 2010; MARTINON; BURNS; TSCHOPP, 2002; ZHOU et al., 2011). Reactive oxygen species (ROS) are oxygen metabolites that, because of their ability to gain and lose electrons, are prone to participate in oxidation-reduction reactions. Mammalian cells have developed various mechanisms to limit the production of ROS, including deactivating them and repairing cell damage. However, when the rate of ROS production increases dramatically and/or the antioxidant defenses are insufficient or fail, oxidative stress occurs. This oxidative stress is a secondary signal for the activation of the inflammasome (DOSTERT et al., 2008b; ZHOU et al., 2010, 2011). In the context of the implemented cellular model, it would be interesting to verify the role of ROS in IL-1β production through the activation of the NLRP3 inflammasome in order to elucidate the underlying mechanisms involved in the activation of the NLRP3 inflammasome induced during

S. aureus-infection in MG-63 cells. Analysis of extracellular ROS generation in MG-63 cells will be measured using the inhibition of cytochrome C reduction by superoxide dismutase (SOD), as described by Johnston and Lehmeyer (JOHNSTON; LEHMEYER, 1976). For the detection of intracellular ROS, MG-63 cells will be infected or not with *S. aureus* strains. After a 24 h incubation period, the 2', 7'-dichlorodihydrofluorescein diacetate (H2DCFDA) is used which is cell membrane permeable and non-fluorescent. In the presence of ROS, this compound is oxidized within the cell and produces a fluorescent compound, the 2', 7'-dichlorodihydrofluorescein diacetate (H2DCFDA), which remains inside the cell. The reading will be performed by flow cytometry (Accuri C6 flow cytometer). Thus, it possibly will identify the formation of ROS in the analysis proposed in MG-63 cells after these stimuli.

Besides, it has recently been discovered that in addition to caspase-1, other caspases, such as caspase-11 and caspase-8, may also be involved in the processing and maturation of IL-1β by the inflammasome (RATHINAM; FITZGERALD, 2016; VANAJA; RATHINAM; FITZGERALD, 2015). Thus, it would be interesting to elucidate the role of non-canonical inflammasome pathways in modulating the immune response during *S. aureus*-infection in MG-63 cell.

Caspase-11, as well as caspase-1, is present in the cytoplasm of cells as a 43 kDa inactive zymogen, which needs to be cleaved (large 23-kDa and small 10-kDa) (RATHINAM; VANAJA; FITZGERALD, 2012). By the technique of western blotting, we could measure the precursor and cleavage forms expressed of caspase-11 after stimulation with *S. aureus* in MG-63 cells. These results could elucidate whether, in addition to the canonical pathway of caspase-1, the non-canonical pathway of caspase-11 is also operating during *S. aureus* infection.

Besides, we could include the evaluation of caspase-8 to further elucidate the existence of an additional non-canonical pathway, since caspase-8 can directly cleave pro-IL-1β as well as initiate caspase-1 in response to different pathogens (MAELFAIT et al., 2008; SHENDEROV et al., 2014). For this, the activity of caspase-8 in the osteoblastic cell lysates will be measured according to the recommendations of the Caspase-Glo 8 Assay kit (Promega, Madison, WI, USA). Summarizing, this assay the signal intensity generated by the luciferase reaction is proportional to the cleavage of the caspase-8 substrate. Thus, we will possibly identify the presence of caspase-8 in MG-63 cells in these analyzes.

The direct interactions of *S. aureus* with osteoblasts are crucial in the pathogenesis of osteomyelitis. The ability of *S. aureus* to invade and gain access to the cytoplasm of non-professional phagocytes, such as osteoblasts, is gaining increasing attention and is now

considered a key factor in refractory infections. Also, the investigation of the underlying mechanisms of the activation of the canonical and non-canonical pathways of the NLRP3 inflammasome in the modulation of the immune response during *S. aureus* infection will be interesting and new knowledge and open up new perspectives for the development of more selective treatments for the intracellular persistence of *S.aureus* in Bone Joint Infection.

Conclusion générale et perspectives

Staphylococcus aureus est une bactérie largement étudiée en raison de son impact important sur la santé humaine et animale ainsi que de son rôle dans l'industrie alimentaire. Le but de cette thèse était d'étudier les contributions de *S. aureus* intériorisé sur l'altération d'événements de défense de l'hôte tels que l'activation de l'inflammasome et la clairance bactérienne dans des cellules MG-63 ressemblant à des ostéoblastes infectés qui sont largement utilisées pour l'étude des infections des os et des articulations (BJI).

Nous avons démontré que les cellules humaines MG-63 ressemblant à des ostéoblastes, traitées avec des activateurs de l'inflammasome, forment des inflammations entraînant l'activation de *IL-1β-converting enzyme* (ICE; caspase-1) et la sécrétion de la cytokine pro-inflammatoire Interleukine-1β (IL-1β). La production d'IL-1β par des cellules ostéoblastiques MG-63 a débuté plus tard et son niveau était beaucoup plus bas que la production d'IL-1β par des cellules ThP1 différenciées qui sont des phagocytes professionnels, des macrophages. Cette divergence est probablement liée à des origines différentes dans les deux types de cellules.

En raison de la capacité du composé inflammasomes dans l'activation de la caspase-1 à traiter l'IL-1β et l'IL-18, nous avons utilisé l'édition de gènes CRISPR/Cas9 pour générer des cellules CASP1$^{-/-}$ MG-63, un outil puissant étude de la fonction des inflammations à étudier. L'augmentation progressive des taux d'IL-1β dans les cellules MG-63 infectées de plusieurs heures à plusieurs jours, contrairement à l'absence de production d'IL-1β dans les cellules CASP1$^{-/-}$ MG-63 infectées, a démontré des mécanismes dépendant de la caspase-1 et d'IL-1β production. En outre, l'effet était fortement dépendant des souches de *S. aureus*. Des résultats similaires ont été obtenus avec des isolats cliniques de *S. aureus* fraîchement isolés: cela souligne la signification biologique de nos résultats. L'absence de production d'IL-1β par les cellules exposées à des bactéries tuées par la chaleur indique que des facteurs associés aux bactéries viables sont impliqués dans l'activation de l'inflammasome NLRP3. L'emploi de mutants PSM de délétion et de complément a démontré le rôle pivot des toxines de *S. aureus* PSM (en particulier PSM-β et PSM-γ) dans les inflammations liées à la production d'IL-1β par les cellules infectées. Cependant, la présence d'autres facteurs ne peut pas être exclue car les mutants complémentés ne restituent pas entièrement le niveau d'IL-1β sécrétée.

L'étude d'un autre événement de défense de la cellule hôte, tel que le contrôle de la prolifération bactérienne intracellulaire, révèle une augmentation considérable de la

prolifération des bactéries internalisées dans les cellules CASP1$^{-/-}$MG-63 de type ostéoblastique. Cette découverte, en plus des rapports précédents démontrant la corrélation de l'absence d'activation de la caspase-1 avec un manque de restriction de la réplication de *S. aureus* à l'intérieur de cellules phagocytaires, indique que la caspase-1 active empêche la réplication intracellulaire exacerbée de *S. aureus* (SA113) chez les phagocytes professionnels et non professionnels.

Ensemble, nos résultats ont démontré que les cellules MG-63 de type ostéoblaste humain jouent un rôle important dans la réponse immunitaire contre l'infection à *S. aureus*. Les ostéoblastes infectés ne sont pas des passants passifs, mais des acteurs actifs de la défense contre l'infection à *S. aureus*. L'issue de l'infection dépend de l'équilibre entre la réponse immunitaire de l'hôte et l'action de multiples facteurs de virulence de *S. aureus* parmi lesquels les PSM jouent un rôle pivot et dont la production peut différer chez les *S. aureus*. Outre nos observations d'activation de l'inflammasome (par mesure de la production d'IL-1β), nous avons démontré que les cellules de type ostéoblastes déficientes en caspase-1 étaient incapables de restreindre la réplication intracellulaire de *S. aureus*, ce qui suggère le rôle central de la caspase-1 dans la clairance des agents pathogènes indépendamment du type de cellules. Ces découvertes méritent d'être approfondies afin d'améliorer notre compréhension des interactions entre *S. aureus* et l'hôte et de découvrir les mécanismes sous-jacents.

Et les prochaines étapes

Le modèle que nous avons développé est un outil puissant pour suivre l'inflammation *in vitro*. Bien que ce modèle soit basé sur une seule lignée cellulaire, il ouvre de nouvelles perspectives pour la génération de modèles similaires de cellules non phagocytaires permettant d'explorer le processus infectieux associé à *S. aureus*.

De plus, nous spéculons sur l'action des autres mécanismes dans la formation de l'inflammasome NLRP3 dans les cellules MG-63 infectées par *S. aureus*. Une fois que ces processus dépendent de la participation active de la bactérie, tels que perturbation de la membrane lysosomale, déséquilibre ionique (efflux de K +) et induction du stress oxydatif, signaux essentiels à l'activation des inflammasomes de NLRP3 (HALLE et al., 2008; JIN; FU, 2019; LATZ, 2010; MARTINON; BURNS; TSCHOPP, 2002; ZHOU et al., 2011). Les *Reactive oxygen species* (ROS) sont des métabolites de l'oxygène qui, en raison de leur capacité à gagner et à perdre des électrons, ont tendance à participer aux réactions d'oxydoréduction. Les cellules de mammifères ont mis au point divers mécanismes pour limiter la production de ROS, y compris leur désactivation et la réparation des dommages cellulaires. Cependant,

lorsque le taux de production de ROS augmente considérablement et/ou que les défenses anti-oxydantes sont insuffisantes ou défaillantes, un stress oxydatif se produit. Ce stress oxydatif est un signal secondaire pour l'activation de l'inflammasome (DOSTERT et al., 2008b; ZHOU et al., 2010, 2011). Dans le contexte du modèle cellulaire mis en œuvre, il serait intéressant de vérifier le rôle des ROS dans la production d'IL-1β via l'activation de l'inflammasome NLRP3 afin d'élucider les mécanismes sous-jacents impliqués dans l'activation de l'inflammasome NLRP3 induit pendant *S. aureus*-infection dans les cellules MG-63. L'analyse de la génération de ROS extracellulaires dans les cellules MG-63 sera mesurée en utilisant l'inhibition de la réduction du cytochrome C par la superoxyde dismutase (SOD), comme décrit par Johnston et Lehmeyer (JOHNSTON; LEHMEYER, 1976). Pour la détection des ROS intracellulaires, les cellules MG-63 seront infectées ou non avec les souches de *S. aureus*. Après une période d'incubation de 24 h, on utilise la sonde *2',7'-dichlorodihydrofluorescein diacetate* (H2DCFDA) perméable à la membrane cellulaire et non fluorescente. En présence de ROS, ce composé s'oxyde à l'intérieur de la cellule et produit un composé fluorescent, le diacétate de *2',7'-dichlorodihydrofluorescein diacetate* (H2DCFDA), qui reste à l'intérieur de la cellule. La lecture se fera par cytométrie en flux (cytomètre en flux Accuri C6). Ainsi, il pourra éventuellement identifier la formation de ROS dans l'analyse proposée dans les cellules MG-63 après ces stimuli.

En outre, il a récemment été découvert qu'outre la caspase-1, d'autres caspases, telles que la caspase-11 et la caspase-8, pourraient également participer au traitement et à la maturation de l'IL-1β par l'inflammation (RATHINAM; FITZGERALD, 2016; VANAJA; RATHINAM; FITZGERALD, 2015). Ainsi, il serait intéressant d'élucider le rôle des voies d'inflammasome non canoniques dans la modulation de la réponse immunitaire lors d'une infection à S. aureus dans une cellule MG-63.

La caspase-11, ainsi que la caspase-1, sont présentes dans le cytoplasme des cellules en tant que zymogène inactif de 43 kDa, qui doit être clivé (gros 23 kDa et petits 10 kDa) (RATHINAM; VANAJA; FITZGERALD, 2012). Par la technique du Western Blot, nous avons pu mesurer les formes de précurseur et de clivage exprimées de la caspase-11 après stimulation avec *S. aureus* dans des cellules MG-63. Ces résultats pourraient élucider si, en plus de la voie canonique de la caspase-1, la voie non canonique de la caspase-11 fonctionne également pendant l'infection à *S. aureus*.

Aussi, nous pourrions inclure l'évaluation de la caspase-8 pour élucider davantage l'existence d'une voie supplémentaire non canonique, car la caspase-8 peut directement cliver la pro-IL-1β et initier la caspase-1 en réponse à différents agents pathogènes (MAELFAIT et al., 2008;

SHENDEROV et al., 2014). Pour cela, l'activité de la caspase-8 dans les lysats de cellules ostéoblastiques sera mesurée selon les recommandations du kit Caspase-Glo 8 Assay (Promega, Madison, WI, USA). En résumé, cet essai l'intensité du signal généré par la réaction de luciférase est proportionnelle au clivage du substrat de la caspase-8. Ainsi, nous étudierons l'implication de voies non canoniques de la caspase-11 et de la caspase-8 dans la libération de IL-1β dans des cellules MG-63 après ces stimuli.

Les interactions directes de *S. aureus* avec les ostéoblastes sont cruciales dans la pathogenèse de l'ostéomyélite. La capacité de *S. aureus* à envahir et à accéder au cytoplasme de phagocytes non professionnels, tels que les ostéoblastes, attire de plus en plus l'attention et est désormais considérée comme un facteur clé des infections réfractaires. En outre, l'étude des mécanismes sous-jacents de l'activation des voies canoniques et non canoniques de l'inflammasome NLRP3 dans la modulation de la réponse immunitaire pendant l'infection à *S. aureus* sera une nouvelle connaissance intéressante et ouvrira de nouvelles perspectives pour traitements plus sélectifs pour la persistance intracellulaire de *S. aureus* dans l'infection des articulations osseuses.

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S. aureus strains description

S. aureus clinical strains isolates were collected with the approval of the French South-East ethics committee (no. CAL2011-21) 22. Three pairs of *S. aureus* strain used (P1, P2, P3) were obtained from patients with initial acute (i) and recurrent (r) staphylococcal bone & joint infections (BJI): isolates from the same patient were named 45i and 46r (P1), 47i and 48r (P2), 51i and 53r (P3) for initial and recurrent BJI, provided by CNR Staphylocoque (Centre National de Référence des Staphylocoques) of Lyon. The choice of these strains relies on the complete characterization of these strains by CNR Staphylocoque (sequencing, cultural characteristics and origin of the sample).

All cultures of *S. aureus* were cultured in Brain Heart Infusion (BHI) and incubated overnight at 37 °C with shaking. The following day, DMEM (Dulbecco's Modified Eagle's medium) was inoculated with the previous day's culture and the strains were incubated at 37 °C overnight before the day of infection. The optical density (OD_{600nm}) of all cultures was adjusted to 0.6.

Annexes

Annexe 1. Review: Comparing bacteriocins with conventional antibiotics as treatment options for *Staphylococcus aureus* infections (Submitted to the peer-reviewed journal PeerJ on 20th May 2019).

Comparing bacteriocins with conventional antibiotics as treatment options for *Staphylococcus aureus* infections

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Keywords: *Staphylococcus aureus*, Antibiotic Resistance, Bacteriocins, Biotechnology use of Bacteriocins.

Abstract

Background: *Staphylococcus aureus* (*S. aureus*) is a highly versatile Gram-positive bacterium that is carried asymptomatically by up to 30% of healthy people, besides being a major cause of healthcare-associated infections, making it a worldwide problem in clinical medicine. The adaptive evolution of bacteria is demonstrated by its remarkable capacity to promptly develop a high resistance to multiple antibiotics, thus limiting treatment choice. Employment of bacteriocins, antibacterial peptides or proteins produced by bacteria allows the development of new approaches and strategies to control the spread or to kill phylogenetically related strains.

Methodology: A comprehensive review of the relevant literature was made through a collection of original articles and review articles that were selected by an academic search engine and academic databases, such as (e.g. https://scholar.google.com/; https://www.ncbi.nlm.nih.gov/pubmed/; http://www.scielo.br/ and http://www.periodicos.capes.gov.br/). We searched the following keywords: "*Staphylococcus aureus*, antibiotic, resistance, bacteriocins, biotechnology and bacteriocins". A total of 65 articles were accumulated and categorized into four main categories. The research criteria adopted in this review took into account important studies that addressed a high degree of observation of resistance to antibiotics by strains of *S. aureus*. As a method criteria of inclusion, we considered the first cases of resistance and evaluated the progress until the present day. Additionally, in order to answer possible ways to deal with this protective advance in bacteria, the activity of bacteriocins was explored in the research studies considered here, as a good substitute capable of replacing in some cases the antibiotics currently used. The criteria for inclusion of possible bactericidal agents were evaluated considering experimental cases published in scientific journals. Our study highlighted some of the cases analyzed experimentally and we also show methods to explore the production of these potential molecules at an industrial scale using protocols from other review works.

Results: Our work reviews the current informative content and brings the applicability of the use of bacteriocins overlapping with the use of conventional antibiotics in the context of *S. aureus* infections. Here we ascertained the main antibiotic-resistance mechanisms and analyzed the current bacteriocins that stand out as strong competitors in the struggle against *S. aureus* infections. At the same time, our work highlights perspectives for the production of these biomolecules with implications at an industrial scale, using aqueous two-phase system (ATPS) and Aqueous micellar two-phase system (AMTPS).

Conclusions: The use of Fermented Plant Extract (FPE) is explored showing itself to be an innovative tool, serving as a carrier for numerous health benefits, which includes the control of microbial activity.

Introduction

Mammal's skin is the main environment for the colonization of members of genus *Staphylococcus* which can facilitate infection in immunologically suppressed individuals, found in cases of *S. epidermis* as reported on a leukemic patient, but not exclusively (Otto, 2010b; Senthilkumar et al., 2001; Weisser et al., 2010). Specifically, *S. aureus* is the main trigger of several diseases such as osteomyelitis (Olson and Horswill, 2013), septic arthritis (Corrado *et al*., 2016), bacteremia (Holland et al., 2014), endocarditis (Fowler *et al*., 2005), pneumonia

(Chickering, 1919) and mastitis (Kiku *et al*., 2017). This bacterium is also the major causal agent of numerous implant infections (Arciola et al., 2018). Bacterial infections have been treated by the use of antibiotics since the beginning of the 40's, but their efficiency tends to decrease due to the high rate of antibiotic resistance developed by several microorganisms, such as S. aureus (Ventola, 2015b). The inappropriate use of antibiotics, particularly their overuse, has been considered one of the most important factors contributing to the development of microbial resistance. This constitutes a serious global public health problem since the frequency of established and emerging infectious diseases has increased because of the ineffectiveness of antibiotics. This augurs a warning about the inefficacy of our current drug arsenal and pushes us to look for new strategies in the struggle against bacterial infection (Ventola, 2015c).

The World Health Organization (WHO) has been monitoring this danger with worldwide alert, since the mechanisms of resistance are emerging and spreading around the world faster than usual. In order to prevent and control the antibiotic resistance, the WHO advised the healthcare industry to invest in the development of new antibiotics and novel strategies to treat bacterial infections. At the same time, it is estimated that close to 80% of all antibiotics sold just in the US are intended for animal use (Martin et al., 2015). One study some years ago estimated that the global average annual consumption of antibiotics in livestock considering cattle, chicken and pigs will increase by 67% from 63,151 ± 1,560 tons to 105,596 ± 3,605 tons (Van Boeckel *et al*., 2015).

In the context of human and animal health, bacteriocins appear to have a different strategy and may act as strong candidates to overlap with or even replace the role of conventional antibiotics. The bacteriocins have been described with potential application as bio-preservatives, but they are mainly defined as proteins and peptides produced by bacteria that inhibit the growth or kill other related and unrelated microorganisms (Balciunas *et al*., 2013).

This review gathers recent information about the possible use of bacteriocins replacing antibiotic treatment during *S. aureus* infection considering the advantages offered by these natural compounds, as well as the differences and comparisons between them.

Survey methodology

A comprehensive review of the relevant literature was made through a collection of original articles and review articles that were selected by an academic search engine and academic databases, such as (e.g. https://scholar.google.com/; https://www.ncbi.nlm.nih.gov/pubmed/; http://www.scielo.br/ and http://www.periodicos.capes.gov.br/). We searched the following keywords: "*Staphylococcus aureus*, antibiotic, resistance, bacteriocins, biotechnology and bacteriocins". A total of 65 articles were accumulated and categorized into four main

categories. The research criteria adopted in this review took into account important studies that addressed a high degree of observation of resistance to antibiotics by strains of *S. aureus*. As a method criteria of inclusion, we considered the first cases of resistance and evaluated the progress until the present day. Additionally, in order to answer possible ways to deal with this protective advance in bacteria, the activity of bacteriocins was explored in the research studies considered here, as a good substitute capable of replacing in some cases the antibiotics currently used. The criteria for inclusion of possible bactericidal agents were evaluated considering experimental cases published in scientific journals. Our study highlighted some of the cases analyzed experimentally and we also show methods to explore the production of these potential molecules at an industrial scale using protocols from other review works.

Mechanisms of Antibiotic Resistance in *S. aureus*

Since the discovery of the first case of resistance to penicillin in mid of 20's, the number of key events of antibiotic resistance to *S. aureus* only increases. At the same time it is unstable, since the speed of response to antibiotics may be a few years or several (Figure 1) (Andriole, 2005; Fleming, 1929b; Humphreys and Mulvihill, 1985; Kaiser, 2000). Considering the broad spectrum of pathogenic bacteria currently observed in nature, *S. aureus* is pointed out as the most worrisome due to its capacity to manifest several diseases, a set of virulence factors (Rozemeijer *et al*., 2015), the ability to survive in different environmental conditions (Mäder *et al*., 2016) and due to the plasticity of response to antibiotics. Presently, *S. aureus* is described as being resistant to a broad class of antibiotics including β-lactams, glycopeptides, quinolones, oxazolidinone etc.

In the class of β-lactam, *S. aureus* can acquire resistance to penicillin and methicillin (Fuda et al., 2005). Penicillin inhibits the formation of peptidoglycan (PG) cross-links in the bacterial cell wall. PG cross-links generate a three-dimensional structure around the cell, which ensures bacterial integrity. Penicillin binds to DD-transpeptidase, the enzyme responsible for the formation of PG cross-links, and prevents its catalytic activity. While a part of PG synthesis is inhibited by penicillin, bacterial enzymes involved in PG turnover remain active such as hydrolases and autolysins. The activity of penicillin that causes an imbalance between PG synthesis and degradation weakens PG and leads to cell death. Penicillin, which was discovered by Alexander Fleming in 1929, was the first antibiotic used to fight *S. aureus*-mediated infections (Fleming, 1929b). Only two years after its introduction, *S. aureus* penicillin-resistant strains appeared. Theses strains contained an enzyme, β-lactamase, able to destroy penicillin (Dietz and Bondi, 1948). The gene *blaZ* together with *blaR1* and *blaI*, are the genetic determinants of the resistance of *S. aureus* to penicillin. They code for the β-lactamase

enzyme, a sensor of the presence of penicillin and a transcriptional repressor of *blaZ* expression, respectively. β-lactamase acts on penicillin outside the cell by changing its molecular structure to an inactive form, penicilloic acid, by the hydrolysis of its β-lactam ring. The expression of *blaZ* is controlled by external penicillin availability. Penicillin interacts with the transmembrane protein BlaR1. This interaction leads to the autocatalytic activation of BlaR1 to BlaR2 (or BlaR1 in active form) that can promote the inactivation of the BlaI repressor and therefore *blaZ* to synthesize enzyme. The three genes are located on a transposable element of a large *S. aureus* plasmid called b-lactamase-encoding transposon Tn552 that shows persistence over time and geographic spread. Furthermore, it was also described to carry cadmium resistance genes, which can strengthen the role of resistance by attributing greater persistence among strains that carry this genetic content (Shearer *et al*., 2011). Like the others β-lactams, methicillin impends the synthesis of bacterial cell walls. It inhibits cross-linkage between the linear peptidoglycan polymer chains, which compose a major component of the cell wall of gram-positive bacteria, by binding to and competitively inhibiting penicillin-binding proteins (PBPs). These PBP molecules can also behave like transpeptidases (D-alanyl-alanine). The mechanism that describes the methicillin-resistance of *S. aureus* (MRSA) proceeds in a similar way to Penicillin. However, several works have discussed that the mecA gene, that is responsible for methicillin resistance, may be a mobile genetic element (MGE), which confers the ability to respond to environmental stresses.

Conversely, *mecA* codes for the enzyme PBP2a, which belong to the class of glycopeptide antibiotics, and acts in exactly the same way as the β-lactamases by hydrolyzing and inactivating Penicillin. Its activation leads to the cleavage of *mecR1* that inactivates *mecI* releasing the operator region to the expression of *mecA*.

Another glycopeptide antibiotic described as susceptible to resistance in *S. aureus* is Vancomycin (Walters *et al*., 2015). Currently, two groups of strains set up the scenario of vancomycin resistance: Vancomycin-sensitive *S. aureus* (VSSA) and Vancomycin-resistant *S. aureus* (VRSA) (McGuinness et al., 2017). Vancomycin alters the peptidoglycan density in the cell wall, being capable of interacting with it forming non-covalent hydrogen bonds, in the exposed D- D-Ala-D-Ala peptides (Hanaki *et al*., 1998) which inhibits cell wall synthesis in the VSSA strains. The mechanism of resistance is associated with either the presence of an enterococcal plasmid vanA or by the transposition of elements related to Tn1546 (Zhu *et al*., 2010). The affinity of vancomycin to the polypeptide is heavily reduced due to the *vanA* operon provided by the conjugation of the plasmid in the VRSA strains, which is able to produce the different polypeptide D-Ala-D-Lac.

S. aureus may also become resistant to the class of quinolones antibiotics (Lowy, 2003; Oizumi et al., 2001; Tanaka et al., 2000). This resistance is achieved due to specific mutations in two

chromosomal genes: grlA coding for a subunit of DNA topoisomerase IV, the primary quinolone target reported (Ferrero *et al*., 1994), and *gyrA* coding for DNA gyrase A subunit. The two proteins are intimately associated with the overlapping and opening of the double DNA strand during DNA replication. Specific mutations in *grlB* and *gyrB* also cause resistance to quinolones (Andriole, 2005). Both proteins have also been described as having the B subunit (*grlB* and *gyrB*) intrinsically associated with resistance in *S. aureus*. These mutations stand out in a region known as quinolone-resistance determining region (QRDR), which trigger several codon alteration in synonymous and non-synonymous amino acid mutations (Tanaka *et al*., 2000). Mutations reduce the affinity of enzyme-DNA complex for quinolones. Figure 4 displays the mechanism of resistance to fluoroquinolones.

Considering the oxazolidinone class, resistance of *S. aureus* to linezolid has also been observed in the last decade (Besier *et al*., 2008). The main feature of the mechanism of action describes the linezolid interaction with the 50S subunit of prokaryotic ribosomes, which prevents initiation factors such as formylmethionyl-tRNA from acting to form the complex with the 30S subunit. This disruption precludes the formation of the 70S complex, which sequentially disrupts protein synthesis (Figure 5) (Swaney *et al*., 1998).

However, some cases of bacteria resistant to linezolid bearing point mutations at the specific targets in 50S, more precisely in the 23S ribosomal portion have been reported (Afşar et al., 2012; Meka et al., 2004). Furthermore, a new mechanism of linezolid resistance, which was first explored in a plasmid that included the gene *cfr*, was detected (Schwarz et al., 2000). Several years ago a novel variant of the phenicol resistance transposon Tn558 was detected on the plasmid pSCFS6 suggesting the ability of horizontal transfer between staphylococci (Kehrenberg et al., 2007). The transcription of *cfr* gene produces a methyltransferase protein, which catalyzes the methylation of 23S rRNA at position A2503 offering resistance to some antibiotics such as chloramphenicol, florfenicol and clindamycin (Kehrenberg *et al*., 2005) and later to linezolid (Toh *et al*., 2007).

Considering all the information explored here, the broad spectrum of antibiotics capable of inhibiting the bacterial growth has been gradually decreasing over the last 70 years. All mechanisms of bacterial resistance to the action of antibiotics are embedded in a complex interactions among genes and its mobile elements and also with their interactions with the bacterial hosts. Moreover, the misuse of antibiotics by humans intensifies the selection pressure, making the resistance even more pronounced.

Bacteriocins: as alternative way in the treatment of *S. aureus* infections

Bacteriocins are molecules usually produced by bacteria, that can be used with biopreservative applications (Bali et al., 2016) but mainly in an antibiotic role (Egan et al., 2017). These biomolecules can be differently classified over the gram-positive and gram-negative bacteria (Table 1).

It is suggested that the primary function of bacteriocins with regard to their killing ability is directly associated to maintaining the population around, in order to reduce the number of nutritional competitors in the environment. The targets for bacteriocins may have a broad spectrum of action similar to those of antibiotics (Table 2), preventing several biologically important phases in the cell. However, so far there has been no report on preventing vitamin biosynthesis (Cavera *et al*., 2015).

In relation to the increased rate of *S. aureus* resistance, bacteriocins show great potential as candidates that are able to overlap the function of a large number of antibiotics (Table 2) (Arumugam et al., 2019; Cavera et al., 2015; Ceotto-Vigoder et al., 2016). Several studies have described the bactericidal activity of bacteriocins that inhibits the growth of *S. aureus*, as observed in the Table 3. Varella *et al* (2017) described the inhibitory activity of seven bacteriocins in 165 strains of *S. aureus* in cases of bovine mastitis, showing a strong inhibition by epidermin (>85%) and a medium inhibition by aureocin A53 (>67%) (Varella Coelho et al., 2017). However, a combination of aureocin 70 and A53 showed a greater inhibitory potential compared to previous results (>91%). Still exploring cases of bovine mastitis, another study (Barboza-Corona *et al*., 2009) highlighted five bacteriocins derivate from *B. thuringiensis* that were tested against 50 strains of *S. aureus* recovered from milk of lactating cows. The results of the study presented data on the resistance of these strains to penicillin, dicloxacillin, ampicillin and erythromycin. However, all strains were susceptible to the five tested bacteriocins, showing them to be useful as an alternative approach to control bovine mastitis.

Currently, one bacteriocin has shown strong relevance in the treatment of bovine mastitis (Ceotto-Vigoder *et al*., 2016). The bacteriocin lysostaphin shows a minimal inhibitory concentration of 3.9 to 50 µg ml-1 compared to the usually used bacteriocin nisin, which was 15.6 to 500 µg ml-1. This study concludes that the treatment using lysostaphin alone or associated with nisin was efficient in promoting bacterial cell lysis.

Others studies investigating the role of biofilm formation in methicillin-resistant *S. aureus* (MRSA) strains, which has been observed to be able to alternate the resistance phenotype and thus attenuate the virulence (Pozzi *et al*., 2012), noted the effects of three bacteriocins. In this study, nisin A showed the highest bactericidal activity against planktonic and biofilm cells, while Lacticin Q showed lower activity. However, the Nukacin ISK-1 just showed bacteriostatic

activity against planktonic cell (Nissen-Meyer *et al*., 2009). Despite this, the results show that the bacteriocins used stand out as potent molecules effective in the treatment of MRSA infections (Okuda *et al*., 2013). Recently, cases of MRSA have been achieving prominence in the search for new bacteriocins. Jiang *et al*. (2017) observed the role of pentocin JL-1 bacteriocin showing effectiveness in both gram negative and positive bacteria (Jiang *et al*., 2017). Moreover, the authors also explored the ability of this bacteriocin to target the cell membrane of MRSA strains leading to the cell death. At the same time, another study devoted to the investigation of the pattern of bacterial strains that are capable of exhibiting antibacterial activity against MRSA, revealed one bacteriocin-like protein with a molecular mass of ~10 kDa produced by *P. aeruginosa* TA6 strain (Arumugam *et al*., 2019). Several analysis have highlighted the fact that, in addition to resistance to high temperature and various chemical compounds, it is a potent antimicrobial efficient against MRSA and a strong candidate for higher yielding and enhancement of bacteriocin production. In a different study trying to comprise the synergy of antimicrobials, a group of bacteriocins and some antibiotics were evaluated considering the two subtypes in order to understand the efficacy of bacteriocins vs antibiotics; these results show the effectiveness of nisin Z, pediocin pa-1, duracin 61 and reuterin in inhibition of MRSA (Hanchi *et al*., 2017). However, in the set of antibiotics used, only vancomycin displays effects against MRSA. Evaluation of synergistic activities of antimicrobial agents showed that the combination of duracin with nisin or pediocin is a strong strategy in the control of bacterial growth, thus demonstrating the important role of duracin 61A as an active bacteriocin against clinical drug-resistant MRSA.

Bacteriocins production: Perspective at the industrial scale

Interest in bacteriocins as well as their production has been growing over the years due to their use in food preservation, which exhibits antimicrobial activity as being an alternative to the use of chemical preservatives. In view of the vast amount of methods capable of purifying these biomolecules, two methods have been gaining more interest and becoming more effective in purifying bacteriocins: the Aqueous two-phase system (ATPS) and the Aqueous micellar two-phase system (AMTPS) (Jamaluddin *et al*., 2018). These systems can be formed by mixing in a solution with various components. ATPS are generally formed when two polymers that are inconsistent, i.e. polyethyleneglycol (PEG) and dextran or sodium sulfate, are diluted in water (Telis, 2012) (Figure 4). There are other types including, ionic liquids and short-chain alcohols. The result of purification from ATPS displays a higher yield (~70%) (Md Sidek *et al*., 2016) compared to the conventional method, for example the single gel filtration chromatography

(~1.0%). Interest has grown around this approach in the academic world, and recent studies have shown better results when the PEG / salt-based ATPS type is used, reaching yield values around 93% (Sabo *et al*., 2018).

In addition, the AMTPS results trying to improve the nisin extraction in the presence of electrolytes, show the advances in using the approach compared to conventional methods (Jozala *et al*., 2013).

Hence, the potential of ATPS and AMTPS as primary recovery methods for bacteriocins from a complex fermentation broth can be explored at an industrial scale, considering the easy handling and speed in obtaining these biomolecules, compared to the production stages already developed in the research laboratory.

A future applicability to *S. aureus*: bacteriocins becoming green?

Recent research on fermented vegetable extract has been shown to be a good strategy and a new application in the use of bacteriocins (Feng *et al*., 2017). Receiving the name of Fermented Plant Extract (FPE), this approach - most commonly performed as a plain liquid manure or plant extract can be used as a tool that assists in obtaining active substances providing a diversity of health benefits (Altay *et al*., 2013; Marsh *et al*., 2014). Some plants of the family Labiatae which contain a diversity of herbs were shown to have antimicrobial activity (Mahboubi *et al*., 2014). FEP are also described as being fermented microorganisms, which includes yeast and bacteria (Blandino *et al*., 2003; Manzanilla *et al*., 2006). This motivates the specific use of bacteria and plants, which may constitute a synergy in the antimicrobial treatment and a better understanding of the control by bacteriostatic phenotypes. The approach describing a composition comprising a bacteriocin and an extract from a plant is reported in a patent of Coyne *et al*. (Coyne *et al*., 2014). This kind of strategy has been gaining strength and are evidenced in numerous cases where the interruption of bacterial growth appears to be ineffective due to the use of these vegetable broths in conjunction with specific microbiotas (Marbun *et al*., 2016). In addition, extracts of tea and soybean showed dose-dependent growth inhibition of pathogens (Zhao and Shah, 2015). This study used phenolic-enriched milk (PEM), fermented with lactic acid bacteria (LAB) and ultrafiltered, concluding that multiple agents, such as bacteriocins secreted by LAB, may exhibit synergistic antibacterial activity. This evidence on the role of FPE reveal their importance and applicability not only considering their use for health, thus presenting a new approach in treatments for *S. aureus* infections, but also the production at an industrial scale, and amongst other considerations, the environment.

Conclusions

Our work concludes that bacteriocins are becoming increasingly important in the fight against infections by several microorganisms, especially *S. aureus*. The mechanisms of resistance to antibiotics explored here show the need to obtain new strategies to combat this pathogen. A number of bacteriocins having latent action spectrum against *S. aureus* are shown to be as effective as or even more so than conventional antibiotics as discussed herein. We believe that the information shown here is remarkably important for animal and human health, as well as to provide a means for production at an industrial scale besides creating new possibilities for future applications such as therapeutic methods.

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Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figure 1 :

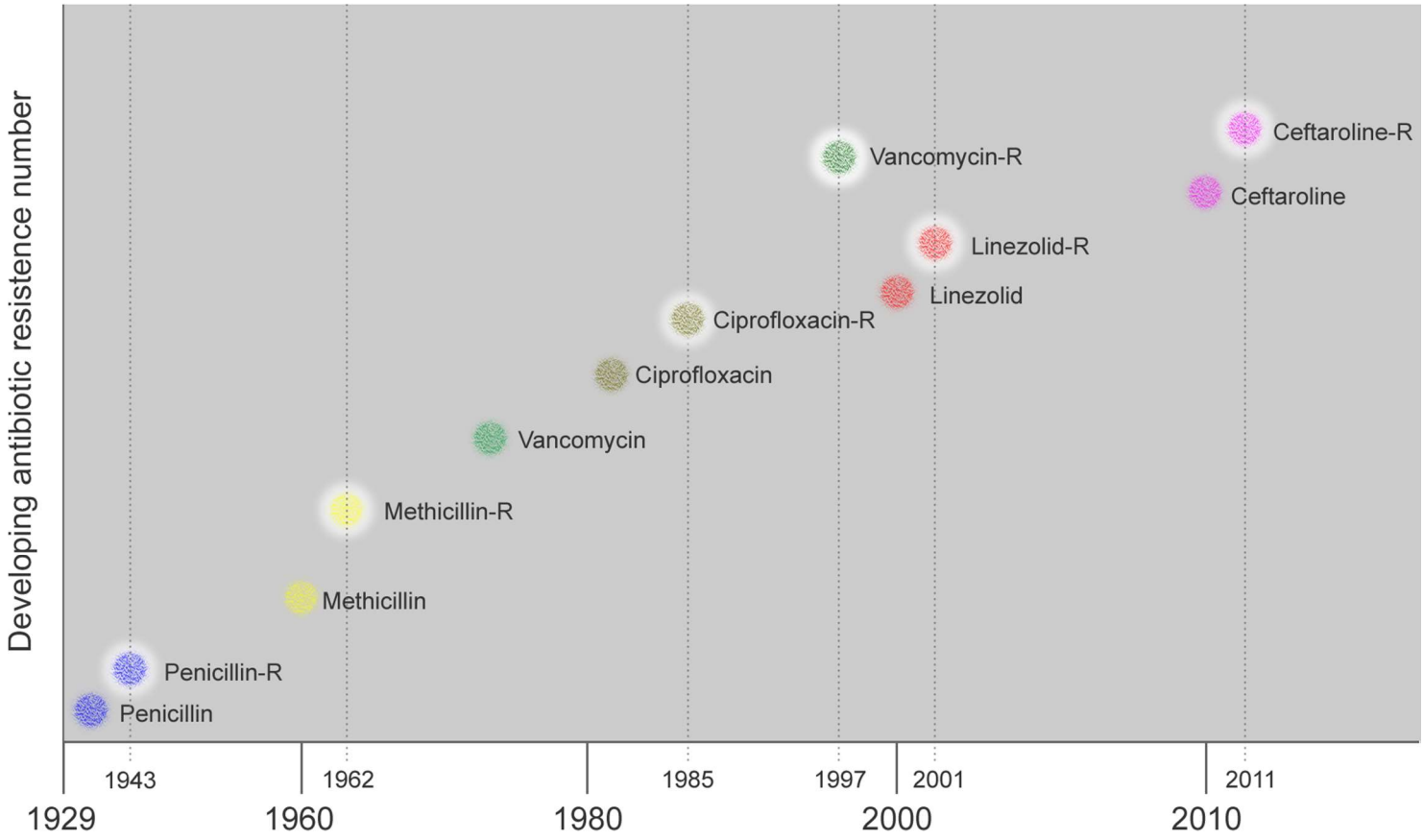
Developing antibiotic resistence number
Penicillin
Penicillin-R
Methicillin
Methicillin-R
Vancomycin
Ciprofloxacin
Ciprofloxacin-R
Linezolid
Linezolid-R
Vancomycin-R
Ceftaroline
Ceftaroline-R
1943
1962
1985
1997
2001
2011
1929
1960
1980
2000
2010

Figure 2 :

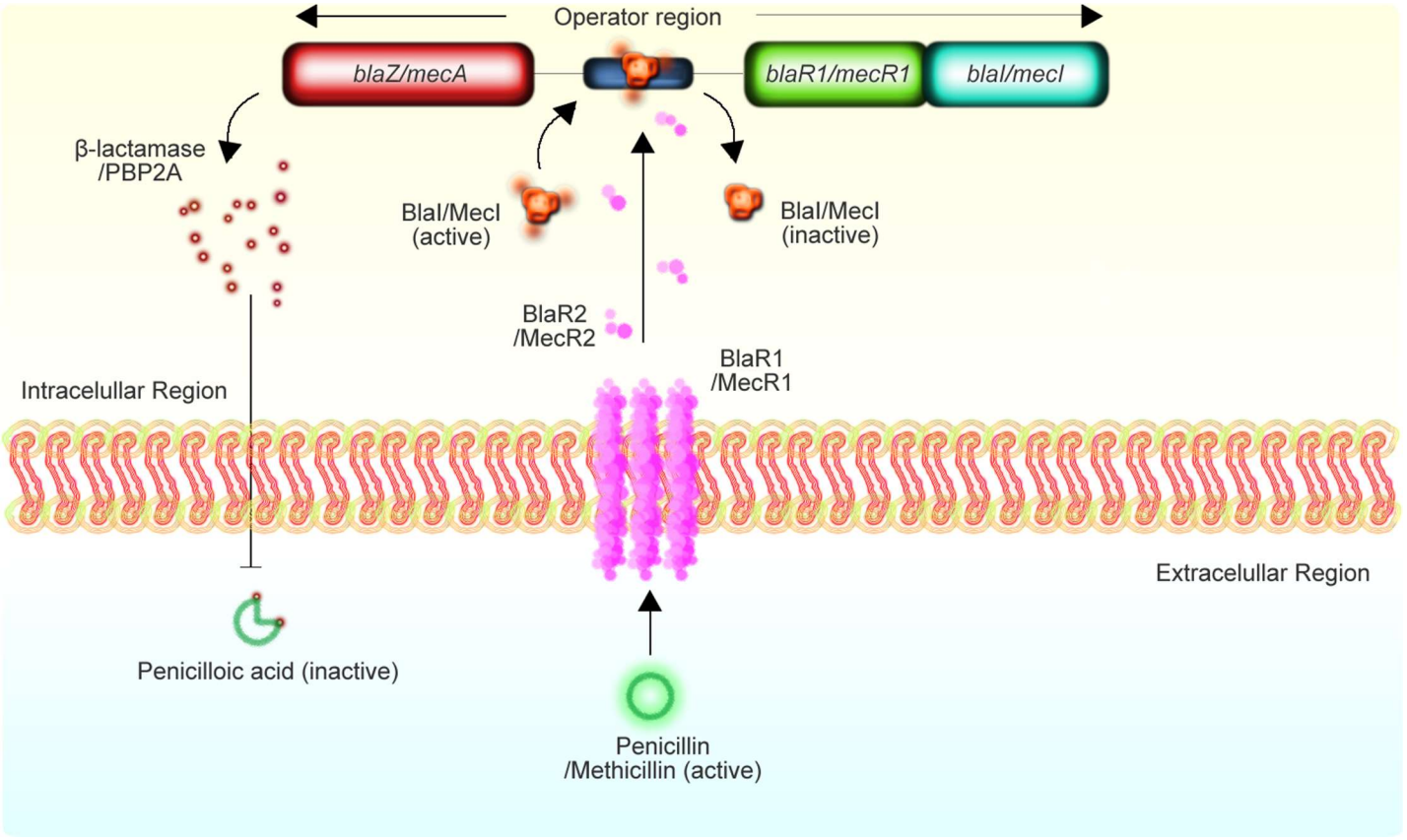
Operator region
blaZ/mecA
blaR1/mecR1
blaI/mecI
β-lactamase
/PBP2A
BlaI/MecI
(active)
BlaI/MecI
(inactive)
BlaR2
/MecR2
BlaR1
/MecR1
Intracelullar Region
Extracelullar Region
Penicilloic acid (inactive)
Penicillin
/Methicillin (active)

Figure 3 :

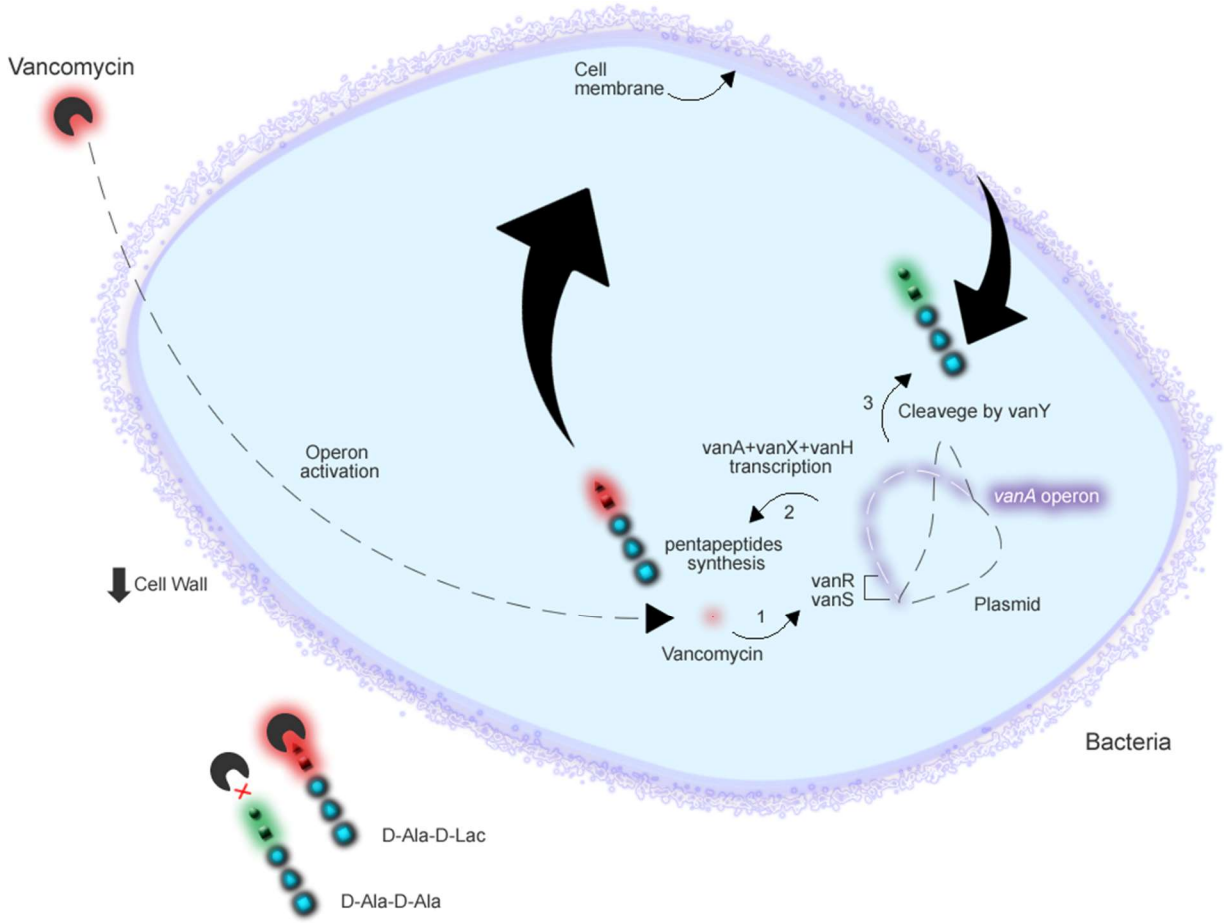
Vancomycin
Cell membrane
Operon activation
Cell Wall
pentapeptides synthesis
vanA+vanX+vanH transcription
2
3
Cleavege by vanY
vanA operon
vanR
vanS
Plasmid
1
Vancomycin
Bacteria
D-Ala-D-Lac
D-Ala-D-Ala

Figure 4 :

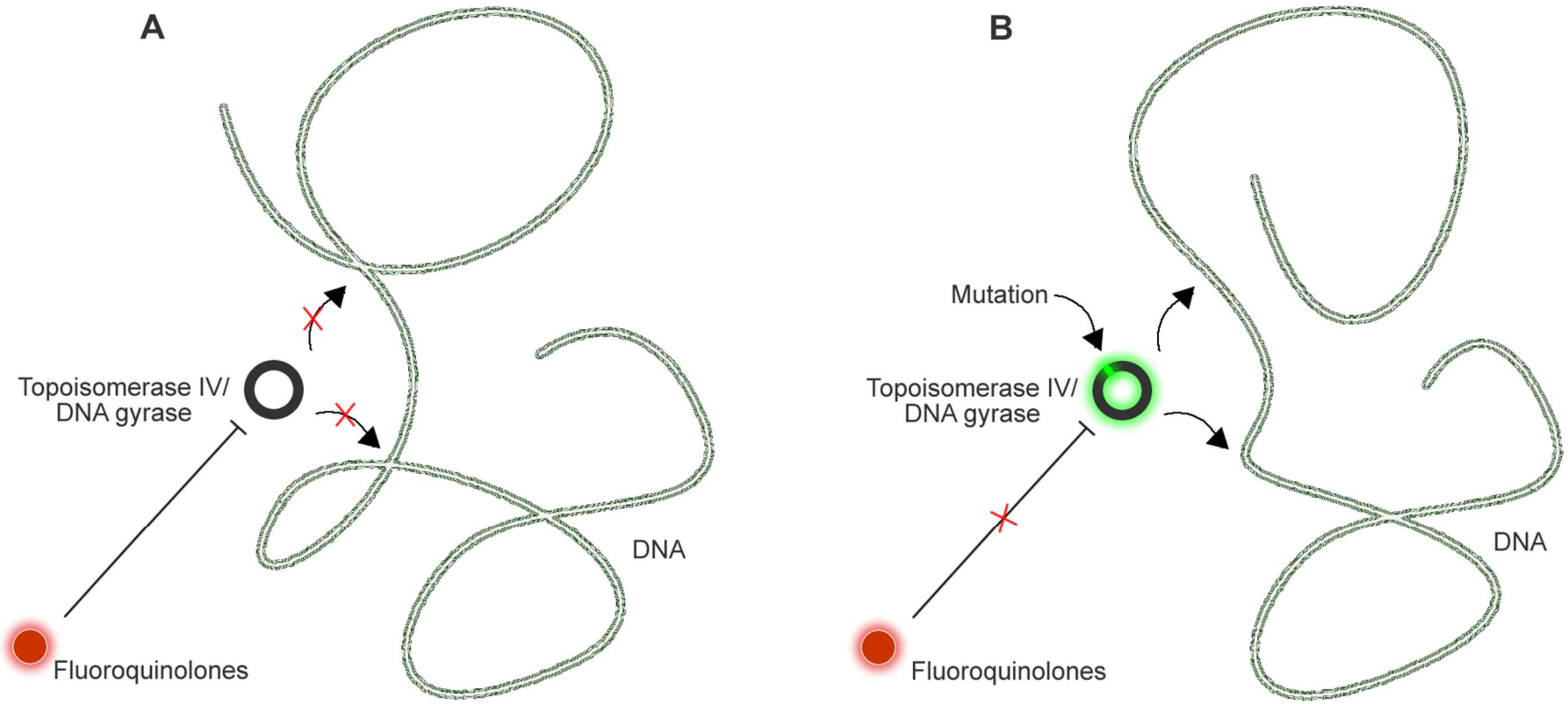
A
Topoisomerase IV/
DNA gyrase
DNA
Fluoroquinolones
B
Mutation
Topoisomerase IV/
DNA gyrase
DNA
Fluoroquinolones

Figure 5 :

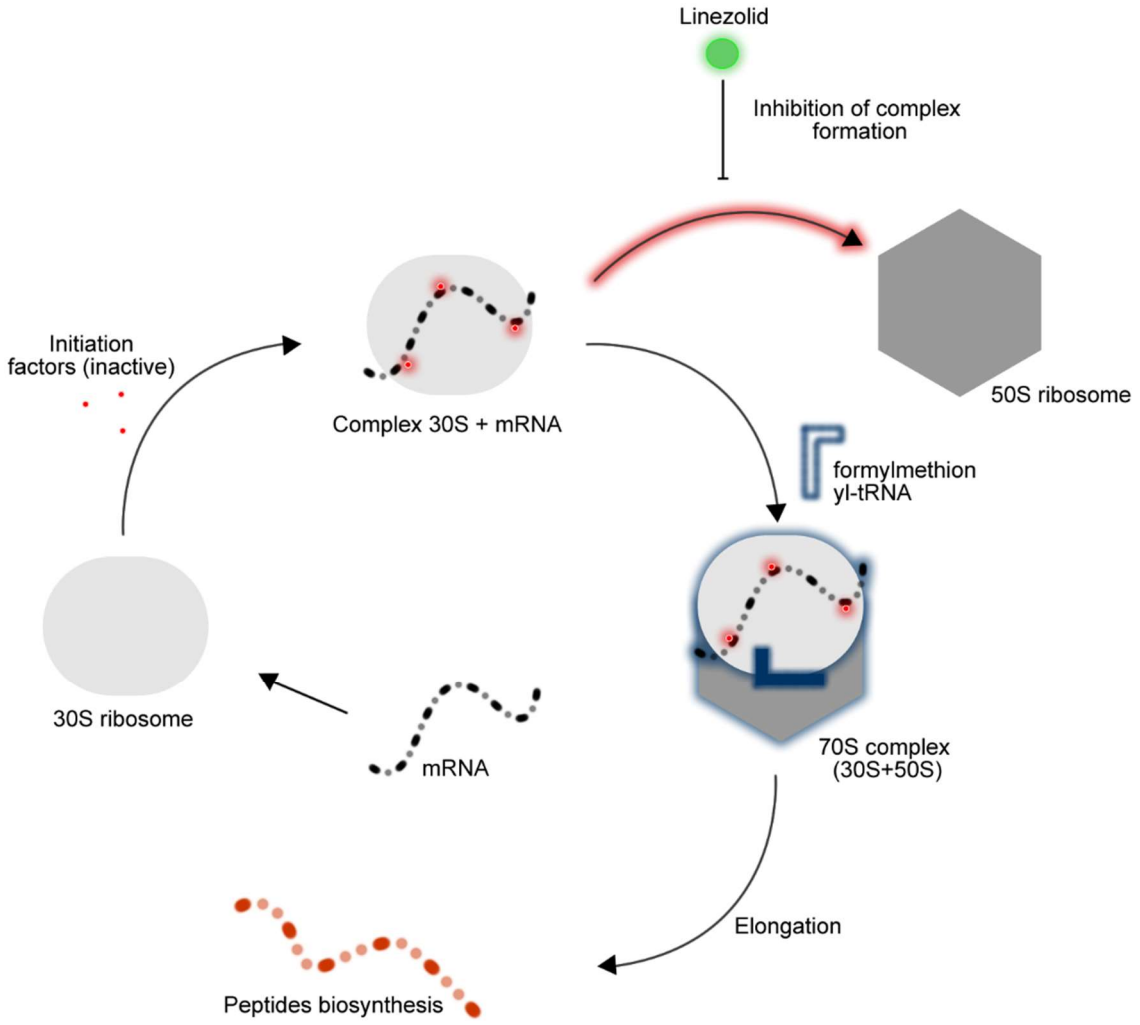
Linezolid
Inhibition of complex formation
50S ribosome
Complex 30S + mRNA
Initiation factors (inactive)
formylmethionyl-tRNA
70S complex (30S+50S)
30S ribosome
mRNA
Elongation
Peptides biosynthesis

Figure 6 :

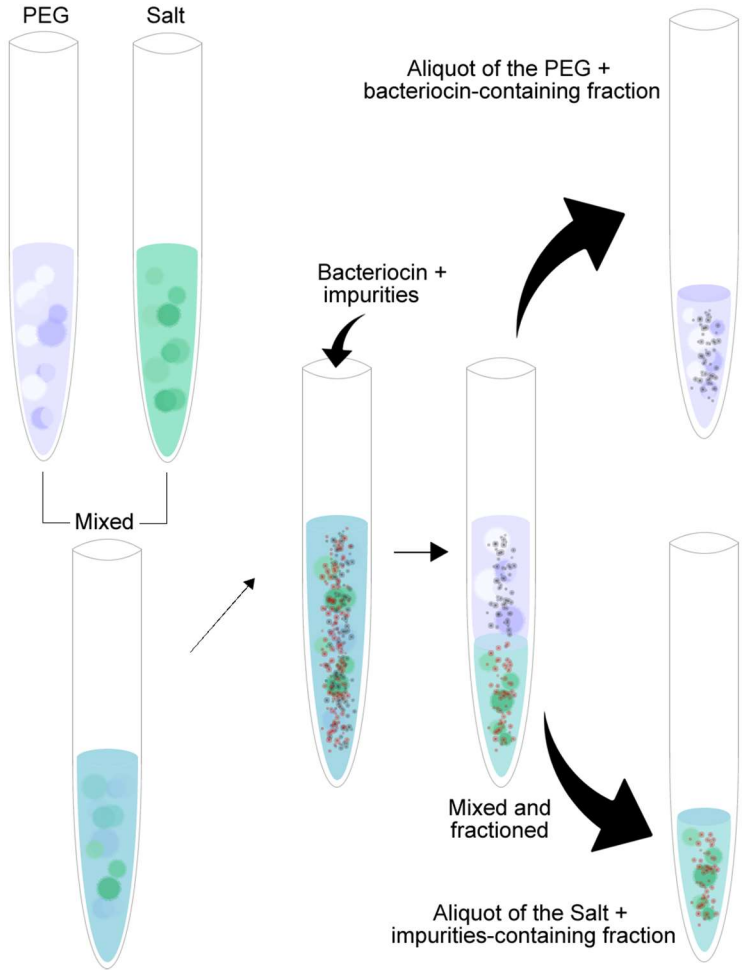
PEG
Salt
Mixed
Bacteriocin +
impurities
Aliquot of the PEG +
bacteriocin-containing fraction
Mixed and
fractioned
Aliquot of the Salt +
impurities-containing fraction

Figure 7 :

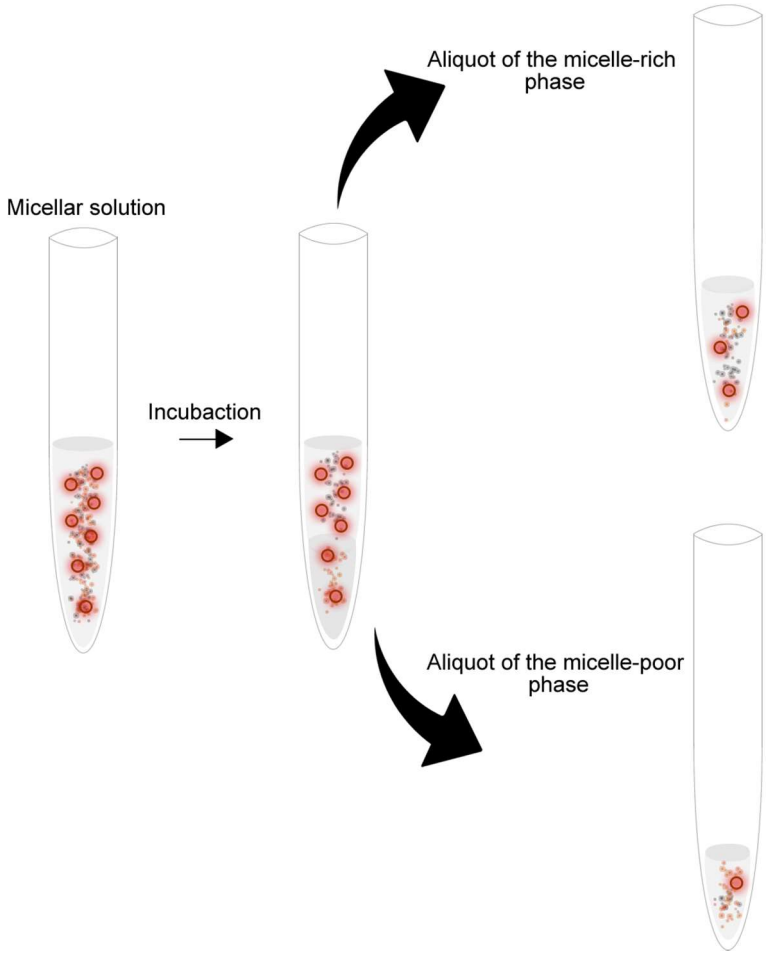
Aliquot of the micelle-rich phase
Micellar solution
Incubaction
Aliquot of the micelle-poor phase

Figure 7 :

Table 1. Classification of bacteriocins produced by gram-positive (Bierbaum and Sahl, 2009; Nissen-Meyer et al., 2009; Coelho et al., 2017) and gram-negative bacteria (Rebuffat, 2011).

	Classification	Features	Subclasses
Gram-positive	Class I (lantibiotics)	Small, heat-stable peptides (<5 kDa), containing modified amino acids (lanthionine, 3-methyl-lanthionine, dehydrated amino acids, S-aminovinyl-cystein, among others)	Type A (linear)
			Type B (globular)
			Type C (two components)
			Type D (reduced antimicrobial activity)
	Class II	Small, heat-stable peptides (<10 kDa), containing no modified amino acids	IIa (linear; pediocin-like)
			IIb (linear; two components)
			IIc (cyclic peptides)
			IId (linear)
			IIe (linear; more than two components)
	Class III	Large, heat-labile proteins	Type IIIa (bacteriolysins)
			Type IIIb (non-lytic)
	Class IV	Small (<10 kDa), circular peptides without posttranslationally modified amino acids and with an amide bond between the N- and C-terminal	-
	Class V	Small (<5 kDa), linear or circular peptides containing extensively posttranslationally modified amino acids with thioether bridges formed between α-carbon of other amino acid residues and the thiol groups of Cys residues	-
Gram-negative	Colicins	High molecular mass modular proteins (30–80 kDa)	-
	Microcins	Low molecular mass peptides (between 1 and 10 kDa)	-

Table 2. Biological functions affected by the action of bacteriocins and antibiotics.

Targeted biological functions	Bacteriocins	Antibiotics
Cell Wall Biosynthesis	Nisin A, nukasin ISK-1, NAI-107	β-lactams, glycopeptides
DNA Replication and Transcription	Microcin B17, colicins, carocin S2	Quinolenes
Membrane Disruption	Geobacillin I, bac-GM17, plantaricins, dysgalacticin, lactococcin, pediocin-like bacteriocins, mesentericin Y105, lacticin Q, nisin A, Uberolysin, AS-48 Bacteriocin	Lipopetides
Protein Synthesis	Colicins, cloacin DF13	Aminoglycosides, tetracyclines, chloramphenicol, macrolides
Septum Formation	Garvicin A, lactococcin 972	Benzamide derivade, N-heterocycles, phenols/polyphenols, carboxylic acids
Vitamins Biosynthesis	-	Sulfonamides

Table 3. Role of bacteriocins on preventing and control of the *S. aureus* growth.

Bacteriocins	Class	Organism source	Reference
Aureocins A70, A53 and 215FN (aureus) Pep5, Epidermin K7 and Epicidin 280 (epidermidis)	II and I	*Staphylococcus aureus* and *Staphylococcus epidermidis*	(Coelho *et al.*, 2007)
Morricin 269, Kurstacin 287, Kenyacin 404, Entomocin 420 and Tolworthcin 524	II	*Bacillus thuringiensis*	(Corona *et al.*, 2009)
Lysostaphin	III	*Staphylococcus simulans biovar staphylolyticus*	(Coelho *et al.*, 2017; SCHINDLER and SCHUHARDT, 1964)
Epidermicin NI01		*Staphylococcus epidermidis*	(Sandiford and Upton, 2012)
Pediocina PA-1	II	*Lactococcus lactis*	(Rodrıguez *et al.*, 2005)
Nisin A, lacticin Q, and Nukacin ISK-1	I	*Lactococcus lactis QU 5* and *Staphylococcus warneri ISK-1*	(Okuda *et al.*, 2013)
Lacticin 3147	I	*Lactococcus lactis subsp. lactis*	(Twomey,Wheelock, Flynn, Meaney, 2000)
Enterocin CCM 4231	II	*Enterococcus faecium CCM 4231*	(Laukova and Czikkova, 1999)
E 50-52 and OR-70	II	*Enterococcus faecium* NRRL B-30746 *and Lactobacillus salivarius* NRRL B-30514	(Svetoch *et al.*, 2008)
Duracin 61A and Reuterin		*Enterococcus durans* 61A and *Lactobacillus reuteri*	(Hanchi *et al.*, 2017)
Pentocin JL-1	I	*Lactobacillus pentosus*	(Jiang *et al.*, 2017)
TA6	II	*Pseudomonas aeruginosa* TA6	(Arumugam *et al.*, 2018)