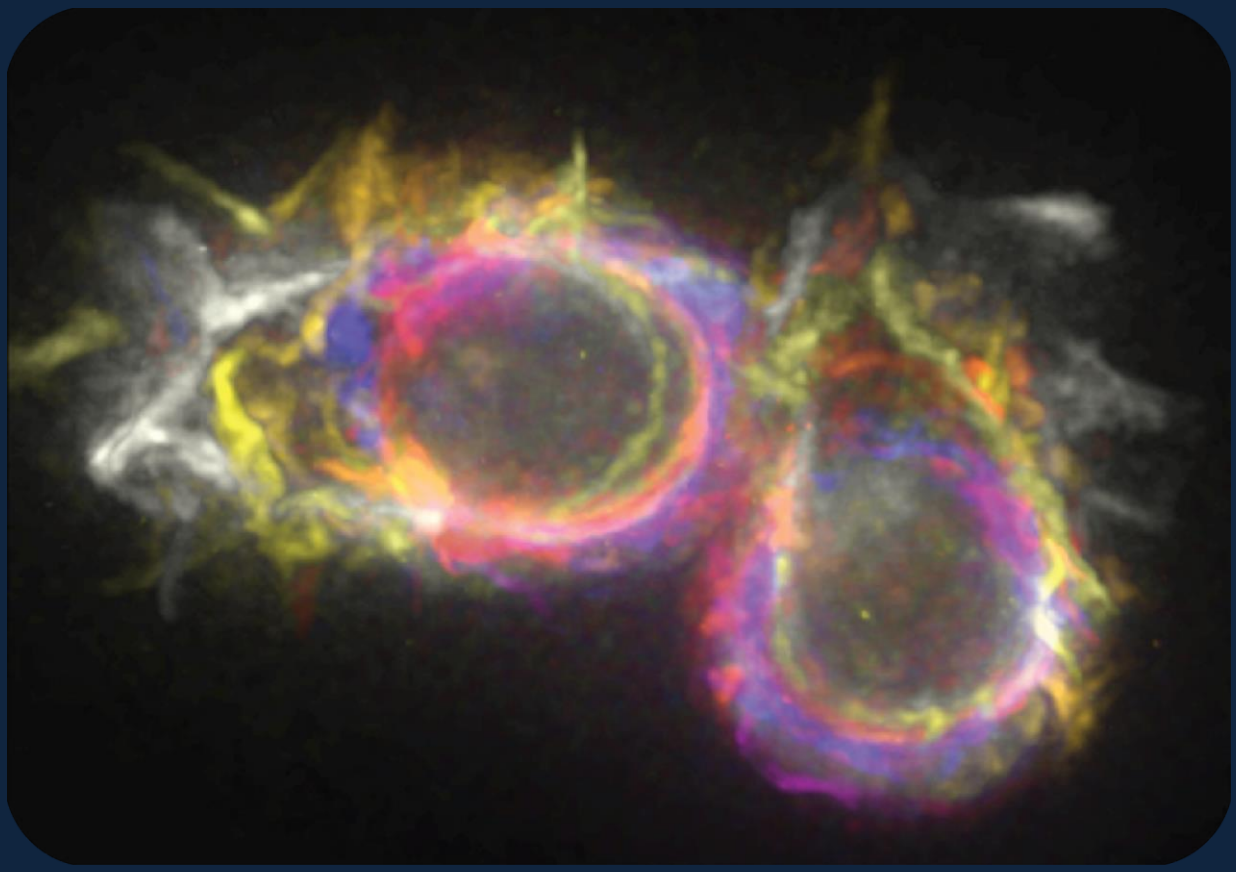
Bacteria present mechanisms to evade cellular and humoral responses mediated through peptidoglycan recognition by PGRP-SA and PGRP-LC

Filipa Baltazar da Costa Vaz



Dissertation presented to obtain the Ph.D degree in Biology

Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

Oeiras,
December, 2016

UNIVERSIDADE NOVA DE LISBOA

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Cover image

Temporal Color Code of *Drosophila melanogaster GFP-Moesin* macrophages phagocytosing *Staphylococcus aureus* NCTC 8325-4 strain.

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Acknowledgements

It seems that in Science great discoveries started with “Eureka!”, “This is interesting…” and similar expressions that translate excitement and curiosity. The first major discovery I did that put me on the track to some of the findings that I present in this thesis started with an expression that translates annoyance. As soon as my supervisor stepped into the lab, I followed him to his office and said “Sergio, I did something wrong”. After I told him the results of my last pull-down assay, without blinking, he looked at me and said “You didn`t do anything wrong, that makes total sense”. As I stared at him from his office door waiting for him to follow me to my bench, he added “I mean, I don´t think you did anything wrong in the experimental procedure”. For not doubting my results, when I am pretty sure everyone else would (and some did), I am profoundly grateful. This is something that even after all these years I had never expressed. It turns out that the following steps were the hardest. Sergio demanded me several experimental controls with repeats. After long and hard hours of work, I had ended any doubts that what I was observing was merely a result that was only reproducible in certain specific conditions. Naturally, I am thankful to Sergio for giving me the opportunity to do my PhD, for all the excellent conditions that he provided me and to allow me to pursue my sometimes (I admit it) crazy and out-of-the-box ideas, without any judgement. Also, I deeply appreciate that he gave me the freedom to perform experiments on my own initiative which sometimes were a little bit out of the aims of the work, just because… Just because I thought something was fun and wanted to try it! At his own terms and ways, with the interaction and working dynamics that I developed with him, I was able to learn from him how to build elegant experiments that give results which are convincing and explicit to others and to anticipate variables that can

interfere and tackle them from the beginning. If others cannot understand your ideas and the reason for presenting certain data, you will most likely not thrive, no matter how good or smart you may be. I hope in the future to continue developing such an exciting work (at least exciting for me) and Sergio certainly has a major impact in helping me to one day become (hopefully) a successful scientist.

Mariana Pinho, "the Lady next door", was the best neighbour I could have encountered. Her outsider-insider role in my work throughout all these years proved to be valuable in several moments. With Mariana I learnt to stay focused on a certain topic, to not allow myself diverge from the "main road", whilst keeping in mind all the observations that I would eventually need to interpret my data and/or to get "unstuck" from a puzzling data. I learnt that the success of a project relies in a sharp and objective mind where at all times it is key to determine very well what I aim to get with a certain experiment. In sum, she showed me that my time is valuable so I might as well spend it properly going straight to point. However, I admit that I still occasionally indulge myself in experiments just for fun and mere curiosity… I guess some things never really change!

I am very grateful to Petros because he invested in me, in my work, he believed in my data and he received me as a member of his Lab. Without him, probably I would have never been able to finish this work the way I did and without his investment in my study of the macrophages I would probably have never been able to find the role of the lytic activity of PGRP-SA. Thank you very much for everything! The faith and enthusiasm that you deposited in me was something that I had never been given before. It was indeed a great experience to go through. I will certainly remember this for my future and I hope to be able to one day provide similar experience to others.

To Maria João Gravato-Nobre, you are a sunshine in the Department of Biochemistry, a great friend and you opened me the doors

to figure out the optimum conditions for the Zymography assays of PGRP-SA. All the optimisations I performed using other extracts and enzymes, had the secret goal to prove the lytic activity of PGRP-SA. I remember your words “I saw and felt that you were living a moment that many people work for many years in Science and never live.” I am glad you could put words into that moment, because even today I still cannot quite describe it. Thank you!

To Dr. Jonathan Hodgkin, thank you for being so welcoming by letting me use your lab space and trusting in me without knowing me at all, that I would be able to do the zymopgraphy experiments for Maria`s paper. Thank you for reading my thesis and all the support for my next adventure!

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suggestions and critics that truly helped me to seat down and improve it to what it is now here presented.

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Thank you all!

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List of abbreviations and acronyms

µL	microlitro
Ala	Alanine
AMP	Antimicrobial peptide
CFU	Colony Forming Unit
CHAP	cysteine, histidine dependent amidohydrolase/peptidase domain
ChBD	Choline binding domain
CM	Cytoplasmic membrane
CW	Cell wall
Cys	Cysteine
DAP	meso-diaminopimelic acid
DD	Death domains
dIAP-2	Drosophila Inhibitor of apoptosis-2
Dif	Dorsal-related immunity factor
DREDD	Apical caspase death-related Ced-3/Nedd2-like protein
Dscam	Down syndrome cell adhesion molecule
dSR-CI	*Drosophila* scavenger receptor class C, type I
ERM	Ezrin, radixin and moesin
FADD	The Drosophila homologue of FAS-associated death-domain protein
Fig.	Figure
GFP	Green Fluorescent Protein
Glc*N*Ac	N-acetylglucosamine
Glu	Glutamic acid
Gly	Glycine
GNBP	Gram-negative binding protein

GroP	Glycerol phosphate
h	Hour
HF	Hydrofluoric acid
HPAEC-PAD	High Performance Anion Exchange Chromatography coupled with Pulsed Amperometric Detection
HPLC	High Performance Liquid Chromatography
IIS	Innate Immune System
IKK	IkB kinase
IM	Inner Membrane
IMD	Immune deficiency pathway
IS	Immune System
kDa	Kilodalton
LA	Luria Agar
LB	Luria Broth
LPS	Lipopolysaccharide
LTA	Lipoteichoic Acids
Lys	L-Lysine
LysM	Lysin motif
LYZ2	Lysozyme subfamily 2
M	Molar
M1	Mutanolysin
MAMP	Microbe Associated Molecular Patterns
Man*N*Ac	N-acetylmannosamine
MDP	muramyl-dipeptide
mg	miligram
mL	mililitro
ModSP	Modular Serine Protease
Moe	Moesin

Mur*N*Ac	N-acetylmuramic acid
MurNAcLAA	N-acetylmuramoyl-L-alanine amidase
MYD88	Myeloid differentiation primary response protein 88
N/A	Not applicable
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NimC4/SIMU	Nimrod C4/Six-microns-under
Nod2	Nucleotide-binding oligomerization domain-containing protein 2
ns	Not significant
O/N	overnight
OD	Optical density
OM	Outer Membrane
OMP	Outer Membrane Protein
PAMP	Pathogen Associated Molecular Patterns
PBPs	Penicillin Binding Proteins
PCD	Programmed Cell Death
PGN	Peptidoglycan
PGN-BLD	PGN-Binding/Lytic domain
PGRPs	Peptidoglycan Recognition Proteins
PPO	Prophenoloxidase Cascade
PRR	Pattern-recognition receptors
PSH	Persephone
RboP	Ribitol phosphate
RHD	Rel Homology domain
RHIM	RIP homotypic interaction domain
RIP	Receptor-interacting protein
ROS	Reactive Oxygen Species
sec	second

seml	*semmelweis*
SH3	Src homology 3
SH3b	Bacterial SH3
SLT	transglycosylases SLT domain 1
SPE	Spatzle processing enzyme
Spz	Spatzle
TAB2	TAK1-associated binding protein 2
TAK1	Transforming growth factor -β- activated kinase 1
TCT	tracheal cytotoxin
TF	Transcription factor
TGL	transglycosylases-like domain
TIR	Toll/interleukin-1 receptor
TLR	Toll-like Receptor
TNF	tumour necrosis factor
TRIF	TIR domain-containing adaptor protein inducing IFNβ
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
WTA	Wall Teichoic Acids

Summary

The Host presents different innate immune components to fight bacterial infections, most of which are evolutionary conserved strategies. Conversely, Bacteria present numerous mechanisms of virulence and evasion, transversal to different bacterial species, that confer resistance or subvert the activity of the Host components. This thesis presents a study on how the Host perceives the Bacteria and reacts to them and how the Bacteria protect themselves from those responses.

Peptidoglycan (PGN) is a ubiquitous cell surface bacterial polymer. It is composed of glycan chains cross-linked through peptide bridges. The residue at the third position of the peptide moiety shows the highest variability among the different PGNs. Nevertheless, most bacteria possess either a lysine (Lys) or a *meso*-diaminopimelic acid (DAP) residue.

The Host components that act on the PGN, range from enzymes that degrade it to receptors that upon recognition initiate downstream signalling cascades leading to the production of antimicrobial peptides. The Peptidoglycan Recognition Proteins (PGRPs) are evolutionary conserved innate immune receptors in Invertebrates and Vertebrates, including Humans. In *Drosophila melanogaster*, PGN is the main bacterial component that triggers an immune response and it is recognised by the PGRPs. According to the current model of bacterial recognition, the PGRPs possess discriminatory ability between Lys- and DAP- type PGN. PGRP-SA, *in vivo*, binds exclusively to Lys-type bacteria, whereas PGRP-LC specifically binds to DAP-type bacteria.

Previous work from the Host Labs[1] has shown that bacteria

[1] Filipe Lab, Laboratory of Bacterial Cell Surfaces and Pathogenesis, Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa.
Ligoxygakis Lab, Laboratory of Cell Biology, Development and Genetics, Department of Biochemistry, University of Oxford.

present components at the cell surface that impair detection of the PGN and thus compromise host survival [1], [2][2]. The wall teichoic acids (WTA) are phosphate-rich polymers of the Gram-positive cell wall that exert stereo hindrance by shielding the PGN from PGRP-SA [1]. Atl is an autolysin that cleaves PGN in order to sustain cell growth and division. In the absence of this enzyme, the bacteria accumulate cell surface exposed PGN which is recognised by PGRP-SA and allows the survival of the Fly [2].

From the Bacteria side, I aimed to understand: 1) whether WTA are a transversal bacterial evasion mechanism that impair PGN recognition by Host receptors; 2) whether other autolysins could impair the binding of PGN by PGRP-SA and contribute to evasion. From the Host side, I aimed to ascertain the discriminatory ability of PGRP-SA and PGRP-LC towards the Lys and DAP residues. Why are two major immune receptors limited in bacterial recognition due to the discrimination of two residues? As the work evolved, the data suggested that PGRP-SA could engage in cellular responses. Thus, the final goal of my work was to evaluate the role of PGRP-SA in phagocytosis.

As an overall, the work here presented asks for a re-evaluation of the current model of bacterial recognition and confirms the importance of cellular responses as a first line of defence, followed by the prolonged and protective humoral responses. In addition, I show that recognition of PGN is paramount for the triggering of efficient responses by both the cellular and humoral responses.

I show that PGRP-SA has access to PGN fragments that are

[2] [1] M. L. Atilano, J. Yates, M. Glittenberg, S. R. Filipe, and P. Ligoxygakis, "Wall teichoic acids of staphylococcus aureus limit recognition by the drosophila peptidoglycan recognition protein-SA to promote pathogenicity," *PLoS Pathog.*, vol. 7, no. 12, 2011.

[2] M. L. Atilano, P. M. Pereira, F. Vaz, M. J. Catalão, P. Reed, I. R. Grilo, R. G. Sobral, P. Ligoxygakis, M. G. Pinho, and S. R. Filipe, "Bacterial autolysins trim cell surface peptidoglycan to prevent detection by the drosophila innate immune system," *Elife*, vol. 2014, no. 3, pp. 1–23, 2014.

temporarily surface exposed during the bacterial cell division. Thus autolysins do play a role in the impairment for PGN detection. If their activity is compromised, there is accumulation of PGN at the cell surface. Particularly, Atl and Sle1 have a great impact in cell division. Besides Atl, Sle1 also contributes to impair PGN detection. In the absence of Sle1, there is the accumulation of newly synthesised PGN fragments at the septum which are recognised by PGRP-SA. However, the amount of PGN that is recognised is fundamental for the triggering of a potent immune response. Therefore, paradoxically, Sle1 does not seem to contribute for virulence regarding PGN recognition. I propose that it is the sum of the autolysins activity that can properly impair the detection of PGN during cell division, albeit the Atl protein presents a more preponderant role

I show that PGRP-SA and PGRP-LC are promiscuous for the Lys and DAP residues of the PGN and both participate in the immune responses upon infection. When the PGN is detected, both PGRPs act as opsonins and engage in the phagocytic and clearance processes. In particular, PGRP-SA has optimal binding ability and presents lytic activity at low pH, indicating that it may act as an enzyme inside the phagolysosomes. After phagocytosis, the macrophages seem to present two ways of clearance: phagocytosis-exocytosis or phagocytosis-apoptosis. Through induction of apoptosis, the macrophages may be able to incorporate the internalised bacteria in the apoptotic bodies which will be phagocytosed. The bacterial destruction can then be achieved through a new phagocytosis process. This strategy of phagocytosis-apoptosis is likely to occur until complete bacterial clearance.

Concomitantly, the clearance is dependent on the degradation of the PGN, which is compromised by the WTA. Therefore, the WTA are a dual strategy of immune evasion. At a first level, they impair the primary recognition by both PGRPs which is crucial to trigger the phagocytic processes (and also the induction of antimicrobial peptides). At a second

level, they impair the processes of clearance by impairing the activity of these PGRPs (and perhaps by other Host components).

I propose that it is the accessibility of the PGRPs through the cell wall that determines the PGN recognition and not the discrimination between the DAP and Lys residues. Furthermore, I propose that the WTA of the Gram-positive bacteria and the outer membrane of the Gram-negative bacteria, are conserved bacterial immune evasion strategies towards the recognition and activity of PGN host receptors. In addition, I propose that the macrophages present two pathways of clearance: phagocytosis-exocytosis and phagocytosis-apoptosis. The activation of either pathway is in turn dependent on the bacterial type and the factors that they possess that interfere with an efficient phagocytosis-clearance process.

Sumário

O Hospedeiro apresenta inúmeras estratégias de resposta inata contra a Bactéria. Estas estratégias estão presentes em diferentes Hospedeiros por serem conservadas ao longo da evolução das espécies. A Bactéria, por seu turno, apresenta diferentes mecanismos de virulência e de evasão que permitem a sobrevivência e o estabelecimento de uma infeção no Hospedeiro. Estas estratégias são transversais a diversas espécies de bactérias. A presente tese de doutoramento descreve um estudo sobre o modo como o Hospedeiro perceciona e reage à Bactéria e o modo com que esta se protege de tais respostas.

O peptidoglicano (PGN) é um polímero ubiquitário da superfície das bactérias. É composto por cadeias polissacarídeas que se interligam por pontes peptídicas. A terceira posição da cadeia peptídica alberga o aminoácido mais variável dentro dos vários tipos de PGNs. Ainda assim, na maioria dos casos, encontra-se ou uma Lisina (Lys) ou um ácido diaminopimélico (DAP).

O PGN é detetado por componentes do Hospedeiro que iniciam respostas imunitárias. Estes componentes compreendem quer enzimas que degradam o PGN, quer recetores que o reconhecem e ativam cascatas imunitárias que conduzem à expressão de péptidos antimicrobianos. As "Peptidoglycan Recognition Proteins" (PGRPs) são recetores evolutivamente conservados do Sistema Imunitário Inato de Invertebrados e Vertebrados, inclusive o Homem.

O PGN é o principal componente bacteriano que inicia respostas imunitárias na mosca *Drosophila melanogaster*. Segundo o modelo de reconhecimento do PGN em *D. melanogaster*, as PGRPs possuem especificidade para os aminoácidos Lys ou DAP. A PGRP-SA, *in vivo*, liga-se exclusivamente à Lys e a PGRP-LC ao DAP.

Os Grupos de Investigação que participaram no presente trabalho[1], verificaram anteriormente que as bactérias apresentam componentes à sua superfície que impedem o reconhecimento do PGN com consequências na sobrevivência da mosca [1], [2][2]. Os ácidos teicóicos da parede das bactérias Gram-positivas (WTA – "Wall teichoic acids") são polímeros de fosfato que impedem o reconhecimento do PGN pela PGRP-SA por comporem uma camada que impede o acesso da proteína ao ligando [1]. A "major autolysin" Atl, cliva o PGN para promover a duplicação e a divisão da célula. Na ausência do Atl, a bactéria acumula à sua superfície fragmentos de PGN que são reconhecidos pela PGRP-SA o que resulta na sobrevivência da Mosca.

Pelo lado da bactéria, o meu trabalho de doutoramento teve dois objetivos: 1) avaliar se o efeito dos WTA é um mecanismo bacteriano conservado que permitem a evasão do reconhecimento do PGN; 2) se outras autolisinas impede o reconhecimento do PGN pela PGRP-SA como mecanismos de evasão. Concomitantemente, procurei averiguar a capacidade discriminatória da PGRP-SA e da PGRP-LC ao PGN. Por que motivo estes recetores centrais à resposta antibacteriana, encontram-se limitados na deteção devido à especificidade a uma molécula? Com o avançar do trabalho, os resultados obtidos levantaram a hipótese de que a PGRP-SA participa em respostas celulares. Com efeito, o trabalho culminou com o objetivo de avaliar a função da PGRP-SA no processo de fagocitose.

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[1] Filipe Lab, Laboratory of Bacterial Cell Surfaces and Pathogenesis, Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa.
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As conclusões do trabalho constante nesta tese pedem que a comunidade científica reconsidere o modelo atual de reconhecimento das bactérias e intensifica a importância das respostas imediatas celulares como primeira linha de defesa, seguidas pelas respostas prolongadas e protetoras humorais. O trabalho aqui presente demonstra ainda que o reconhecimento do PGN é crucial para a ativação de respostas imunitárias celulares e humorais eficientes contra as bactérias.

O meu trabalho demonstra que a PGRP-SA reconhece fragmentos de PGN que são temporariamente expostos à superfície durante a divisão das células. Por conseguinte, as autolisinas assumem um papel importante na protecção do reconhecimento desta molécula. Quando a actividade das autolisinas é comprometida de tal modo que a célula não consegue controlar a exposição do PGN durante a divisão, fragmentos de PGN recentemente sintetizados não são prontamente incorporados no polímero da macromolécula e por isso acumulam-se à superfície. Particularmente, a ausência do Atl e do Sle1 têm um forte impacto na divisão da bactéria. Com efeito, para além do Atl, também o Sle1 assume um papel importante contra o reconhecimento pela PGRP-SA. Na ausência do Sle1, há acumulação de fragmentos de PGN recentemente sintetizados na região septal que são reconhecidos pela PGRP-SA. Porém, a quantidade de PGN que é reconhecida revela-se crucial para a ativação de uma resposta eficiente. Por conseguinte, paradoxalmente, o Sle1 não contribui para a virulência da bactéria através da evasão ao reconhecimento do PGN. Por fim, eu proponho um modelo que se baseia no todo das atividades das autolisinas que eficientemente conseguem impedir que uma grande quantidade de PGN esteja acessível ao reconhecimento, sendo que o Atl assume um papel preponderante.

Eu demonstro que a PGRP-SA e a PGRP-LC reconhecem quer a Lys quer o DAP do PGN e que ambas participam nas respostas imunitárias contra os dois tipos de bactérias. Aquando da deteção do PGN, ambas as

PGRPs atuam como opsoninas e participam nos processos de fagocitose e degradação da bactéria. A PGRP-SA demonstrou ser uma potencial enzima lítica que atua em condições acídicas, indicando que deverá exercer atividade no fagolisosoma. Após a fagocitose, os resultados sugerem que os macrófagos seguem uma de duas estratégias para a degradação da bactéria: fagocitose-exocitose ou fagocitose-apoptose. Através da indução da apoptose, os macrófagos potencialmente podem conter as bactérias dentro dos corpos apoptóticos que serão por seu turno fagocitados. As bactérias serão eliminadas ao sofrerem uma nova fagocitose. É provável que este processo de fagocitose-apoptose se repita até se atingir a destruição total das bactérias.

Por outro lado, a degradação do PGN é essencial para a destruição da bactéria e os WTA comprometem e dificultam a clivagem do PGN. Assim sendo, os WTA atuam a dois níveis contra as defesas do Hospedeiro. Primariamente, eles impedem o reconhecimento pela PGRP-SA e PGRP-LC e, por conseguinte, a ativação do processo de fagocitose (e a indução da expressão de péptidos antimicrobianos). Quando o Hospedeiro ultrapassa esta barreira de reconhecimento, os WTA atuam contra a segunda linha de resposta que é a destruição da bactéria após fagocitose, ao impedirem a ação da PGRP-SA e PGRP-LC (e provavelmente de outros componentes).

Em suma, eu proponho um modelo de reconhecimento segundo o qual é a acessibilidade das PGRPs através da parece celular que determina o reconhecimento do PGN. Eu proponho que os WTA das bactérias Gram-positivas e a "outer membrane" das bactérias Gram-negativas são estratégias bacterianas conservadas de evasão ao Sistema Imunitário. Por fim, proponho que possivelmente os macrófagos apresentam duas vias de degradação de microrganismos: fagocitose-exocitose e fagocitose-apoptose. A ativação de uma das vias está por sua vez dependente do tipo de bactéria e de fatores que possuem que dificultam a eficiente destruição por parte dos macrófagos.

Thesis at a glance

Chapter I. The Bacteria and the Host

- Are autolysins redundant in impairing access to surface exposed peptidoglycan by PGRP-SA?
- Are PGRP-SA and PGRP-LC specific towards Lys and DAP residues of the peptidoglycan?
- Are WTA a transversal Gram-positive mechanism to shield different peptidoglycans from different PGRPs?
- Is PGRP-SA involved in phagocytosis?

Chapter II. Recognition of peptidoglycan determines efficient antibacterial responses

- PGRP-SA recognises PGN that is temporarily exposed at the cell surface during cell division, which can be impaired by the temporal and spatial activity of the autolysins.
- Sle1 appears to be crucial for cleavage of septal PGN to allow septum re-shaping upon splitting of the cells.
- Recognition of peptidoglycan is key for efficient antibacterial responses.

Chapter III. PGRP-SA and PGRP-LC recognise both Lys and DAP residues of the peptidoglycan *in vivo*

- *In vitro* assays showed promiscuity of the PGRPs towards Lys and DAP residues.
- *In vivo* assays showed that the recognition of both PGN types by both PGRPs is paramount for host survival.
- WTA impair recognition of both peptidoglycan types by both PGRPs.

Chapter IV. PGRP-SA is involved in bacterial clearance

- PGRP-SA and PGRP-LC participate in phagocytosis and clearance.
- PGRP-SA has lytic activity towards different peptidoglycans.
- Apoptosis is a mechanism to promote clearance.

Chapter V. Bacteria present mechanisms to evade cellular and humoral responses mediated through peptidoglycan recognition by PGRP-SA and PGRP-LC

- The access through the cell wall to the peptidoglycan determines the detection by PGRPs.
- PGRP-SA and PGRP-LC play dual roles in phagocytosis and humoral responses.
- Bacteria possess conserved mechanisms that impair accessibility to the PGN.

CHAPTER I

The Bacteria and the Host

The event of the first cell was paramount in shaping all the subsequent steps of Evolution of Life. The first cell was the first individual and self-sufficient structure that presented defined boundaries, raising the concepts of intra- and extracellular environment. Thus, it constituted the primordial individualized entity that clearly presented a "self" and therefore a "non-self" – the surrounding environment and other cells that were grown from it, identical but not the same individuality.

Colonial organisms evolved to multicellularity, which can be described as whole organisms that in their own self are composed of many non-self-individualities. Evolution of multicellularity culminated in organisms composed of interconnected systems and finally the idea of self and non-self was fully stablished and thus shaped the evolution of the Immune System (IS).

As an integrated system in an organism, like the other systems, the IS guarantees and maintains homeostasis, *i.e.* it helps to maintain a state of equilibrium in the organism. Particularly, the IS plays the role of protecting the organism against danger [1]. As the scientific knowledge grew, we became aware of the two branches of the IS – the Innate and the Adaptive. Whereas only the Vertebrates possess an Adaptive Immune System, the most basic mechanisms of an Innate Immune System (IIS) are found to some extent in almost all life forms across the Eukarya Domain. The IIS is a constant vigilant of danger signal and is the first line of defence in an organism.

The surveillance and detection of invading microorganisms relies on an evolutionary conserved system of receptors that are constitutively expressed, non-clonal and independent of immunological memory. They

are collectively known as Pattern-recognition receptors (PRR) and recognise specific structures in the microorganisms, *i.e.* they detect danger through discrimination of non-self [2]. Whether the detection of danger is followed by an immune response is dependent on the confirmation of the danger signalling, which is in turn dependent on how the microorganism is perceived. It is within these interactions that two concepts became implemented: a "Host organism"[1] and a "pathogenic organism"[2].

These evolutionary conserved PRR detect conserved microbial structures that are specific of a class of microbe and absent in the Host. These microbial structures are designated as Pathogen Associated Molecular Patterns (PAMP) [2], however nowadays they are also commonly referred to as Microbe Associated Molecular Patterns (MAMP), since these structures are not specific of pathogenic microorganisms [3], [4]. As PAMP are not present in the Host and are conserved in a class of microbes, it is the logical that they are crucial for the microbial life. Hence, the pathogens present various mechanisms to avoid the detection of their PAMP by PRR. The Host-pathogen interaction can be viewed as a battlefield that in extreme cases, upon failure of efficient recognition and response, can culminate with either the survival of the Host or of the pathogen.

As the definition of boundaries in a cell, the cell surface, was of extreme importance to evolution of Life, again we find that it is at the surface of the cells that most immune responses begin. Upon breaching of the Host surface, many of the first encounters of a PRR and a pathogen happen mainly at the level of the microbial cell surface. Thus, many PRR are specific for PAMP that are components of the outer surface of the pathogens – viruses, bacteria, fungi and parasites.

[1] An organism that harbours another organism to which provides nutrients and shelter.
[2] An organism that can cause disease in a Host.

The basic mechanisms underlying Innate Immunity are conserved among Plants, Invertebrates and Vertebrates, including Humans [5]. Moreover, the virulence determinants and strategies that bacteria employ against Host defences are also conserved among a group of pathogens, such as Bacteria [6], [7]. Therefore, it is possible to study fundamental questions in one organism and extrapolate to others. Hence, the concept of model organisms became a foundation for studying processes and mechanisms both in Microbiology and Immunology.

The work presented in this thesis describes a study on Host-Bacteria interactions. As an overall, the work aimed to understand how a certain type of PRRs – the Peptidoglycan Recognition Proteins (PGRPs) – recognise a ubiquitous bacterial component, the peptidoglycan, and how Bacteria can prevent this recognition. The study of the bacterial factors that evade the IS was focused in the bacteria *Staphylococcus aureus* and *Bacillus subtilis* and the *in vivo* studies of the PGRPs recognition were conducted in the fruit fly *Drosophila melanogaster*. In this chapter, I address the current knowledge on the structure and biology of the bacterial surface, particularly the peptidoglycan, and a brief state of the art of the *Drosophila* immune responses. Finally, I present the goal of my PhD project and an overview of the contents of each chapter of the thesis.

The Bacterial Cell Wall

Bacteria present several molecules that "coat" and cover the outer surface of the cytoplasmic membrane. The arrangement of these "coating" molecules forms the cell wall or cell envelope. The cell wall (CW) is a complex and dynamic structure that protects the cell and helps in the adaptation to different environmental conditions. In broad and simple terms, the CW components can be divided in three types: 1) a ubiquitous bacterial component, the peptidoglycan; 2) components that are characteristic of either Gram-positives, Gram-negatives or of Gram-positive Corynebacteria; 3) components that may be present in both Gram-types but are often species- or strain-specific (capsular polysaccharides and S-layers).

The CW comprises many bacterial components that are in contact with the external environment which are determinant for infection. It comprises PAMP, virulence factors and components that function in immune evasion strategies. Indeed, most current models of bacterial recognition are based on how certain CW factors confer resistance to Host responses. Thus, the knowledge of the CW composition is crucial to understand how bacteria interact with the Host. In addition, peptidoglycan is a major PAMP and PRRs that specifically recognise it can be found from Plants to Humans. Moreover, along with PRR, the Host also produces lytic enzymes capable of degrading the peptidoglycan such as Lysozymes and produces antimicrobial peptides[3] (AMPs). The PRR, the lysozyme proteins and the AMPs are the most conserved immune strategies found in almost all Animals.

[3] Innate Immunity cationic peptides. They are expressed in response to pathogen detection and control the microbial growth by participating in killing and clearance.

Most cell biology studies on bacteria rely on microscopy techniques that use specific staining methods. Any staining method is inherently dependent in either or both chemical and physical properties of the bacterial CW. The most widely used staining is the Gram stain, which was first described by Carl Friedlander in 1883 for detecting *Streptococcus pneumoniae* in autopsy of lung tissues [8][4]. It was his colleague, Christian Gram, who published it in 1884 and soon it started being gradually stablished as a routine stain in Hospital settings (Roux 1886) [8][5]. The original procedure was subsequently improved and published as we know it today by Hucker in 1921 [9], [10]. It has been known for years that this differential stain which discriminates Gram-positive from Gram-negative bacteria, is a function of cell surface characteristics. However, only in the past decades have we achieved some understanding behind its mechanism [11], [12].

The first step of the Gram-stain is with the crystal violet, a basic positively charged dye that stains all bacterial surfaces (*i.e.* it stains negatively charged cells). The next step is an iodine-iodide mixture which serves as a mordant, so it forms a complex with the primary dye thereby fixing the dye to the cells. Through the use of a solvent, usually ethanol, some cell types lose the dye-mordant complex and the purple colour given by the crystal violet. Hence, any cell that retains the dye is designated Gram-positive and any cell that loses it is designated Gram-negative. For practical visualization, the final step includes a counterstaining with safranin that stains Gram-negative bacteria in red. It was long presumed that the CW of Gram-positive bacteria has physical properties regarding thickness and porosity that are able to retard the efflux of the dye-mordant complex and confer higher resistance to the solvents. Indeed, it is known

[4] Original reference: C. Friedlander, "Die Mikrokokken der Pneumonie," *Fortschr. Med.,* vol. 1, pp. 715-733, 1883.

[5] Original reference: G. Roux, "Sur un procede technique de diagnose des gonococci," *Arch. Gen. Med.* Vol. 2, 1886.

today that these bacteria, in contrast to the Gram-negatives, possess a thick peptidoglycan layer, a macromolecule that covers the entire cell surface nearby the cytoplasmic membrane and that it accounts for most of the physical properties of the cells.

In this thesis, the description of the Gram-negative CW and peptidoglycan takes as a reference *Escherichia coli*, because it is the main bacterial model organism thus the most well studied, including *in vivo* Host responses. Regarding the Gram-positives, two model organisms are here referred which were used for the work presented in this thesis: *Bacillus subtilis* and *Staphylococcus aureus*. *B. subtilis* is the major Gram-positive model organism and the *S. aureus*` CW has been extensively studied because its pathogenicity is highly associated with the surface components.

The structure of the Gram-negative CW is relatively uniform among the bacterial species, whereas the Gram-positive bacteria show two types: the typical and what is commonly referred to as "Gram-positive CW" and the CWs of *Corynebacterineae*, that although also capable to retain the Gram dye, they have a distinct wall structure from the classical Gram-positive bacteria.

Cell Wall architecture – layered vs. mesh-like structure

The Gram-negative CW is structurally well defined by three distinct layers (Fig. 1): the external layer called the outer membrane (OM), the middle layer harbouring the peptidoglycan (PGN) and finally the cytoplasmic or inner membrane (IM). The thin PGN layer is enclosed by the OM and IM, such that it forms an outside cellular compartment, the periplasm. The OM and the periplasm are not found in the Gram-positive CW.

In contrast, the Gram-positive CW presents itself as a 3D mesh-like structure (Fig. 1). A very thick layer of PGN is decorated by wall components that apart from proteins, they are characteristic and exclusive to Gram-positives, such as the teichoic acids – wall teichoic acids (WTA) and Lipoteichoic acids (LTA). Since these walls do not possess an external membrane covering the PGN, there is no cellular compartment like a periplasm. It is presumed that the thickness of the PGN compensates for the lack of a protective OM [13]. Indeed, in Gram-positives the PGN accounts for 30-70% of total CW whereas it accounts for only 10% in Gram-negative bacteria [14]. In the same line of thought, it can also be presumed a protective role for the teichoic acids as they can represent over 60% of CW mass, they extend beyond the PGN layer thus concealing it and are great contributors for the wall structure and function in various processes [15].

Peptidoglycan – the common feature of every cell wall

The word "peptidoglycan" describes the chemical composition of the molecule. Historically, it has received many names and nowadays it is also commonly referred to as "murein", that derives from the Latin *murus* meaning "wall" and was introduced in analogy to "protein" by Weidel and Petzer [16]. The PGN may be understood as somewhat equivalent to the Insects exoskeleton, since it is a rigid structure responsible for cell shape and integrity. However, it is simultaneously a flexible and dynamic structure as it is involved in growth and cell division, can reversibly expand in response to pressure changes and serves as a scaffold for anchoring CW components [17], [18].

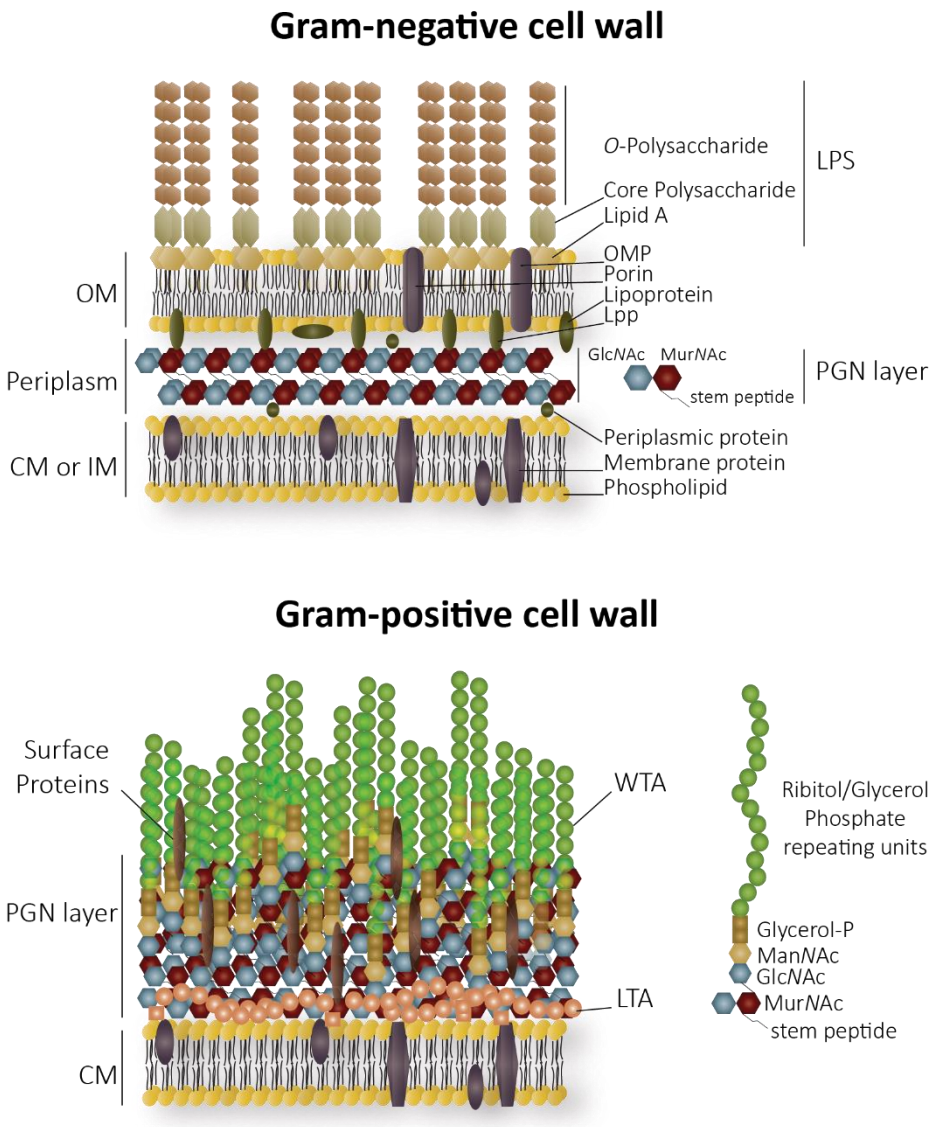

Figure 1. Schematic representation of the Gram-negative and Gram-positive bacterial CWs. OM – Outer Membrane. CM – Cytoplasmic membrane / IM – Inner Membrane. LPS – Lipopolysaccharide. OMP – Outer Membrane Protein. PGN – Peptidoglycan – composed of Glc*N*Ac (*N*-acetylglucosamine) and Mur*N*Ac (*N*-acetylmuramic acid) that form linear glycan strands linked through the stem peptides. WTA – Wall Teichoic Acids. LTA – Lipoteichoic Acids. Man*N*Ac – *N*-acetylmannosamine. Glycerol-P – (two) Glycerol-Phosphates. The WTA repeating units are one of two types: glycerol or ribitol.

The PGN is exclusive and ubiquitous to Bacteria, albeit there are exceptions. PGN and its biosynthetic genes have not been detected in some bacteria: *Mycoplasma* spp., and *Orientia tsutsugamushi* [19]. The PGN has been detected in non-bacterial organisms, the glaucophytes algae, as a component of the photosynthetic organelles [20] and, its biosynthetic genes (but not PGN) have been found in two Plants and are presumed to participate in chloroplast division – *Arabidopsis thaliana* and *Physcomitrella patens* [21].

The PGN macromolecule is formed by linear glycan strands of repeating disaccharide units of *N*-acetylglucosamine (Glc*N*Ac) and *N*-acetylmuramic acid (Mur*N*Ac), linked to a peptide chain, the stem peptides, which cross-link one linear strand to another (Fig. 1). Hence, it is a large polymer that forms layers around the entire cell surface. The chemical structure of the PGN, described in the "Peptidoglycan composition and Host recognition" section, is quite similar in both Gram-positives and Gram-negatives. However, regarding the architecture there are two main differences between the CW types: the thickness of the layers and the presence/absence of certain wall-covalently linked polymers. The Gram-negative PGN is only a few nanometres thick, which can comprise only one to a few layers and the Gram-positive PGN is 30–100 nm thick because it is composed of many layers (Fig. 1) [22]. In addition, Gram-positives typically present surface polymers covalently attached to the PGN, such as the wall teichoic acids (WTA), that are absent in the Gram-negatives (Fig. 1). Although the thickness varies among these two bacterial types, the pores have similar average sizes [23]. It is estimated that globular, uncharged proteins with molecular weights of 22–24 kDa can penetrate the isolated, relaxed PGN [24]. Interestingly, the length of the strands does not correlate with the thickness. *S. aureus* that presents a very thick layer has short strands with an average length of about 18 disaccharide units [25], [26]. Both in *Bacilli* and *E. coli*, the average length varies within

strains and growth conditions [27] but it is estimated to be 50-250 disaccharide units in *Bacilli* [26], [28], [29] and up to 30 disaccharide units in *E. coli* [30].

Components of the Gram-negative cell wall

The Outer Membrane – LPS and OMPs

The OM is essential for the survival of *E. coli* and its only known function is to serve as a protective barrier. Like other biological membranes, the OM is a lipid bilayer but in contrast, it is asymmetrical: the inner leaflet is composed by phospholipids and the outer leaflet is composed by proteins, glycolipids and mainly of lipopolysaccharide (LPS) [31]. Both LPS and proteins participate in the selective permeability of the OM [32]. LPS forms an effective barrier for hydrophobic molecules, the protein porins limit diffusion of hydrophilic molecules >700 Daltons [32] whilst other type of proteins – the outer membrane proteins (OMPs) – allow the passive diffusion of small molecules such as disaccharides and amino acids. The OM is linked to the PGN as if it was stapled, through a lipoprotein called Lpp, or murein lipoprotein, or Braun's lipoprotein, and it is the most abundant protein in *E. coli* [33].

LPS is the molecule responsible for the endotoxic shock associated with septicemia by Gram-negative bacteria thus it is also known as "endotoxin" [34]. It is composed of a lipid portion, the lipid A, which is anchored at the outer leaflet of the OM. The lipid A is linked to a polysaccharide core that connects to the outer surface polysaccharide chain, the *O*-polysaccharide or *O*-antigen, which carries the antigenic specificity and its composition varies among bacteria (Fig. 1) [34].

OM proteins can be divided into lipoproteins and β-barrel proteins. Whereas the latter are transmembrane proteins, lipoproteins are

not and seem to be embed in the inner leaflet by the lipid moiety. There are about 100 OM lipoproteins in *E. coli*, still the functions of most of them are unknown [35]. Nearly all of the integral transmembrane proteins assume a β-barrel conformation and are designated Outer Membrane Proteins (OMPs). Some OMPs, such as OmpA from *E. coli*, can be non-covalently linked to the PGN.

The Periplasm

The periplasm can sequester potentially harmful compounds thus it has been proposed as an evolutionary precursor of the lysosomes in eukaryotic cells [36]. It is densely packed with proteins such as enzymes involved in CW biosynthesis and periplasmic binding proteins that function in chemotaxis and transport of sugars and amino acids [37].

Components of the Gram-positive cell wall

The teichoic acids – WTA and LTA

The teichoic acids (TA) are long anionic polymers that are divided into Wall Teichoic Acids (WTA) and Lipoteichoic Acids (LTA). Although neither of them are essential, it is not possible to delete genes from both pathways because they are synthetic lethal and their presence appears to be crucial for proper CW architecture and integrity [38], [39].

The major distinguishing feature between LTA and WTA, is that the WTA are covalently attached to the PGN, whereas the LTA are anchored to the cytoplasmic membrane (Fig. 1 and Fig. 3) [18]. The WTA are attached via a phosphodiester linkage to the hydroxyl group at position 6 of Mur*N*Ac and they have been propose to extend perpendicularly through the PGN mesh [40], [41], into what has been characterized as a "fluffy" layer [18], [42], [43]. Generally, WTA are composed of a conserved

linkage unit to which is appended a chain of either polyribitol phosphates (polyRboP) like in *S. aureus* and some *B. subtilis* strains, or polyglycerol phosphates (polyGroP) as in most *B. subtilis* spp.. The RboP or GroP repeats are in turn commonly tailored with D-alanyl esters and glycosyl moieties. The nature and extent of these tailoring significantly affect the properties and functions of WTA as they introduce positive charges along the polymer backbone [18]. Accordingly, *S. aureus* strains lacking D-alanine esters are more susceptible to the cationic AMPs and to Host lytic enzymes [44]–[46].

The TA present several functions, some of which may be species-specific. They have been described as a "continuum of anionic charge" which seems to be of vital for the cell [18]. Because they are anionic they play major roles in cation homeostasis which in turn influence the rigidity and porosity of the CW. Indeed, the presence of covalently attached glycopolymers is a hallmark of the Gram-positive CW. Gram-positives lacking WTA like *Micrococcus luteus* present other polymers such as teichuronic acids (repeating units of the disaccharide *N*-acetylgalactosamine - D-glucuronic acid). Similarly, when unable to produce WTA, bacteria can compensate by producing other polymers instead. For instance, under phosphate limiting conditions *Bacillus* spp. can produce teichuronic acids [47].

Surface Proteins

The surface of the Gram-positive bacteria is decorated with a variety of proteins, some of which are analogous to proteins found in the periplasm [17]. They can be inserted in the CM, covalently attached or tightly associated with the PGN, or even bound to the TA [48]. Most of these proteins play major roles as virulence factors. In *S. aureus* the presence of surface proteins is highly dependent on environmental changes and growth conditions [49] and they have great impact in pathogenicity. Furthermore, *S. aureus* relies both in TA and in surface proteins, generally

called adhesins, to successfully stablish colonization on Host tissues [50]–[52]. In addition, many proteins have been implicated in iron acquisition, which is necessary for pathogenesis since it is a requisite for the function of many bacterial enzymes [53]–[55].

The Gram-positive cell wall of *Corynebacterineae*

The Family *Corynebacterineae* includes the major pathogens *Mycobacterium tuberculosis* and *Mycobacterium leprae* and the complexity of their walls substantially contributes to virulence. Similar to the typical Gram-positive wall, the PGN is composed of several layers and contains covalently attached glycopolymers, the arabinogalactan, which is covalently attached to mycolic acids [22][6]. Both components are unique to these bacteria and the mycolic acids are responsible for their acid-fast resistance. Similar to the Gram-negative bacteria, they possess an OM, but in contrast it appears to be symmetrical. Moreover, the porin proteins seem to be structurally different from the typical OM porins of the Gram-negatives. In sum, these walls comprise features of both Gram-positive and Gram-negative bacteria. Accordingly, a genome-based phylogeny places them in between the two types [56].

[6] Original reference: D. E. Minnikin. Lipids: Complex lipids, their chemistry, biosynthesis and roles, p. 95–184. In Ratledge C., Stanford J. (ed.). The biology of the mycobacteria, vol 1. Physiology, identification and classification. *Academic Press*, Inc., New York, NY. 1982.

Peptidoglycan composition and Host recognition

As the name intuitively suggests, PGN is a macromolecule composed of sugars and peptides where linear glycan strands are connected to one another, *i.e.* they are cross-linked by short peptide bridges, the stem peptides [57] (Fig. 2 and Fig. 3). The glycan strands are composed of repeating units of disaccharides made of Glc*N*Ac residue linked to Mur*N*Ac residue through *β*-(1,4) glycosidic bonds. The Mur*N*Ac sugar is exclusively found in the Bacteria Domain (apart from the glaucophytes algae), whereas Glc*N*Ac composes the chitin present in the Fungi CW and in the exoskeleton of Insects and Crustaceans. Regarding the stem peptides, they are pentapeptide chains that are covalently linked through the N-terminus to the lactyl group in the position 3 of the Mur*N*Ac residues (Fig. 2 and Fig. 3). The pentapeptide chain contains alternating L- and D- amino acids, being the latter a typical feature of PGN. Whereas the glycan backbone is highly conserved among bacteria, the peptide moiety shows a great degree of variability in composition [14]. Apart from the third position, the amino acids in all other positions of the pentapeptide are quite conserved among Gram-positive and Gram-negative bacteria. The amino acid at the third position is most commonly either L-Lysine (Lys) or *meso*-diaminopimelic acid (DAP) [14]. Generally, the Gram-negative bacteria like *E. coli* and the rod shape Gram-positives like *Bacilli* and *Listeria* spp. and the Gram-positive mycobacteria present a DAP-type PGN. In contrast, most of the other Gram-positive bacteria present a Lys-type PGN (Fig. 2). As for the stem peptides, typically, Gram-positive bacteria present several types of peptide bridges that link one stem peptide to another, in contrast to the Gram-negatives which have a direct cross-link. Thus, the composition and structure of PGN is quite uniform among Gram-negatives, but shows great variability among the Gram-positives [14]. Accordingly to being the most conserved PGN moiety,

Gram-positives and Gram-negatives present the same type of sugar modifications after the PGN biosynthesis. The differences rely on modifications in the Gram-positives due to the attachment of glycopolymers and the typical presence of an anhydrous form of the Mur*N*Ac in the Gram-negatives (Fig. 3).

The differences in the PGN composition at the pentapeptide bridge between the Gram- positives and –negatives have been accounted for determining different innate immune responses. In addition, bacteria present modifications of the PGN backbone that render them resistant to Host immune strategies. Therefore, understanding the impact of the PGN composition on the interaction with the Host is crucial to unravel bacterial immune evasion mechanisms and how PRR perceive and recognise the PGN. The triggering of immune responses by PGN-derived compounds was discovered more than 30 years ago and the muramyl-dipeptide (MDP) was the first minimal inflammatory PGN fragment to be identified (Fig. 2) [58]. However, the study of the PGN molecular structure has been a challenging topic, particularly when it comes to the Gram-positive CW due to the multi-layered PGN decorated with wall polymers coupled with inter-species variabilities, such as the ones found in the *Bacillus* Genus.

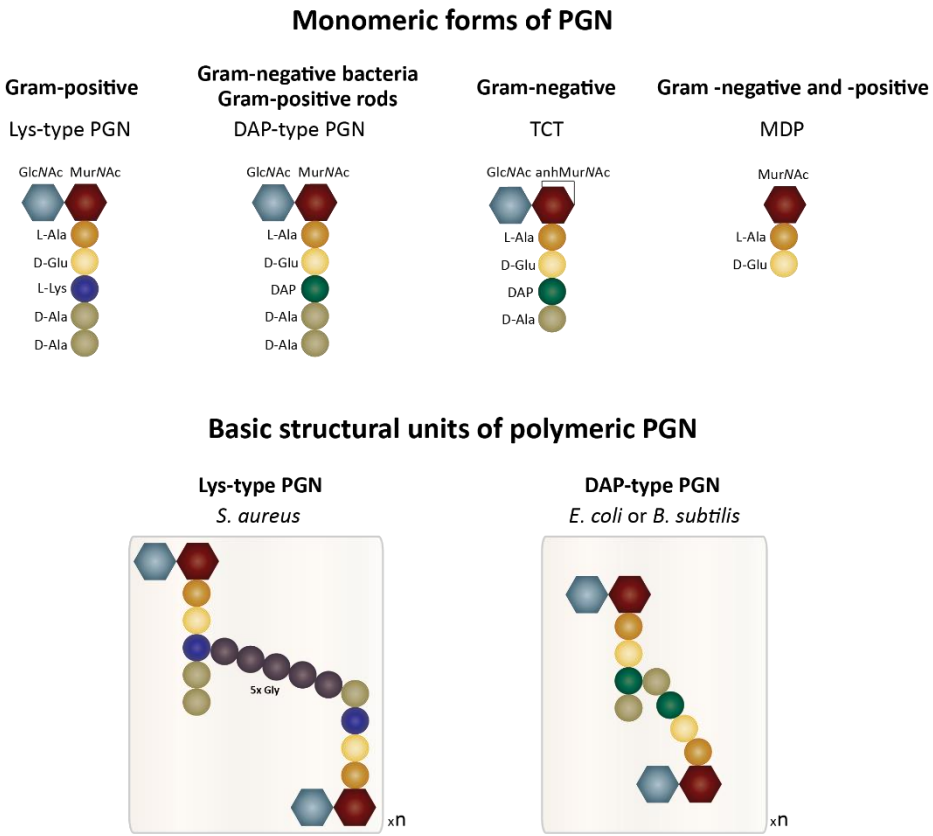

Figure 2. Schematic representation of PGN monomeric species and polymeric PGN. The basic unit of the PGN is the disaccharide-pentapeptide. The Gram-positive bacteria typically present at the third position of the pentapeptide a Lys *vs*. a DAP residue present in Gram-negatives and some Gram-positives. TCT – tracheal cytotoxin – is a muropeptide produced by Gram-negative bacteria. Some bacteria such as *Bordetella pertussis* do not retain it at the CW upon growth and division, thus they release it to the medium and trigger potent immune responses. MDP – muramyl dipeptide – is the minimal PGN-component to be identified as immunostimulatory. Regarding the polymeric composition of PGN, *S. aureus* presents an indirect cross-linking, connecting the glycan strands through peptide side chains, typical of Gram-positives. In contrast, Gram-negative bacteria and some DAP-type Gram-positives, show a direct cross-linking between the pentapeptides.

The modifications in the glycan strands

To date, there are no bacterial species known that in their mature PGN possess unmodified sugar chains [59]. As a matter of fact, the attachment of components to the PGN happens mostly via the sugars. The sugar backbone is structurally modified during synthesis or after insertion into the CW. These secondary modifications happen either by enzymes that directly modify the sugar residues or by the attachment of polymers. There are two types of sugar modifications found both in Gram-positives and Gram-negatives: *N*-deacetylation and *O*-acetylation (Fig. 3). Deacetylation happens in both sugars, whereas *O*-acetylation has only been identified in Mur*N*Ac residues. A third type of sugar modification is *N*-glycolylation where there is a glycolyl group instead of the acetyl group at the amino group on the second position of Mur*N*Ac (Fig. 3). This is a hallmark of most bacteria containing mycolic acids. Regarding Gram-negative bacteria, they present a 1,6-anhydro ring at the terminal Mur*N*Ac residue of the strand that is the result of the cleavage by specific bacterial PGN hydrolases during the processes of turnover and cell division (Fig. 3). As for Gram-positive bacteria, specific modifications happen through linkage of WTA (Fig. 3), capsular polysaccharides (and arabinogalactans) that bind via phosphodiester bonds either to Mur*N*Ac or Glc*N*Ac. Although these PGN alterations have been known for a long time, their role in bacterial biology remains to be fully understood. It is thought that they control cell division, PGN turnover and, as it will be here briefly discussed, they are also involved in the pathogenicity of the bacteria.

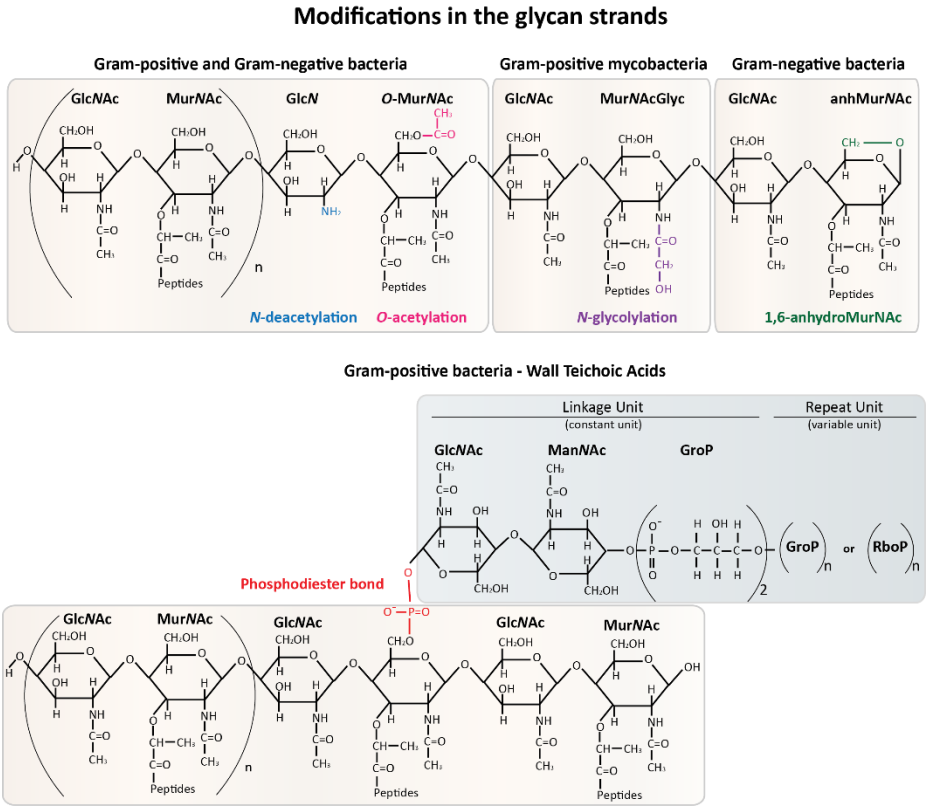

Figure 3. Modifications in the PGN glycan strands. The top panel shows modifications at the sugar level in the different bacterial types. The bottom panel shows the modifications of the Gram-positive PGN by the attachment of the WTA. *S. aureus* has RboP WTA similar to some *B. subtilis* strains. Most *B. subtilis* strains and *Staphylococcus epidermidis* possess GroP WTA. Note the difference between the terminal Mur*N*Ac residue on the PGN chain represented at the top with the PGN chain at the bottom. Gram-negative bacteria possess a number of lytic transglycosylases that cleave between the Mur*N*Ac and Glc*N*Ac residues (same bond as the muramidases) and originate 1,6 – anhydroMur*N*Ac (see Chapter II). In contrast, these termini are rarely found in the Gram-positive PGN, thus the Mur*N*Ac terminal residues present a reducing end. *N*-deacetylation can also occur in the Mur*N*Ac residue in both Gram-positive and Gram-negative bacteria (not depicted).

The deacetylation introduces positive charges into the CW, thus it potentially affects the binding of proteins and other components to the PGN. An increase in the positive charge is more likely to increase the resistance of the bacteria to the activity of the cationic AMPs. In addition, the deacetylation affects bacterial clearance through the activity of

Lysozymes and through phagocytosis. Lysozyme is a muramidase, *i.e.* it cleaves the β-(1,4) glycosidic bond between Mur*N*Ac and Glc*N*Ac and it is ubiquitous in Bacteriophages, Bacteria, Fungi and Mammals. Deacetylation of Glc*N*Ac or Mur*N*Ac strongly decreases the activity of lysozymes [60]–[64] and a *Listeria monocytogenes* mutant lacking deacetylated residues was very sensitive and rapidly killed by macrophages [65].

O-Acetylation of the PGN is more frequent than *N*-deacetylation and occurs in many important pathogens. In fact, most of the known muramidases have decreased or no activity against *O*-acetylated PGN, particularly the Host Lysozymes [59]. To date only the *N,O*-diacetylmuramidase of *Chalaropsis* [66] and mutanolysin from *Streptomyces globisporus* [67] are known to cleave *O*-acetylated PGN. There is a strong correlation between pathogenicity, lysozyme resistance and the occurrence of *O*-linked acetate in the PGN of staphylococcal species [68]. Immune strategies are generally keen in killing and lysing the bacteria, followed by clearance of bacterial debris. However, even when there is efficient killing, it has been observed the persistence of PGN with a high degree of *O*-acetylation [69]–[71].

In Gram-positive bacteria, WTA contribute to virulence in several ways. At a first level, as they protrude through the PGN layers to the outer surface they impair the direct recognition by Host receptors. Work from the Host Labs[7], has shown that they exert stereo-hindrance in Gram-positive pathogens, including *S. aureus*, from a PGRP (PGRP-SA) in *D. melanogaster* [72]. Moreover, it is known that the WTA affect *O*-acetylation which in turn confers resistance to Lysozymes [73]. Also, the tailoring of the WTA, for instance with D-Ala as in *S. aureus* [45] together

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with the deacetylation of the PGN, are two ways of introducing positive charges that can protect the bacteria against the activity of the AMPs.

Additionally, the Host Labs identified another CW factor that can dampen the recognition and compromise efficient immune responses. We identified the activity of the major autolysin protein Atl[8] in *S. aureus* [74]. The loss of virulence is attributed to the recognition by PGRP-SA of PGN fragments exposed at the surface of the cells. We observed a similar effect for the *Streptococcus pneumoniae* major autolysin LytA. Autolytic PGN hydrolases are ubiquitous in Bacteria, thus it can be inferred that perhaps other autolysins from other bacterial species may impair detection of PGN by other PGRPs or by other PGN receptors.

In Gram-negative, the glycan strands do not terminate with a reducing Mur*N*Ac residue but with a non-reducing 1,6-anhydroMur*N*Ac (Fig. 3) [30], [75], [76]. This is due to the activity of the lytic transglycosylases (Fig. 4). They are PGN hydrolases that cleave at the same site as muramidases but form a 1,6-anhydro ring at the Mur*N*Ac and are particularly abundant in Gram- negative species. Interestingly, the lytic transglycosylases cannot cleave the glycan strands at *O*-acetylated Mur*N*Ac residues, thus it has been proposed that *O*-acetylation and de-*O*-acetylation regulate the activities of the lytic transglycosylases [77]–[79].

In sum, the activity of PGN hydrolases/autolysins, the presence of WTA and the *N*- and *O*- dea-/acetylations, modify the glycan backbones and contribute to bacterial virulence [61]–[64], [72], [73], [80]–[84]. Similar to Lysozymes, PGRPs are found in almost all animals, possess affinity to PGN and their recognition is affected by modifications like the WTA and autolysins. However, the effect of *N*-deacetylation, *N*-

[8] Autolysins are enzymes that degrade a component belonging to the cell or tissue where they are produced. Autolysins or PGN hydrolases cleave the PGN in order to sustain cell growth – PGN present at the cell surface – and to split the daughter cells – PGN present at the septum – prior to separation of the daughter cells. These enzymes are the topic of study of Chapter II.

glycolylation and *O*-acetylation on the PGRPs recognition has not yet been addressed.

Variability in the peptide moiety

The peptide moiety of the PGN is highly variable among Bacteria. However, unlike the modifications of the glycans, the impact of the natural variability and modifications of the amino acids on the Host recognition is not so well understood. Paradoxically, current models of Host-bacterial recognition are almost exclusively based in the amino acid composition of the PGN.

Variability in the stem peptides

The variations of the stem peptides can be divided into two categories: those due to the specificity of the Mur ligases, the enzymes responsible for their biosynthesis and incorporation into the nascent PGN chain, and those occurring afterwards upon insertion of the amino acids [85], [86]. The first amino acid is usually L-Alanine and in rare cases, Glycine or L-Serine (Fig. 2). At the second position there is always the incorporation of D-Glutamic acid and any modification that may happen occurs at a later step (Fig. 2). The greatest variations regarding both incorporation and post-modifications happen at the position 3 where it is generally found a diamino acid (Fig. 2). The most prevalent is DAP which is present in probably all Gram-negatives and many Gram-positives (*Bacilli*, *Clostridia*, *Lactobacilli*, *Corynebacteriales*, *Rickettsiae*) and in the glaucophytes algae [14]. The diamino acid Lys is the second most common and it is found in most all other species of Gram-positive bacteria [14]. Less commonly, it can be found other diamino acids, such as L-Ornithine or *meso*-lanthionine and also monoamino acids (L-Homoserine, L-Alanine or

L-Glutamic acid) [13]. The two last amino acids of positions 4 and 5 are added as a dipeptide, in most cases D-Alanine-D-Alanine, and the last D-Alanine residue is lost in the mature PGN (Fig. 2).

Variations of the incorporated amino acids, mainly at the second and third positions, can happen by amidation, hydroxylation, acetylation, attachment of amino acids or other groups, and attachment of proteins. In fact, the stem peptide is an anchoring point for proteins in both bacterial types [17]. In *E. coli*, Lpp is the only known protein covalently attached to PGN (at the DAP residue) [87], [88], whereas the Gram-positive bacteria contain many surface proteins that are involved in pathogenicity. One of the most common post-modifications at the third position is the amidation of DAP (Fig. 4). This modification happens in *B. subtilis* and *Bacillus licheniformis* and not in *E. coli* or *Bacillus megaterium* – all of which are bacteria commonly used in the *Drosophila* literature as bacterial organisms to study Host immune responses.

Current models of PGN recognition by Innate Immune receptors are based on their discriminatory ability between DAP- vs. Lys- PGN. As evolutionary conserved receptors, it is intuitive to consider that PGRPs recognition may rely to great extent on the binding to the conserved sugar moiety, still they have been reported to possess such discriminatory ability at the peptide level. It is postulated, particularly in *Drosophila* immunity, that PGRPs can discriminate Gram-positives vs. Gram-negatives and *Bacilli* through the residue at the third position [89], [90] and therefore they differentially activate distinctive immune pathways [91]. Indeed, *B. subtilis* and *E. coli* present a similar PGN composition, albeit they mainly differ in the percentage of muropeptide species and the amidated DAP in *B. subtilis* [92]. Importantly, the amidation of DAP has been pointed out as the reason for literature reports detecting *Drosophila* PGRP-SA binding to PGN of *E. coli* and *B. megaterium* but not to *B. subtilis* [89][90].

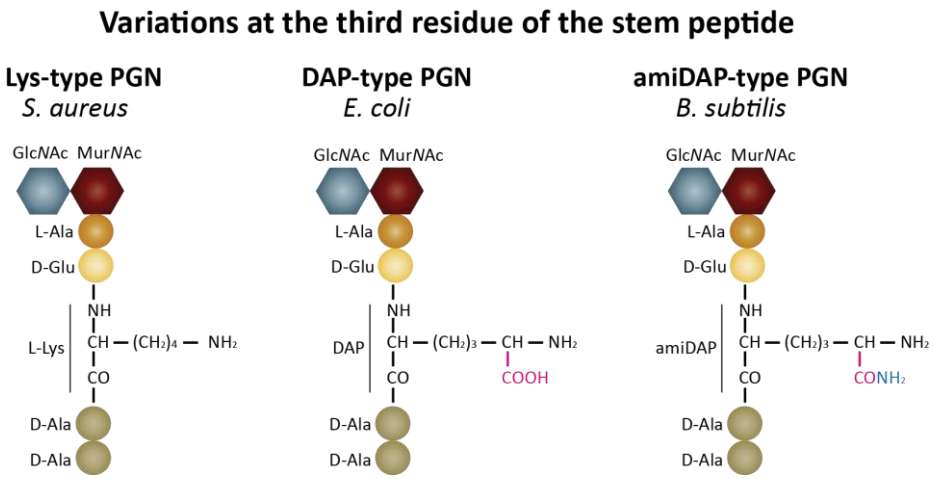

Figure 4. Differences between the amino acids L-Lys, DAP and amiDAP. It is presented the basic structural PGN units with Lys, DAP and amiDAP. The difference between the Lys and the DAP amino acids is the presence of a carboxyl group in the latter (depicted in pink). Unlike *E. coli*, *B. subtilis* suffers a post-incorporation modification of the DAP residue, whereby the extra carboxyl group not present in Lys, that is not engaged in the peptide bond with D-Ala, is amidated (depicted in blue).

Variability in the peptide cross-linking bridges

The bond between two stem peptides from different glycan chains is designated by cross-linking. The nature and the degree of cross-linking show the highest variations in the types of PGNs. It is the differences at the level of the peptides that allow the taxonomic distinctions sometimes to the species level. As such, although there are many differences among Gram-positive organisms, the most notable differences are found at the level of the cross-linking [13], [59].

Most commonly, the cross-linking happens between the amino acid at position 3 of one stem peptide and the D-Ala at position 4 in another stem peptide from another glycan strand (Fig. 2). The link between these amino acids can either be direct or indirect when the linkage happens through an interpeptide bridge (Fig. 2). Most Gram-negative bacteria show direct cross-linking like *E. coli*, although *B. subtilis* also shows direct cross-linking (Fig. 2). In the indirect cross-linking the peptide bond between Lys

and D-Ala occurs via a peptide chain, thus these are called "branched stem peptides". The composition of the peptides in the branch shows substantial variation and these branched stem peptides serve as attachment sites for covalently-associated proteins, such as protein A from *S. aureus* which plays different roles in the pathogenicity of the bacterium [93].

There is also considerable variation in the degree of cross-linkage, *i.e.* the number of cross-links between the strands in one PGN macromolecule. In terms of muropeptide content, the soluble fragments released upon muramidase digestion, it translates into a low cross-linking like in *E. coli*, where the PGN units appear mostly as monomers or dimers, accounting for 20% of total cross-linking [57]. In contrast, in *S. aureus* most PGN units are present as oligomers with a degree of cross-linking over 93% [94]. The degree of cross-linking is also associated with the susceptibility of PGN to lytic enzymes. In *S. aureus* the high cross-linking aids to the *O*-acetylated Mur*N*Ac and the presence of WTA on the resistance to Lysozyme [73]. However, we did not observe a correlation between the cross-linking and the binding of *Drosophila* PGRPs (data shown in Chapter III).

The *Drosophila melanogaster* Immune System

In order to study Human-pathogen interactions, several animal models have been developed. Due to logistical, ethical and financial reasons, the use of mammals is difficult and time consuming. Therefore, non-mammalian models of infection for Human pathogens have been developed. The conservation of signalling pathways for the activation of antimicrobial responses suggests that some innate immune components share an ancient origin in metazoan evolution, making non-vertebrate animal models suitable for infection studies [95]. Moreover, virulence factors for disease in one host, also contribute to disease in non-natural hosts [96].

The fruit fly *Drosophila melanogaster* has shown to be a highly adequate invertebrate model to understand mammalian biology [95], [97], [98]: the organs systems are analogous to that of Vertebrates and the signalling pathways and transcriptional regulators that control development, metabolism and immunity share evolutionarily conserved components [99]. In addition, there is a wide array of genetic tools coupled with the sequenced genome [100], [101]. Regarding Host-pathogens interactions and fundamental innate immune mechanisms, flies have proven to be one of the most suitable organisms to date. They are naturally infected by bacteria, fungi and viruses and can be experimentally infected with human pathogens [72], [74], [102]–[104]. Moreover, the mechanism of bacterial recognition and one of the outcomes of it which is the expression of AMPs, is similar and probably common to all animals including Humans [95]. The discovery of the AMPs induction upon infection [105] helped to put *D. melanogaster* as a model organism for the study of conserved innate immune mechanisms. Besides the genes encoding for the AMPs being conserved sequences both in insects and mammals, their regulation is under the control of the conserved nuclear

factor kappa-light-chain-enhancer of activated B cells (NF-κB) signalling cascades. The D. melanogaster conserved NF-κB pathways are the TOLL and IMD (Immune Deficiency) pathways (Late Immune Responses section and Fig. 6) [106].

Early-Constitutive and Late-Induced Responses

D. melanogaster possesses multiple innate defence mechanisms, many of which similar to mammals [107]–[110]. The systemic responses are divided into constitutive responses and induced responses [111]. Upon an infection, these responses can be temporally classified as immediate/early responses and late responses, respectively. The constitutive early responses are always present and rely on cellular responses through the haemocytes, the insect blood cells [112]. These responses are paramount for clearance of the pathogens and they are followed by the induced late responses which are protective responses that rely mainly on the antimicrobial activity of the AMPs [113].

Early Immune Responses

The boundaries of an organism are vital for its integrity and survival. The epithelia are the first barrier in an organism against invading pathogens. A breach on the cuticle initiates a series of reactions against infection – production of Reactive Oxygen Species, coagulation, melanization and local inducible AMPs by the IMD pathway. In addition, there is constitutive expression of AMPs in specific tissues.

The cellular responses play major roles in phagocytosis and encapsulation of microorganisms and are crucial for clearance of the pathogen [114]. These responses act immediately upon injury and are maintained for as long as it is necessary until clearance of the pathogen.

Local expression of AMPs

This synthesis of AMPs is referred to as the local immune response as opposed to the systemic response. They can either be constitutive or inducible. The AMPs constitutively expressed in a defined tissue are not upregulated during microbial infection, *i.e.* they are not regulated by the NF-kB pathways. However, the inducible local AMPs` gene expression is triggered upon natural infection with DAP-type bacteria, mediated through PGRPs and regulated by the IMD pathway. To date, no implication of the TOLL pathway in the local immune response has been demonstrated and there is no evidence that AMPs are induced in the epithelia in response to neither Lys-type bacteria or fungi [95].

Production of Reactive Oxygen Species

Both in Mammals and *Drosophila*, natural infections with bacteria induce rapid synthesis of Reactive Oxygen Species (ROS). Similar to the IMD pathway, ROS responses are crucial in gut immunity.

Coagulation

Upon breach of the cuticle there is a rapid reaction to control the bleeding and initiate wound healing called coagulation. In this process there is immobilization of the bacteria at the local wound, thus preventing the spreading into the haemolymph [95], the *Drosophila* equivalent to the Vertebrate blood. Since Insects possess an open circulatory system, the trapping of the bacteria is crucial against septic infections and it also helps in the killing by ROS and phagocytosis.

Melanization

Melanization is observed at the site of injury and also at the surface of the pathogen. It is the *de novo* synthesis and deposition of melanin, seen

by blackening of the wound site and it is regulated by the prophenoloxidase cascade [115]. Particularly, melanization helps in the encapsulation of pathogens that are too large to be phagocytosed [116]. It is thought to play important roles in arthropod defence reactions such as wound healing, encapsulation, sequestration of microorganisms and production of toxic intermediates that are toxic to the microorganisms [117], [118].

Cellular responses

Phagocytosis is paramount to eliminate apoptotic bodies, bacteria and fungal spores. The *Drosophila* haemocytes are the functional equivalents of the human blood cells. There are three types of functional haemocytes – plasmatocytes, crystal cells and lamellocytes. Plasmatocytes are monocyte-like cells involved in phagocytosis of apoptotic bodies and pathogens. Crystal cells are required for melanization and thus participate in the engulfment of parasites whose size is too large to follow a canonical phagocytosis route. These two types of haemocytes circulate in the haemolymph and/or can be found sessile. Lamellocytes participate in the encapsulation of foreign bodies that are too large to be phagocytosed and are only differentiated in response to specific immune challenges, such as wasp parasitism.

Late Immune Responses

The late responses are systemic humoral responses via humoral molecules, including the AMPs. These systemic responses happen through production of AMPs by the fat body, a functional equivalent to the mammalian liver. These inducible AMPs are controlled by the immune pathways TOLL and IMD and are activated by the recognition of pathogens by the PRRs, in particular by PGRPs. In contrast, the local constitutively

expressed AMPs in specific tissues are controlled by transcription factors independent of these pathways.

The AMP responses are a hallmark of Insect Immunity [95]. The nature of the AMP immune responses and its temporal expression upon infection, has been well characterised in *Tenebrio molitor* and *Bombus terrestris* [114], [119]–[122]. In *Tenebrio molitor*, the late induced AMPs start to increase after 99.5% clearance of the bacteria [114]. It is estimated that induced AMPs take at least 1-3h to originate and 12-48h to reach peak levels [119]. This induced response can persist for weeks [119], [121]. The induced systemic AMPs are late responses because their primary role is to protect against the bacteria that persist within their body, rather than to clear the infection [114]. This expression is prolonged in order to prevent the enrichment of resistant bacteria, as it has been shown that bacteria which survived the early/constitutive responses were more resistant to AMPs [114]. As a consequence, the induced AMPs are also believed to protect against reincidence [120], [122]. It is presumed that the role of AMPs is conserved among Insects. However, in *D. melanogaster* it seems that haemocytes rather than the AMPs are responsible for protection against secondary infection [123]. Priming of adult flies with heat-killed *Streptococcus pneumoniae* cells, conferred resistance to infection in an AMP-independent manner [123]. Furthermore, the resistance remained for the life-time of the Fly and it was specific for *S. pneumoniae* infection.

The *Drosophila* immune cascades – TOLL and IMD

In *Drosophila*, there are two main immune pathways that control the inducible expression of the AMPs, which are evolutionarily conserved NF-κB signalling pathways– the TOLL and the Immune Deficiency (IMD) pathways (Fig. 5). The TOLL signalling is similar to the MYD88[9]-dependent

[9] Myeloid differentiation primary response protein 88.

Toll-like receptor (TLR) pathway in mammals and share conserved membrane receptors with a TIR domain[10] (Fig. 5) [124], [125]. The IMD signalling is similar to both the tumour necrosis factor (TNF) pathway and the TRIF[11]-dependent TLR pathways (Fig. 5).

The NF-κB family of transcription factors (TFs) is found in the cytoplasm of virtually all cell types in most Animals. Proteins of this family share a 300 amino acid Rel homology domain (RHD) at the N-terminus, which mediate dimerization, binding to DNA and interaction with a regulatory family of inhibitor proteins, IkB. The NF-κB TFs regulate the expression of hundreds of genes that are associated with diverse cellular processes including innate (and adaptive) immune responses. The NF-κB signalling cascade is probably the most frequently targeted intracellular pathway by anti-immune modulators that are encoded by a wide spectrum of pathogens. This is an indication of the relevance of this pathway in immunity and evolutionary conservation mechanisms.

The NF-kB signalling pathways happen through three main steps that take place in different subcellular localisations (Fig. 5). First there is the activation of a transmembrane receptor upon recognition (directly or indirectly) of the PAMP on the extracellular environment. Second, the activation of the transmembrane receptor triggers the formation of cytoplasmic signalling complexes through conserved adaptor molecules [126]. These adaptor proteins contain death domains (DD) that are homotypic protein interaction modules, thus they stablish protein-protein interactions. In non-stimulated conditions, the NF-κB dimers are sequestered in the cytoplasm by the IkBs through their ankyrin repeats[12] that mask the nuclear localization signals of NF-κB. Upon activation of the

[10] Toll/interleukin-1 receptor (TIR) homology domain.

[11] TIR domain-containing adaptor protein inducing IFNβ.

[12] Ankyrin repeats are tandemly repeated modules of ca. 33 amino acids and represent one of the most common protein-protein interaction motifs found in nature.

signalling cascades, IkB is phosphorylated by the IκB kinase (IKK) thus it is degraded and the NF-κB dimers are activated to translocate into the nucleus. Finally at the nucleus, the TFs bind to the promoter regions of the responsive genes containing the NF-κB sequences.

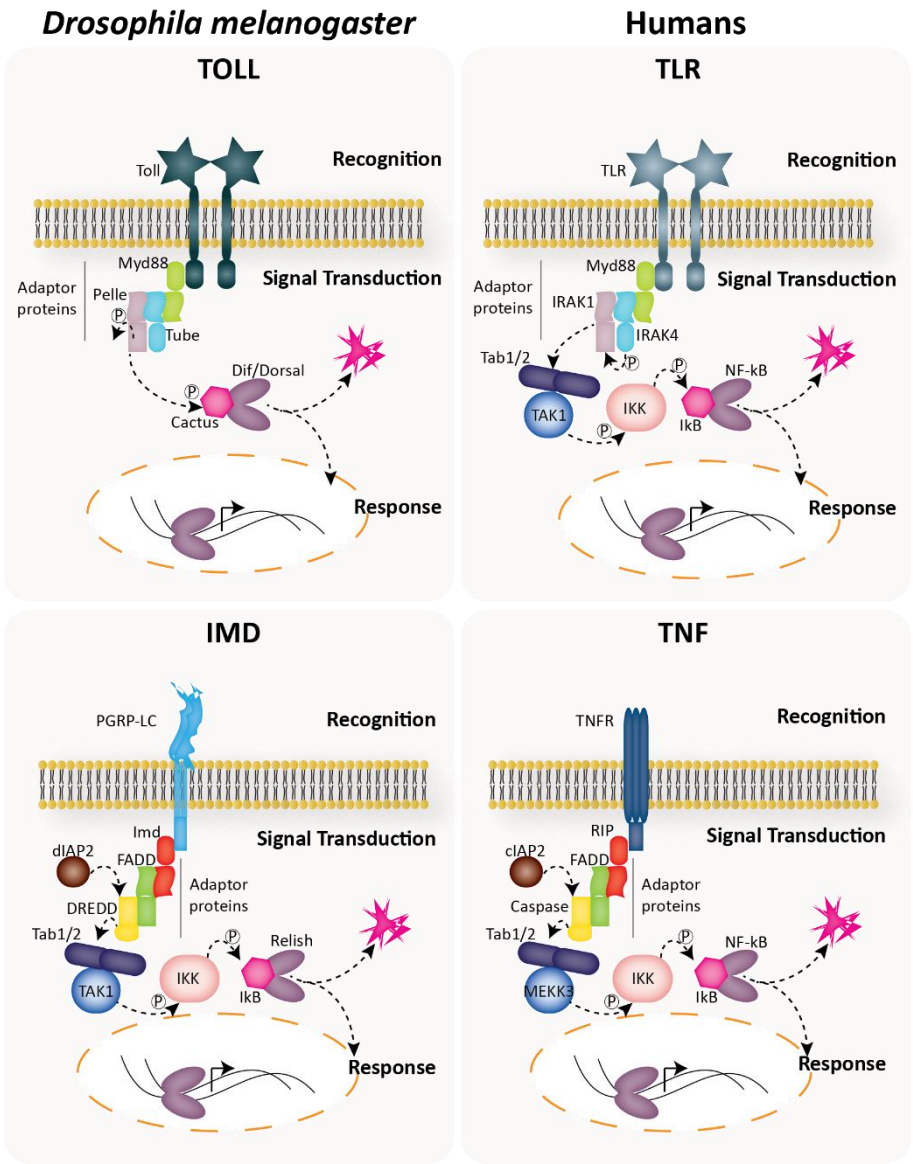

Figure 5. Summary of the similarities across the NF-κB signalling pathways. The pathways share a conserved molecular mechanism whereby a set of adaptor molecules transduce the signal upon activation of the transmembrane receptors and trigger the downstream signalling events. The TOLL and TLR pathways share a conserved membrane receptor containing cytoplasmic TIR domains. In the TOLL pathway, an IKK complex has not been yet identified as in IMD, however it is known that the IkB Cactus undergoes phosphorylation and degradation similarly to the other cascades. *Drosophila* has three NF-κB TFs: Dorsal and Dif that regulate TOLL-responses and Relish that regulates IMD-responses.

Final remarks and thesis overview

My PhD project aimed to understand Host-Bacteria interactions through the recognition of PGN. For that I followed two different approaches – one from the side of the Host and another from the side of the Bacteria. Through the Host side, I aimed to study how PGRPs can distinguish different types of PGN. Through the Bacteria side, whether the presence of WTA could impair recognition from different PGRPs and whether the activity of different autolysins can prevent the recognition of cell surface exposed PGN fragments. The rationale behind this dual strategy was that data collected from both sides would be complementary and thus help to achieve a wider understanding of these complex interactions.

I aimed to study the role of autolysins in the *S. aureus* virulence. I enquired whether the dramatic impact of the absence of the major autolysin Atl [74], would extend to the other autolysins from *S. aureus*. I studied the binding of PGRP-SA to different autolysins mutants and their virulence in *D. melanogaster*.

To understand the PGRPs-PGN interactions, I started by studying the reason behind the reported PGRP-SA specificity to Lys-type PGN, using *Staphylococcus aureus* as a model pathogen [89], [90]. Because I aimed to study *in vitro* the biological relevance of the binding of PGRP-SA to the PGN, I tried that my *in vitro* assays would be as close as possible to *in vivo* conditions. This made me start the work by optimizing experimental procedures. As I was finishing my optimizations and testing several controls, I made a striking discovery that shaped the following steps of my PhD work. I detected binding of PGRP-SA to the *B. subtilis* amiDAP PGN. This binding had never been previously detected [89], [90]. This observation suggested that PGRP-SA is promiscuous towards the amino acid of the third position of the stem peptide, which if true, it should

possess a biological role in the Host upon infection. Furthermore, if PGRP-SA proved to be important for a response towards different bacterial PGN types, then PGRP-LC as also a major immune receptor, should also be promiscuous for the PGN-types. Consequently, I aimed to ascertain the binding specificities of PGRP-SA and PGRP-LC towards the Lys and DAP amino acids. In parallel, I aimed to understand whether the WTA in the Gram-positive CW could impair recognition of different PGN types by both PGRPs. Thus, I contacted Eric Brown and asked for the *Bacillus subtilis* Δ *tagO* mutant (unable to produce WTA) with the respective wild-type strain and I used them as model organisms of DAP-type Gram-positive bacteria. In parallel, as the work evolved, the data suggested that PGRP-SA was likely to be involved in cellular responses. The final goal of my PhD project, was to study the role of PGRP-SA in phagocytosis by both Lys- and DAP-type PGN bacteria.

After describing the variability in PGN composition, I aimed in this introduction to demonstrate how variable the peptide moiety is and open the readers` mind to the fact that conserved immune receptors may perhaps target more conserved regions of the PGN. Accordingly, I also called the attention that although being the most conserved regions among PGNs, the glycans present modifications that effectively protect the bacteria against Host strategies. Moreover, I intended to be clear for the reader that independently of the CW type, PGN is the common denominator of bacteria. Hence it is logical that it is a target for Host receptors and intuitive to realise that Bacteria may present common strategies to avoid its recognition, such as the WTA and the autolysins.

Briefly, in Chapter II, I present novel data regarding the recognition of PGN by PGRP-SA and how autolysins play simultaneously a role in cell division and evasion to the Host responses. In Chapter III, I present data that invalidate the specificity of PGRPs towards the PGN peptide moiety as the reason to the reported differential activation between the TOLL and

the IMD pathways [125]. Moreover, I show that the WTA can impair PGN recognition by both PGRP-SA and PGRP-LC and that these two PGRPs are important for an efficient response upon infection of Lys- and DAP- type PGN bacteria. Chapter IV presents work which shows that both PGRP-SA and PGRP-LC engage in cellular responses through phagocytosis and clearance of the bacteria. Finally, Chapter V – conclusion of the thesis – presents an integration of all of these data on the comprehension of the interaction between the Bacteria and the Host.

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CHAPTER II

Recognition of peptidoglycan determines efficient antibacterial responses

Contributions to this Chapter:

Filipa Vaz[1,2] designed and performed the experiments: constructed the bacterial mutant strains, performed the characterisation of the mutants, zymography, pull-down assays, microscopy, haemolysins assays and purification of the proteins. Performed the *in vivo* studies of survivals and qPCRs. Analysed the data.

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Magda Atilano[1] performed the infections of the flies for survivals and qPCRs. Helped performing the qPCRs and in the methodology to analyse the data.

Sergio Filipe[2] and Petros Ligoxygakis[1] designed the experiments and analysed the data.

Publications in this chapter:

M.L. Atilano*, P.M. Pereira*, **F. Vaz**, M.J. Catalão, P. Reed, I.R. Grilo, R.G. Sobral, P. Ligoxygakis, M.G. Pinho and S.R. Filipe, "Bacterial autolysins trim cell surface peptidoglycan to prevent detection by the Drosophila innate immune system**,"** e*Life*, 1;3:e02277, 2014.

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F. Vaz and S.R. Filipe, "Preparation and Analysis of Crude Autolytic Enzyme Extracts from *Staphylococcus aureus*," *Bio-protocol,* vol. 5, Iss 24, pp. 1-12, 2015.

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Summary

The work presented in this Chapter is a follow-up upon the discovery of the role of the major autolysin Atl in the S*taphylococcus aureus* virulence [1]. Atl impairs the accumulation of cell surface exposed peptidoglycan (PGN) which can be recognised by the *Drosophila melanogaster* PGRP-SA. The recognition of this PGN results in a drastic loss of virulence and survival of the Fly. I aimed to ascertain whether the role of Atl was exclusive or if the absence of other autolysins can also result in the accumulation of PGN that can be detected by PGRP-SA.

I present data that show that during bacterial cell division, PGN present at the septum is accessible to PGRP-SA. In the absence of Sle1 there is accumulation of newly synthesised PGN at the septum that cannot be incorporated into the PGN mesh. This is due to the stiffness of the septal PGN which is not resolved by the specific activity of Sle1. PGRP-SA can recognise these fresh PGN fragments, however the levels of PGN accumulation are not sufficient to aid in an efficient immune response. I propose that PGRP-SA recognition plays with the probability of finding a dividing cell at a certain moment that presents exposed accumulated PGN in a particular subcellular region – the septum. In turn, the interplay between the activity of the autolysins and the incorporation of new PGN material into the polymer mesh, determines the level of PGN that is accumulated at the septum. Concomitantly, the amount of PGN that can be detected is determinant for the triggering of efficient immune responses.

Overall, the results show that detection of PGN is paramount for the triggering of efficient immune responses. Recognition of a certain amount of PGN at the cell surface, can efficiently combat an infection from a major pathogen such as *S. aureus*. This in turn suggests that the virulence factors can only effectively play their roles for as long as the bacteria can protect their PGN recognition.

Introduction

As unicellular organisms, bacteria depend exclusively on themselves to survive and propagate. A single tiny cell has to manage by its own to get nutrients, to keep its internal homeostasis, to protect itself from environmental conditions and replicate. A key factor for bacterial survival and replication is the maintenance of the cell wall (CW) and peptidoglycan (PGN) integrity. When a bacterial cell divides, there is the coordination of several processes which involve the duplication of the bacterial components and culminates with the separation of the two daughter cells. For cell growth and separation to occur, old PGN needs to be degraded. This is mediated by the activity of PGN hydrolases, commonly referred to as autolysins [2]. Maintenance of the cellular integrity is achieved through a coordinated process of synthesis and degradation of PGN in order to avoid cell lysis. The last stage of PGN synthesis is catalysed by the Penicillin Binding Proteins (PBPs) and the cleavage of old PGN by the autolysins opens up space for the insertion of new material. Thus, regulation of the autolytic activity is of uttermost importance, in which autolysins act in a temporal and spatial well-orchestrated manner.

The PGN hydrolases compose a vast and diverse group of enzymes that cleave the PGN sacculi or PGN soluble fragments. They are classified into two main types– the glycosidases and the amidases (Fig. 1) [3]. To the bacterial PGN hydrolases involved in the bacterial cell division, I will refer to them as autolysins, to distinguish from PGN hydrolases produced by other organisms which can cleave another organism` PGN, e.g. mutanolysin from *Streptomyces globisporus* and lysostaphin from *Staphylococcus simulans* that can cleave *Staphylococcus aureus* PGN, and they do not participate in cell growth and division.

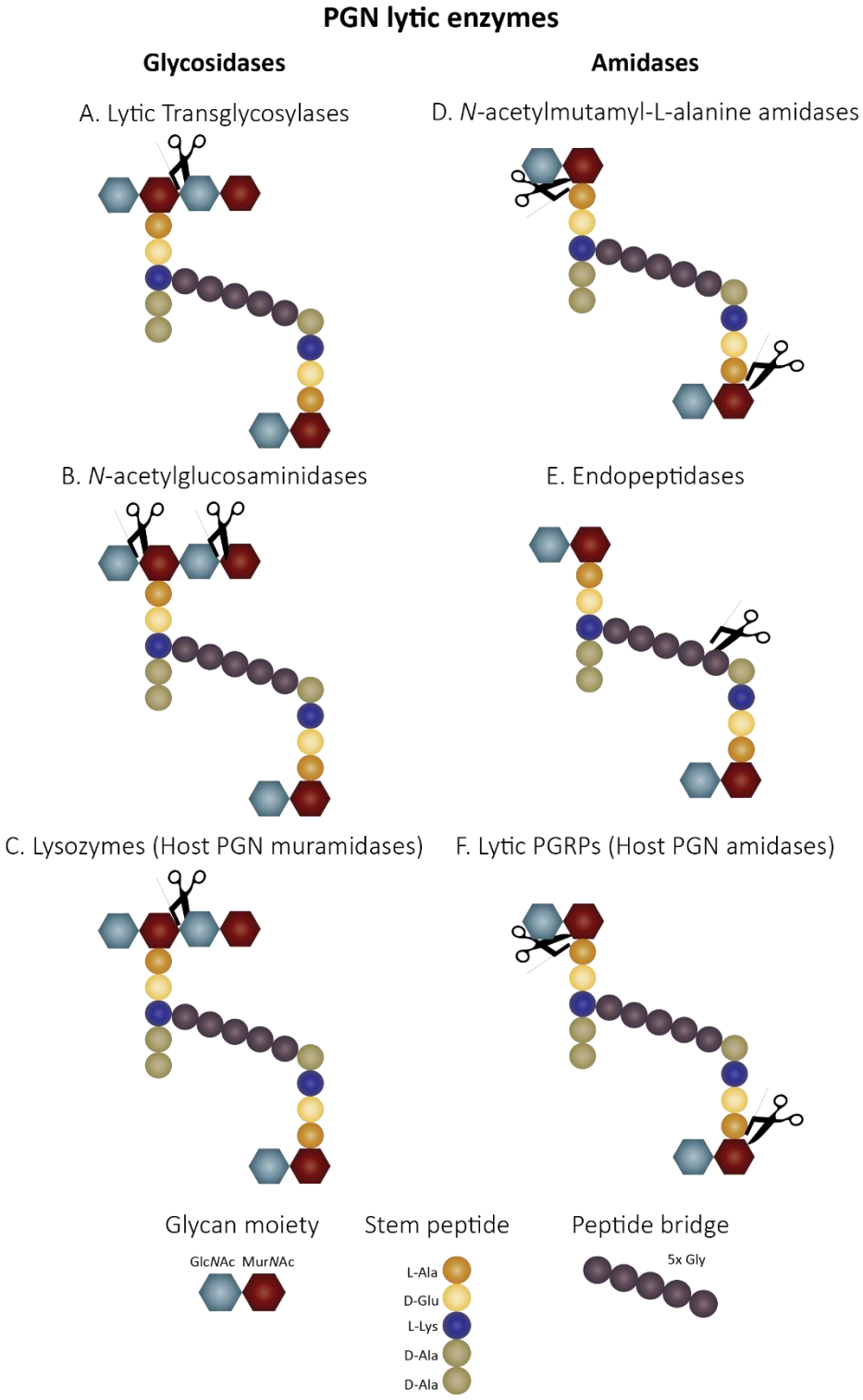

PGN lytic enzymes
Glycosidases
Amidases
A. Lytic Transglycosylases
D. N-acetylmutamyl-L-alanine amidases
B. N-acetylglucosaminidases
E. Endopeptidases
C. Lysozymes (Host PGN muramidases)
F. Lytic PGRPs (Host PGN amidases)
Glycan moiety
GlcNAc MurNAc
Stem peptide
L-Ala
D-Glu
L-Lys
D-Ala
D-Ala
Peptide bridge
5x Gly

Figure 1. Representation of the PGN hydrolytic activities in an *S. aureus* PGN model. The glycosidases cleave the glycan backbone and there are three types: lytic transglycosylases (A), glucosaminidases (B) and muramidases (C). Lytic transglycosylases (A) and muramidases (C) are also collectively known as *N*-acetyl-β-D-muramidases and they cleave between the Mur*N*Ac and Glc*N*Ac residues. They are differentiated by their reaction products. Muramidases (C) generate terminal reducing Mur*N*Ac residues and lytic transglycosylases (A) generate a 1,6-anhydro ring (Fig. 3, Chapter I). The *N*-acetylglucosaminidases (B) hydrolyse Glc*N*Ac residues from adjacent sugar residues. The amidases cleave the peptide moiety and are divided into *N*-acetylmuramyl-L-alanine amidases (D and F) and endopeptidases (E). *N*-acetylmuramyl-L-alanine amidases hydrolyse the amide bond between Mur*N*Ac and L-alanine separating the glycan strands from the peptides. Endopeptidases cleave the bonds in the stem peptides and peptide bridges. In particular, carboxypeptidases cleave the C-terminal amino acid (D-alanine) of the peptide chain (not depicted in the figure).

For every type of bond present in the PGN, there is a PGN hydrolase able to cleave it [2]. However, a bacterium may not possess all autolysins` types. Autolysins comprise different PGN hydrolytic domains which confer a certain lytic activity, which is often specific or optimal for the PGN of the bacteria that produce it [1], [4]. However, it is often difficult to assign a distinct specific function to an autolysin, mainly because many bacteria possess a high number of autolysins which appear to have redundant roles [5].

As autolysins are responsible for PGN cleavage, which in turn is associated with several physiological processes, these enzymes present numerous roles. They are central for CW/PGN growth, PGN turnover and recycling, separation of the daughter cells, autolysis, remodelling of the sacculus to determine cell shape, assembly of cell surface organelles, sporulation and germination, resuscitation of dormant cells, biofilm formation and bacterial lysis in species competition [6]–[14]. In addition, autolytic activity can result in the release of PGN turnover fragments into the surrounding medium [8]. These turnover products can act as signalling molecules [10] and/or be detected by PGN Host receptors, such as the detection of muramyl-dipeptide (MDP) by Nod2 [15] and tracheal cytotoxin (TCT) by PGRP-LC [16].

Autolysins in *Staphylococcus aureus*

The Host Labs have discovered that when the major autolysin Atl of *Staphylococcus aureus* is absent, PGRP-SA from *Drosophila melanogaster* can recognise the accumulated PGN fragments at the cell surface [1]. This recognition renders the *atl* mutant significantly less virulent and highly susceptible to the Host responses. After participating in this study, I aimed to understand if other *S. aureus* autolysins could also contribute to impair bacterial detection and dampen the immune responses by accumulation of high amounts of PGN fragments that are accessible to PGRP-SA.

I searched the genome of NCTC 8325 *S. aureus* strain for genes encoding for proteins containing a PGN-Binding/Lytic Domain (PGN-BLD) which is associated with an autolytic activity. I excluded the bacteriophages genes and identified 19 bacterial genes which codify for proteins that are or might be autolysins (Fig. 2). These 19 proteins fall into five hydrolytic activities (Fig. 3).

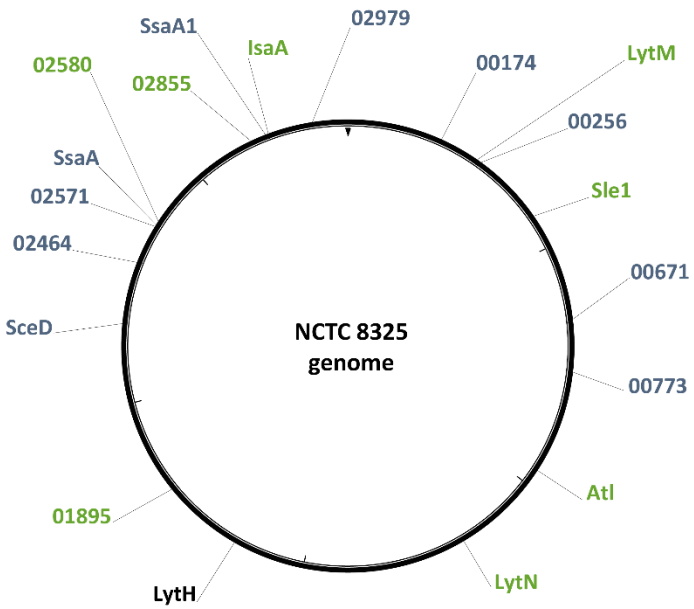


Figure 2. Chromosomal distribution of the bacterial PGN-BLD proteins associated with autolytic activity in NCTC 8325. 19 bacterial genes were identified and found to be distributed throughout the staphylococcal genome. The genes encoding proteins which have not been named in previous reports are designated by their locus number. The names depicted in green are the genes that I deleted from the NCTC 8325-4 background (expect the *atl* mutant).

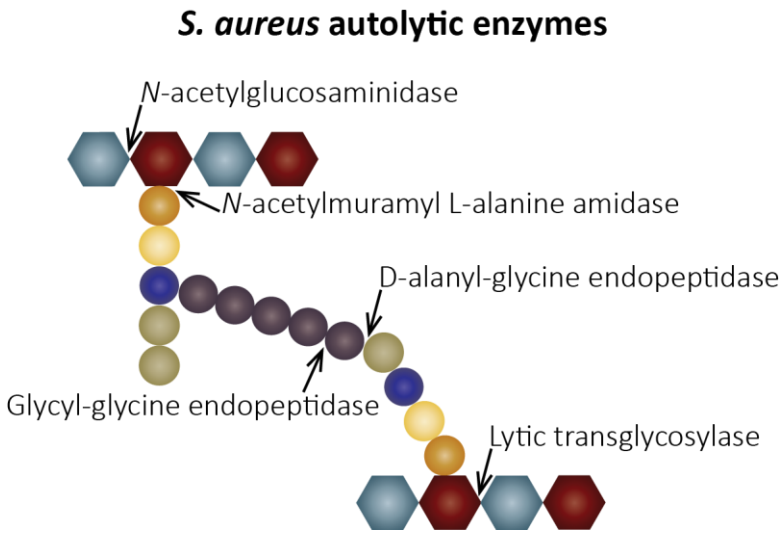


Figure 3. Cleavage sites of the *S. aureus* autolysins. The *S. aureus* PGN is degraded at the glycan backbone by *N*-acetylglucosaminidases and lytic transglycosylases. The peptide moiety suffers cleavage from the glycans through the *N*-acetylmuramyl-L-alanine amidases and the peptides are cleaved by endopeptidases at the cross-bridge either between two glycine residues or between the glycine and the D-alanine.

The presence of a great number of PGN-BLD proteins with a possible function in hydrolysis, led us to enquire whether a certain activity given by a specific domain could have an effect in the virulence of the bacteria. Thus, I constructed six single null mutants of the genes depicted in green in Fig. 2 in the NCTC 8325-4 background (reference strain from R. Novick).

The domains associated with glycosidase activity are[3]: LYZ2[4] – mannosyl-glycoprotein endo-β-*N*-acetylglucosaminidase-like domain (IPR002901), SLT – transglycosylases SLT domain 1 (IPR008258) and TGL – transglycosylases-like domain (IPR010618). The domains associated with amidase/endopeptidase activity are: CHAP – cysteine, histidine dependent amidohydrolase/peptidase domain (IPR007921), PGRP – peptidoglycan recognition protein family domain, metazoa/bacteria (IPR006619), M23 – peptidase M23 domain (IPR016047) and MurNAcLAA – *N*-acetylmuramoyl-L-alanine amidase, catalytic domain (IPR002508). The LysM (Lysin motif) domain (IPR018392) and the SH3b (SH3 (Src homology 3) -like domain, bacterial type) domain (IPR003646) are usually present in autolysins with other domains and their main function appears to serve as binding and adherence domain [2], [17], [18]. The distribution of these domains in the 19 *S. aureus* autolysins is represented in Fig. 4.

[3] EMBL-EBI interpro database.

[4] Lysozyme subfamily 2, SMART accession number: SM00047.

Figure 4. Representation of the PGN-BLD domains from each of the 19 proteins identified. The genes to be deleted were selected based on the proteins` proposed function according to the activity domain. At least one protein of the more prevalent domains – LYZ2, CHAP and M23 – was selected to enquire if one specific domain could be more associated with the impairment of PGN detection. The proteins represented on the top panel are the ones of which the genes were deleted to construct single null mutants.

Overview of the deleted gene products.

The major autolysin Atl.

Atl is a 137 kDa enzyme with a PGRP and LYZ2 domains which renders the protein with both activities of *N*-acetylmuramyl-L-alanine amidase and *N*-acetylglucosaminidase, respectively (Fig. 3 and 4) [9], [19]. The protein undergoes proteolytic processing and generates two extracellular forms: a 62 kDa amidase (AM) and a 51 kDa glucosaminidase (GL) [20]. Both enzymes participate in the splitting of the daughter cells. Each processed form presents binding repeats – R1, R2 (AM) and R3 (GL) – which seem to be involved in targeting to the septum region [21]. It has been proposed

that the presence of WTA mediate the binding of AM and GL through an exclusion strategy mechanism [22]. Atl seems to be involved in different processes that aid in the establishment of infection. Atl can mediate bacterial internalization by non-professional phagocytes [23] and AM can bind to host matrix proteins such as vitronectin [9]. In addition, in the absence of Atl, accumulated cell surface PGN is recognised by PGRP-SA, resulting in a dramatic loss of virulence in a *D. melanogaster* model [1].

The amidases.

The CHAP domain can be found in phages, bacteria and eukaryotes and is associated with two types of PGN cleavage: *N*-acetylmuramyl-L-alanine amidase and D-alanyl-glycyl endopeptidase (Fig. 3) [24]–[27]. Bacterial cell surface binding domains frequently encountered in the CHAP superfamily of proteins are LysM, CW- binding 1 for choline binding (ChBD) and SH3 [26]. The LysM domain is one of the most common attachment modules in bacterial cell surface proteins [17], [26]. The ChBD (choline-binding domain) is responsible for the noncovalent anchoring of the choline binding proteins to the choline moieties of both teichoic and lipoteichoic acids, such as *Streptococcus pneumoniae* hydrolases LytA, LytB and LytC [28]–[32]. The SH3 domain was first identified in eukaryotes [18]. The bacterial SH3 domain, SH3b, is thought be involved not only in PGN binding but also in the binding to Host receptors [18].

LytN is a ~42kDa protein presenting an YSIRK/GS-type signal peptide, a LysM domain and a CHAP domain (Fig. 4). LytN possesses *N*-acetylmuramol-L-alanine amidase and D-alanyl-glycine endopeptidase activities (Fig. 3). The CHAP domain contributes to both lytic activities [33]. The LysM domain is responsible for binding to the PGN [34]. The YSIRK/GS-type signal peptides direct the deposition of secreted proteins

into the cell division site [34]–[36]. LytN appears to be distributed all over the cell surface, including the cell division site [33].

The autolysin Sle1 or Aaa is an ~32 kDa *N*-acetylmuramyl-L-alanine amidase composed of 3 LysM domains which have binding properties to the PGN and a CHAP domain that confers the lytic activity (Fig. 3 and 4) [34], [37]. This autolysin is found at the cell surface and it participates, together with Atl in cell splitting [37]. Similar to Atl, it appears to have a role in pathogenesis as both domains mediate the adherence to the extracellular matrix proteins fibrinogen, fibronectin, and vitronectin [38], [39].

02855 is a ~17 kDa uncharacterized protein with a CHAP domain, thus it is predicted to be an autolysin with amidase activity.

LytM is an ~34.4 kDa glycylglycine endopeptidase with significant homology to lysostaphin (Fig. 3) [40], [41]. It has been found both at the CW and in the extracellular medium [42], [43]. At the CW, LytM is distributed over the cell surface, and thus it has been proposed be involved in CW plasticity rather than cell division [41], corroborating recent findings by Monteiro and colleagues [44] (see Discussion). Nevertheless, the levels of *lytM* are highest during the early exponential phase and almost undetectable in the stationary phase [45], which indicates that this enzyme participates in cell growth processes. It possesses an M23 domain, belonging to the lysostaphin-type metallopeptidases [46], [47] (Fig. 4). However, unlike lysostaphin from *S. simulans*, LytM does not lyse S*. aureus* cells, rather it cleaves the pentaglycine bridges altering CW thickness without compromising its integrity [41], [44], [47]–[49].

The glycosidases.

02580 is a 24.4 kDa uncharacterized protein which possesses a LYZ2 domain like Atl, hence it is expected to have an *N*-acetylglucosaminidase activity.

The immunodominant secretory antigen A (IsaA) is a 29 kDa extracellular protein first identified as an immunodominant protein candidate for the development of antibody based vaccines [49] and its immunogenicity was corroborated later on [50]. IsaA is a soluble lytic transglycosylase present both at the cell surface and in the supernatant of medium cultures [51]. The SLT domain belongs to a superfamily of glycosidases ubiquitous in phages, bacteria and eukaryotes [52]–[54]. In bacteria, the lytic transglycosylase activity is commonly found in autolysins of Gram-negative bacteria. The IsaA SLT motif shows a high similarity to the soluble lytic transglycosylase Slt70 of *Escherichia coli* [55]–[57]. The lytic activity was detected by a Zymography assay and it is non-covalently bound to the CW [58]. In particular, it was found to locate at the septum region and it is presumed to be involved in the cell splitting [51]. In accordance, IsaA was detected in the supernatant of exponentially growing cells and its concentration increased until the early stationary phase [59]. As for the cell surface, IsaA was predominantly found in growing bacteria and the levels decreased proportionately with cell proliferation, *i.e.* the reach of stationary phase [59].

Results

Muramidase treatment only allowed detection of processed forms of Atl.

Zymogram assays of SDS crude CW extracts showed five major lytic bands (Fig. 5), typical for the major autolysin Atl [60]. Atl activity can be detected both in *Micrococcus luteus* cells and in *S. aureus* cells, which indicates that the AM and GL activities are not specific for the *S. aureus* PGN. On the other hand, the lytic activity of Sle1 could only be detected when I used *S. aureus* cells as substrate. As an overall, it can be concluded that some Atl forms and Sle1 are present in the CW through a non-covalent manner.

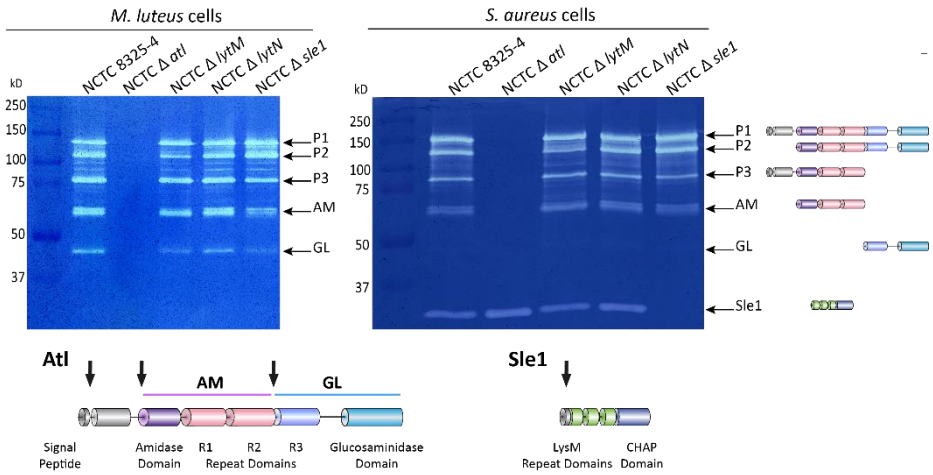


Figure 5. Zymography analysis of SDS crude autolytic enzyme extracts of *S. aureus* strains. The autolytic enzymatic extracts were ran both in *M. luteus* and *S. aureus* substrates. Five different forms of the major autolysin Atl were seen with either substrates. However, only the *S. aureus* substrate cells allowed detection of Sle1 activity.

In order to detect the activity of other autolysins, the CWs of exponentially growing bacteria were treated with the muramidase mutanolysin (M1). The autolytic extract was analysed by zymogram assays

with *S. aureus* cells as substrate (Fig. 6). Only Atl forms could be detected, as these are not present in the *atl* mutant. The molecular weight of the bands suggests that AM and GL can be detected. However, there are a subset of bands between the predicted AM and GL forms, that are not detected with SDS extraction. Furthermore, it can visualised bands with lower molecular weights (< 37 kDa), which although it is suggestive that one of them might be Sle1, both bands can be detected in the NCTC Δ *sle1* extract. It is possible that one of the bands is M1 which has a molecular weight of 23 kDa. In addition, when both SDS and M1 extracts were run in NCTC Δ *atl* substrate cells, it was not observed differences in the lytic bands (right bottom gel with M1 extracts of NCTC 8325-4 and NCTC Δ *isaA*).

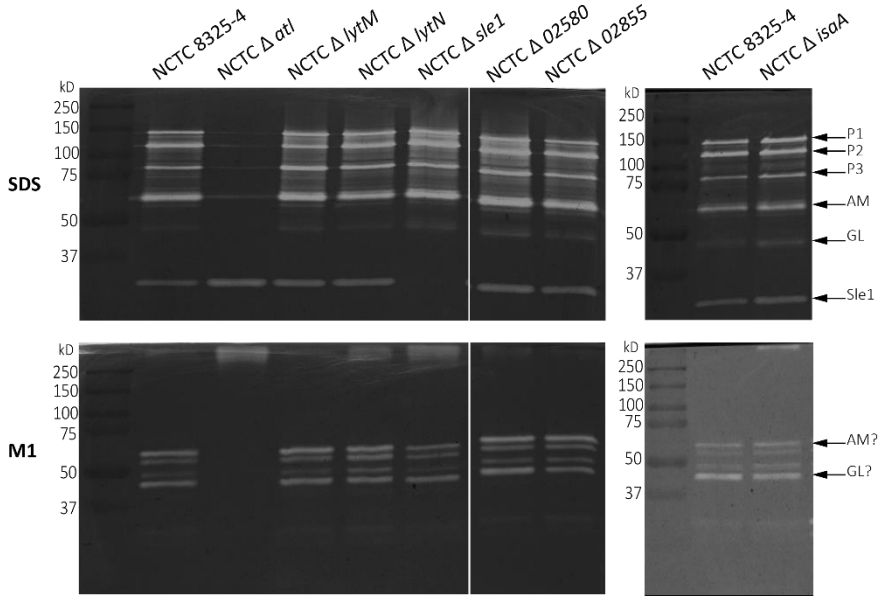


Figure 6. Zymography analysis of M1 crude autolytic enzyme extracts of *S. aureus* strains. Autolytic M1 extraction only allows the detection of Atl processed forms which cannot be detected with SDS extraction. The extracts were run in NCTC 8325-4 substrate cells, except the right bottom M1 extract gel, which was ran in NCTC Δ *atl* cells.

NCTC Δ *sle1* shows binding of mCherry_PGRP-SA at the septum/cell-splitting region.

To determine whether the absence of each autolysin would result in the accumulation of cell surface exposed PGN recognised by PGRP-SA, I performed co-precipitation assays of live cells with an mCherry fluorescent tagged-rPGRP-SA [61]. Cells in early exponential phase were harvested, washed and incubated with mCherry_PGRP-SA. The amount of mCherry_PGRP-SA that bound to the cells was visualised by fluorescent microscopy (Fig. 8). These data are qualitative, since the clustering of the mutants impairs the precise quantification of the fluorescence signal.

NCTC Δ *atl* is the mutant that shows better binding by mCherry_PGRP-SA . In the absence of Sle1, mCherry_PGRP-SA bound exclusively at the splitting septum. Although in the other mutants (and in the wild-type strain), it was observable binding in spots near the cell septum, only in NCTC Δ *sle1* was it possible to visualise the entire division septum through binding of mCherry_PGRP-SA. This pattern of binding is different from the NCTC Δ *atl* cells, to which the whole surface is recognised by mCherry_PGRP-SA [1]. The absence of other CHAP domain containing proteins, 02855 and LytN, did not show the same effect on the recognition of mCherry_PGRP-SA, nor did the absence of the 02580 autolysin (possessing the LYZ2 domain like Atl) and the lytic transglycosylase IsaA.

A.

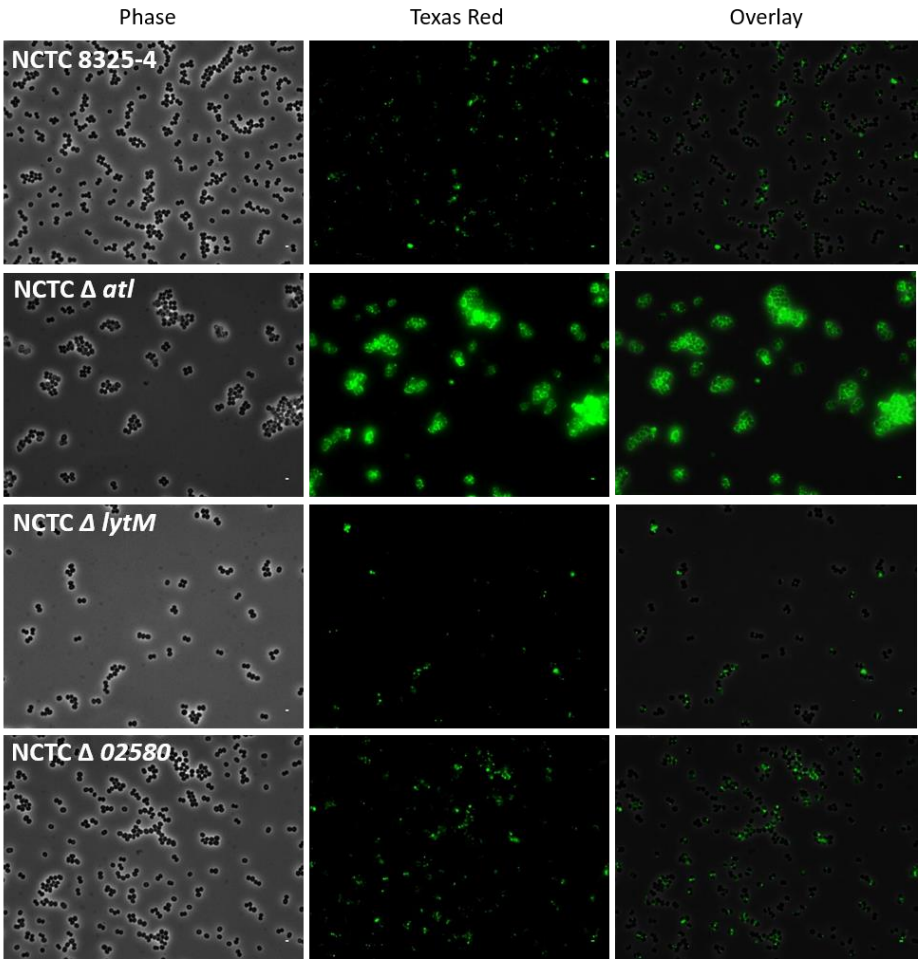


Figure 7. Co-precipitation of live cells with mCherry_PGRP-SA. Cultures in early exponential phase of the parental and mutant strains were incubated with mCherry_PGRP-SA. The protein bound to the cells was pulled down with them and the binding was visualised by fluorescence microscopy. Scale intensities were adjusted to equalise NCTC Δ *atl* – mCherry_PGRP-SA. Pannel A. NCTC 8325-4, NCTC Δ *atl*, NCTC Δ *lytM* and NCTC Δ *02580*. Pannel B. NCTC Δ *isaA*, NCTC Δ *sle1*, NCTC Δ *lytN* and NCTC Δ *02855*.

B.

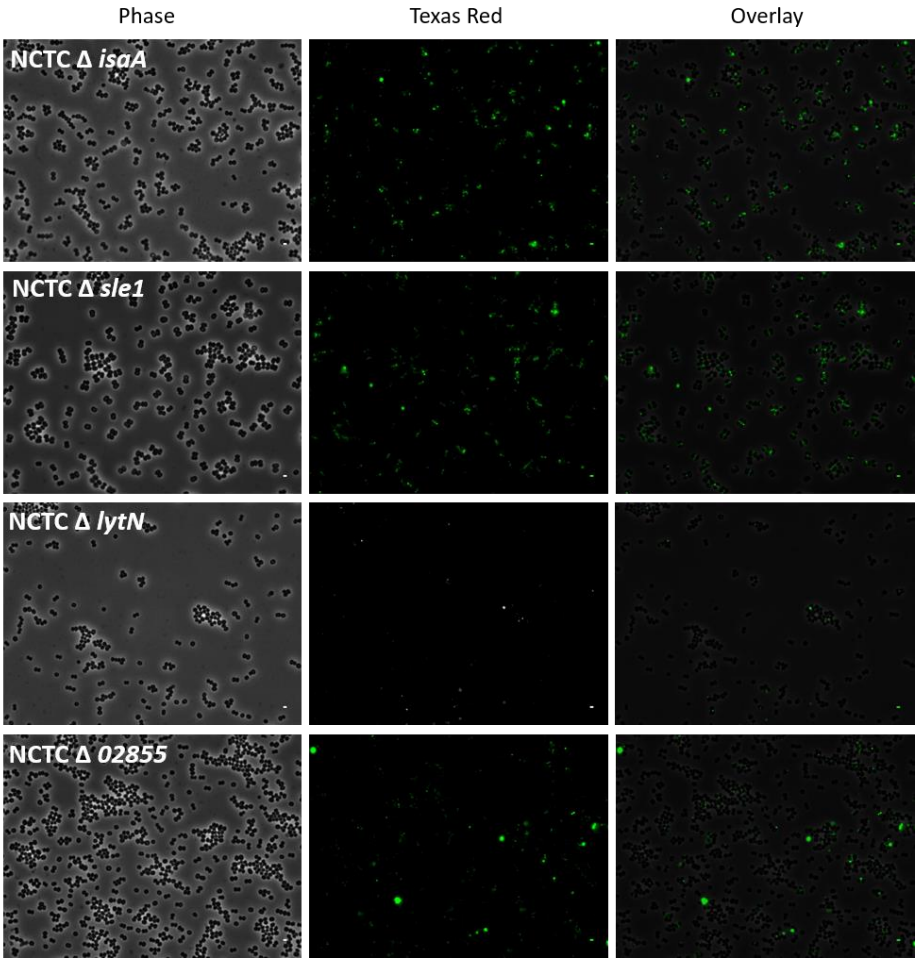


Figure 7. Co-precipitation of live cells with mCherry_PGRP-SA. Phase images show that NCTC Δ *atl* and NCTC Δ *sle1* are the mutants that show a more dramatic phenotype of impaired cell division. Because of the clustering of cells, it was not possible to properly quantify the signals.

mCherry_PGRP-SA binding is lower in cells at the stationary growth phase.

In the wild-type cells, the binding of mCherry_PGRP-SA is very low (Fig. 7). Nevertheless, in some cells mCherry_PGRP-SA binds close to or at the septum/cell division site. In the mutants with a low binding by mCherry_PGRP-SA, it seems that they are also recognised mainly at this region. This suggests that dividing cells are more easily recognised than non-dividing cells. Accordingly, the binding seems to be associated with cell division and the activity of the autolysins, since the recognition of cells in the stationary growth phase is greatly reduced (Fig. 8).

Figure 8. Co-precipitation of live cells in exponential and stationary growth phase with mCherry_PGRP-SA. Cultures in stationary phase of the parental, *atl* and *sle1* strains were incubated with mCherry_PGRP-SA. Scale intensities were adjusted to equalise mCherry_PGRP-SA binding to NCTC Δ *atl* in stationary phase. The binding is lower when the cells are in stationary growth phase.

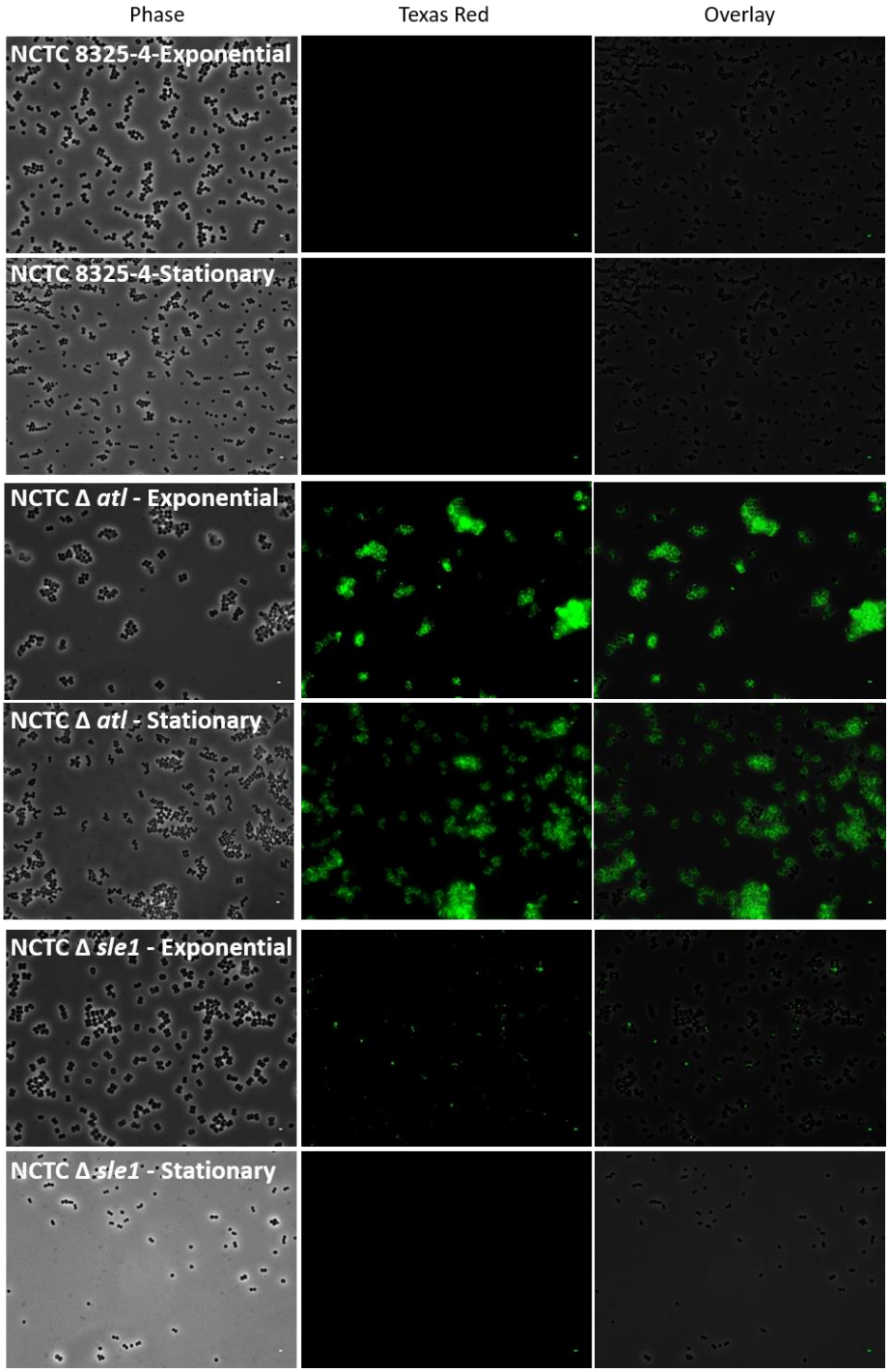
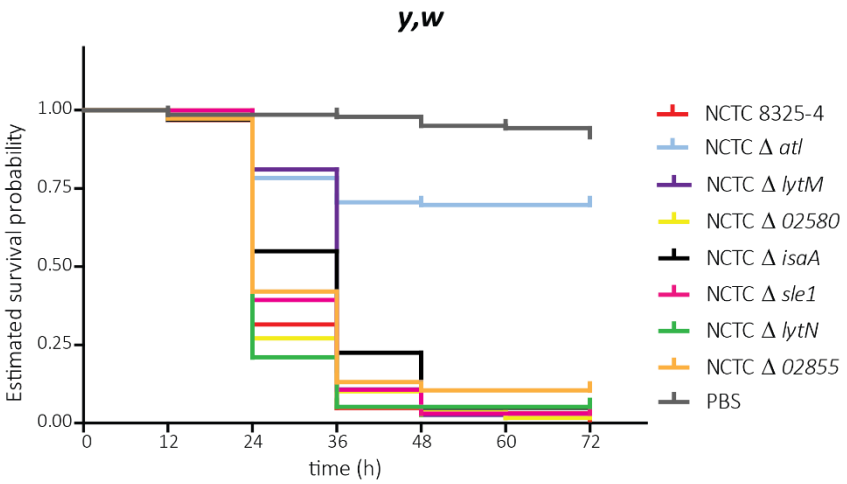


Figure 8. Co-precipitation of live cells in exponential and stationary growth phase with mCherry_PGRP-SA. (Legend on page 81).

Atl is the main autolysin associated with bacterial virulence.

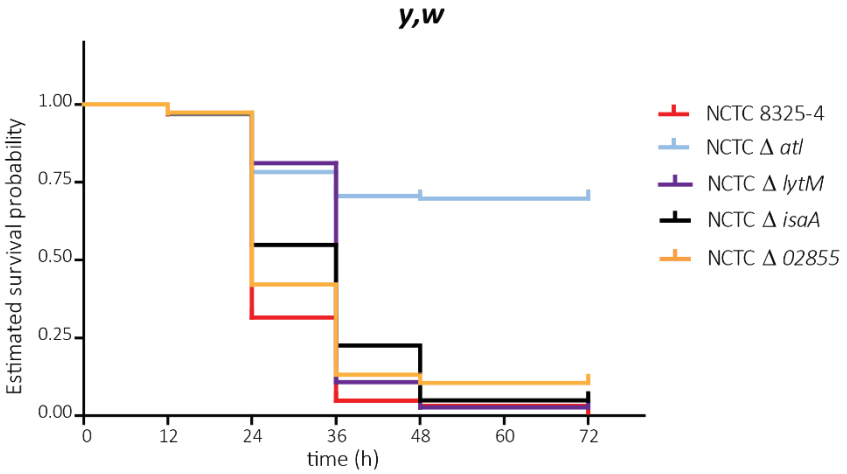
In order to study the virulence of the autolysin mutants, *in vivo* assays were conducted in *Drosophila melanogaster*. The *y,w* flies – immune wild-type flies – were infected with each bacterial strain (Fig. 9). The null mutants *atl*, *lytM*, *isaA* and *02855* showed to be less virulent than the parental strain. Nevertheless, NCTC Δ *atl* is the mutant that shows less virulence (Fig. 9 and 10A). The mutant NCTC Δ *sle1*showed to be as virulent as the wild-type strain (Fig. 9 and Fig. 10B), together with NCTC Δ *02580* and NCTC Δ *lytN.*



Statistical analysis	NCTC Δ *atl*	NCTC Δ *lytM*	NCTC Δ *02580*	NCTC Δ *isaA*	NCTC Δ *sle1*	NCTC Δ *lytN*	NCTC Δ *02855*
NCTC 8325-4	***	***	ns	***	ns	ns	*

Figure 9. Survival curves of *y,w* flies infected with the parental strain and the autolysins mutants. NCTC Δ *atl* is the mutant that shows the most significant loss of virulence compared to NCTC 8325-4. NCTC Δ *atl* is followed by both NCTC Δ *lytM* and NCTC Δ *isaA* which are followed by NCTC Δ *02855*. Whilst *y,w* flies infected with NCTC 8325-4 succumb after 24h p.i. (post infection), the time of highest probability of death is delayed to 36h p.i. with either NCTC Δ *lytM*, NCTC Δ *isaA* and NCTC Δ *02855*.

A.



Statistical analysis	NCTC Δ *atl*	NCTC Δ *lytM*	NCTC Δ *isaA*	NCTC Δ *02855*
NCTC Δ *atl*	N/A	***	***	***
NCTC Δ *lytM*	***	N/A	ns	ns
NCTC Δ *isaA*	***	ns	N/A	ns
NCTC Δ *02855*	***	ns	ns	N/A

B.

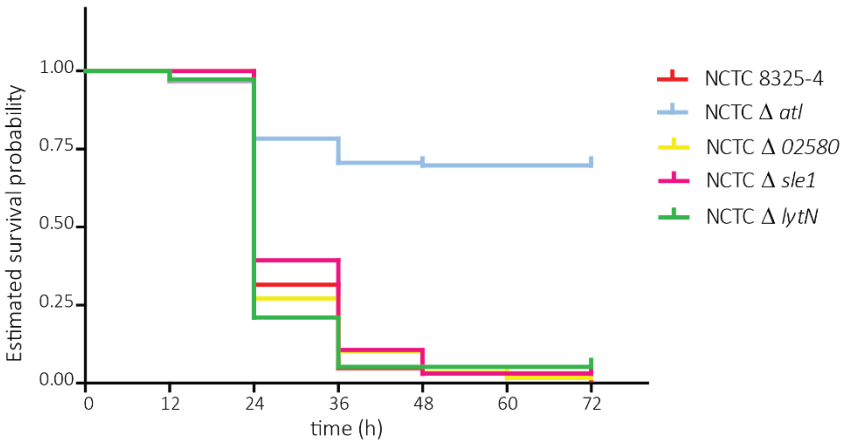
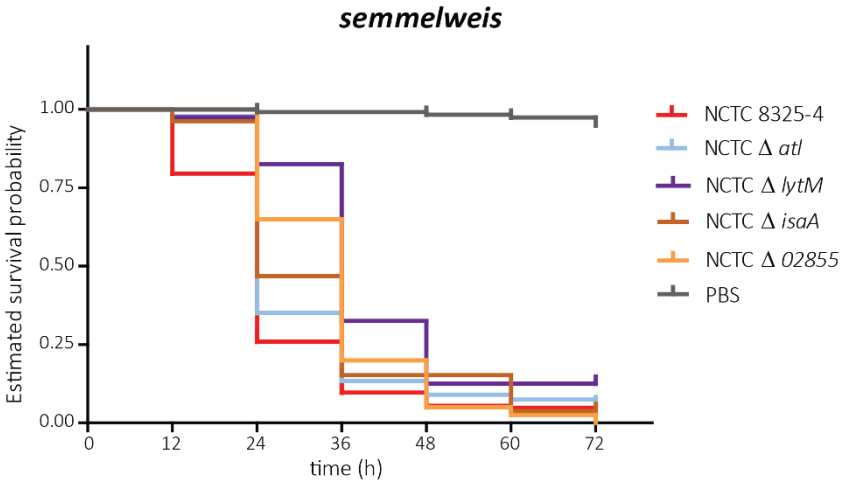


Figure 10. Survival curves for *y,w* flies. The survival graphs show the wild-type strain NCTC 8325-4 and the NCTC Δ *atl* as references for virulent and non-virulent bacteria, respectively. A. Survival curves of the mutants that are significantly less virulent than the wild-type strain. When compared to NCTC Δ *atl*, infection with either NCTC Δ *lytM*, NCTC Δ *isaA* or NCTC Δ *02855*, these mutants are more virulent. The survival curves of these three mutants are in turn indistinguishable between them. B. Survival curves of the mutants that are not less virulent than the wild-type strain. The survival curves of these mutants are indistinguishable between them.

The null mutants *lytM*, *isaA* and *02855* are equally virulent in a *y,w* and a *semmelweis* background.

The response to Lys-type PGN bacteria, particularly *S. aureus* bacteria, is highly dependent on PGRP-SA [1], [61]–[63]. Thus, we infected *semmelweis* flies of the *y,w* background [62], which are unable to produce a functional PGRP-SA, with the bacterial mutants that showed reduced virulence (Fig. 9 and 10A).

When *semmelweis* flies were infected with each of the strains, interestingly NCTC Δ *atl* was the most virulent mutant in this background (Fig. 11). The wild-type strain NCTC 8325-4 was so virulent that it is not possible to distinguish the survivals of *y,w* and *semmelweis* flies (Fig. 12). However, the *semmelweis* flies are highly susceptible to the non-virulent *atl* mutant (Fig. 13). Regarding NCTC Δ *lytM*, NCTC Δ *isaA* and NCTC Δ *02855*, the survival curves of *y,w* and *semmelweis* are not distinguishable (Fig. 14-16).



Statistical analysis	NCTC Δ *atl*	NCTC Δ *lytM*	NCTC Δ *isaA*	NCTC Δ *02855*
NCTC 8325-4	**	***	**	**

Figure 11. Survival curves of *semmelweis* flies infected with the parental strain and the less virulent mutants. The wild-type strain NCTC 8325-4 is the more virulent bacteria, opposite to NCTC Δ *lytM*.

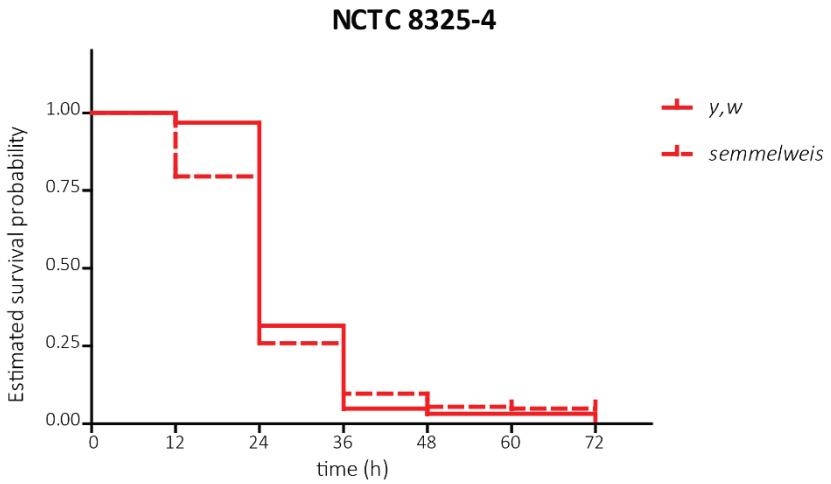


Figure 12. Survival curves upon infection with NCTC 8325-4. The survival curves of *y,w* and *semmelweis* flies are indistinguishable (P = 0.1394).

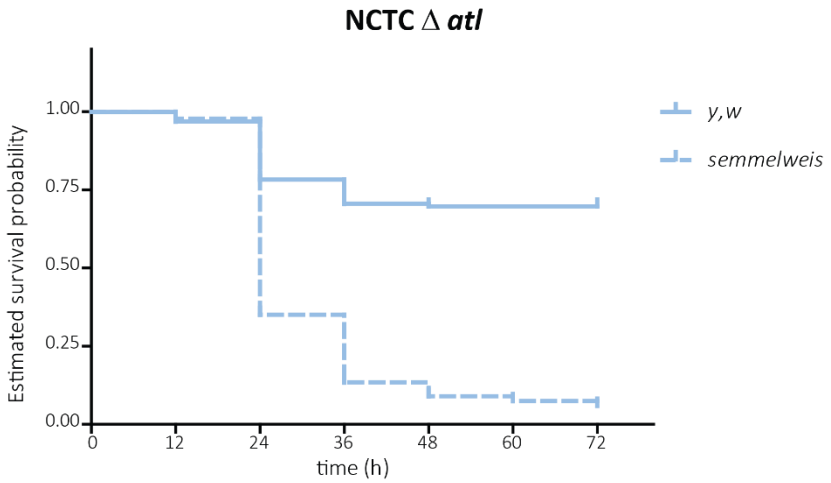


Figure 13. Survival curves upon infection with NCTC Δ *atl*. *semmelweis* flies are significantly more susceptible than *y,w* flies (P<0.0001).

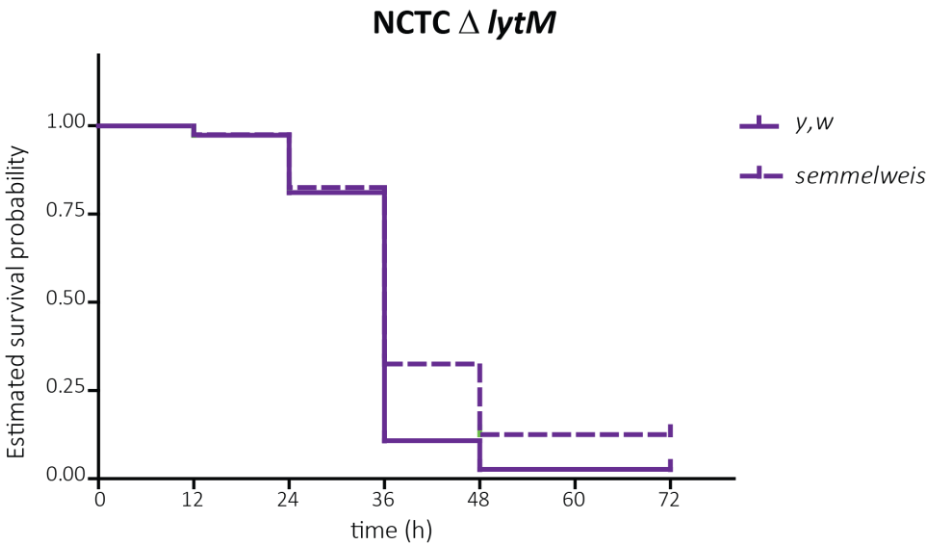


Figure 14. Survival curves upon infection with NCTC Δ *lytM*. The survival curves of *y,w* and *semmelweis* flies are indistinguishable (P = 0.0691).

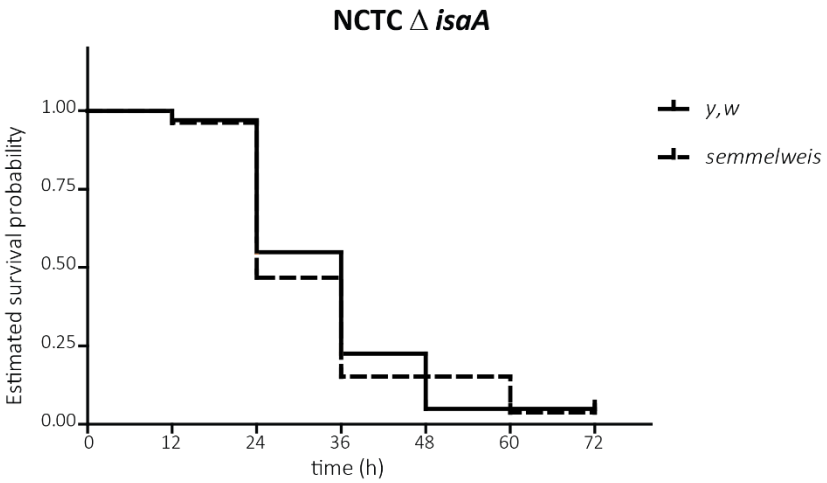


Figure 15. Survival curves upon infection with NCTC Δ *isaA*. The survival curves of *y,w* and *semmelweis* flies are indistinguishable (P = 0.1394).

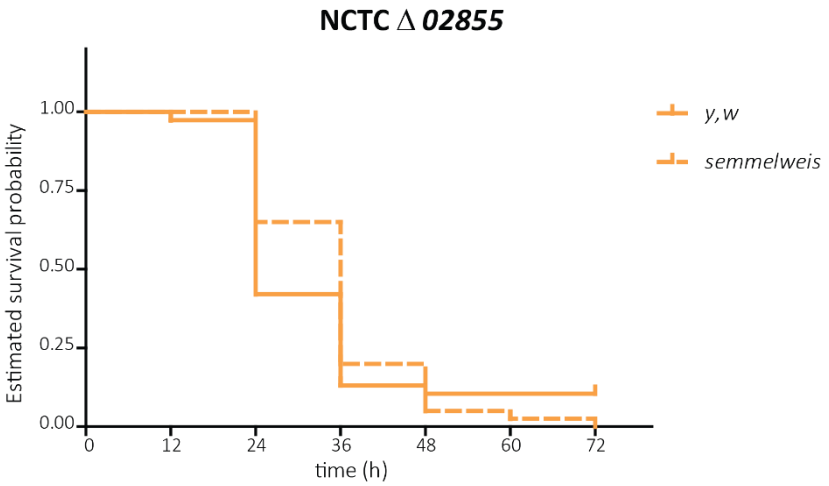


Figure 16. Survival curves upon infection with NCTC Δ *02855*. The survival curves of *y,w* and *semmelweis* flies are statistically indistinguishable (P=0.6089).

Sle1 does not significantly contribute to virulence, albeit binding of PGRP-SA to the NCTC Δ *sle1* splitting septum.

Since NCTC Δ *sle1* showed increased binding of mCherry_PGRP-SA (Fig. 7) but failed to show less virulence in *y,w* flies (Fig. 9 and 10A), we enquired about the susceptibility of the *semmelweis* flies. The survival curves of *y,w* and *semmelweis* were not distinguishable (Fig. 17). The observed *in vitro* binding of mCherry_PGRP-SA to the septum and cell division site (Fig. 7) seems to not be sufficient to promote an efficient immune response by the Fly.

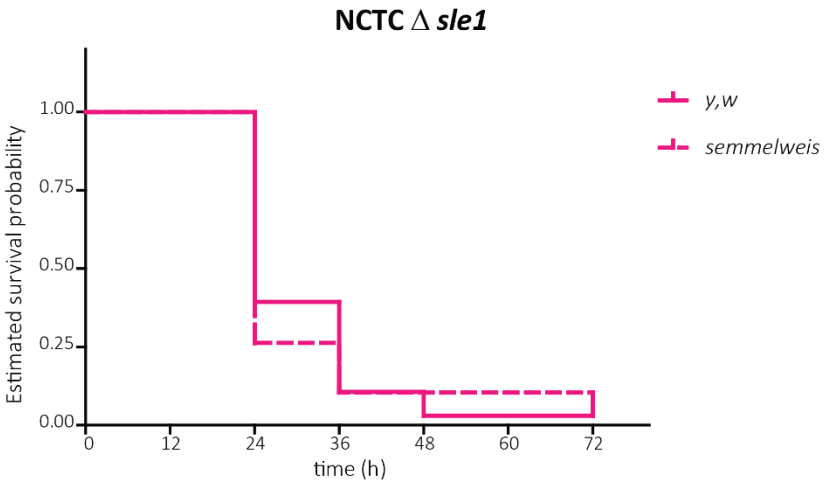


Figure 17. Survival curves upon infection with NCTC Δ *sle1*. The survival curves of *y,w* and *semmelweis* flies are indistinguishable (P = 0.7087).

Subsequently, in order to evaluate whether Sle1 contributed for virulence in the Fly, I constructed a double mutant background NCTC Δ *atl* Δ *sle1*. The double mutant shows a more dramatic impairment in cell division than both single mutants (Fig. 18) in accordance to previous reports [37]. In addition, mCherry_PGRP-SA could bind to the whole surface of the bacteria, similar to NCTC Δ *atl*, and not exclusively to the splitting septum as NCTC Δ *sle1* (Fig. 18).

The double mutant NCTC Δ *atl* Δ *sle1* showed to be as virulent as the single mutant NCTC Δ *atl* (Fig. 19A) and to equally dependent on PGRP-SA recognition (Fig. 19B). Consequently, the loss of virulence of the double mutant is attributed to the lack of the major autolysin Atl and the absence of Sle1 does not significantly contribute for virulence upon septical infection.

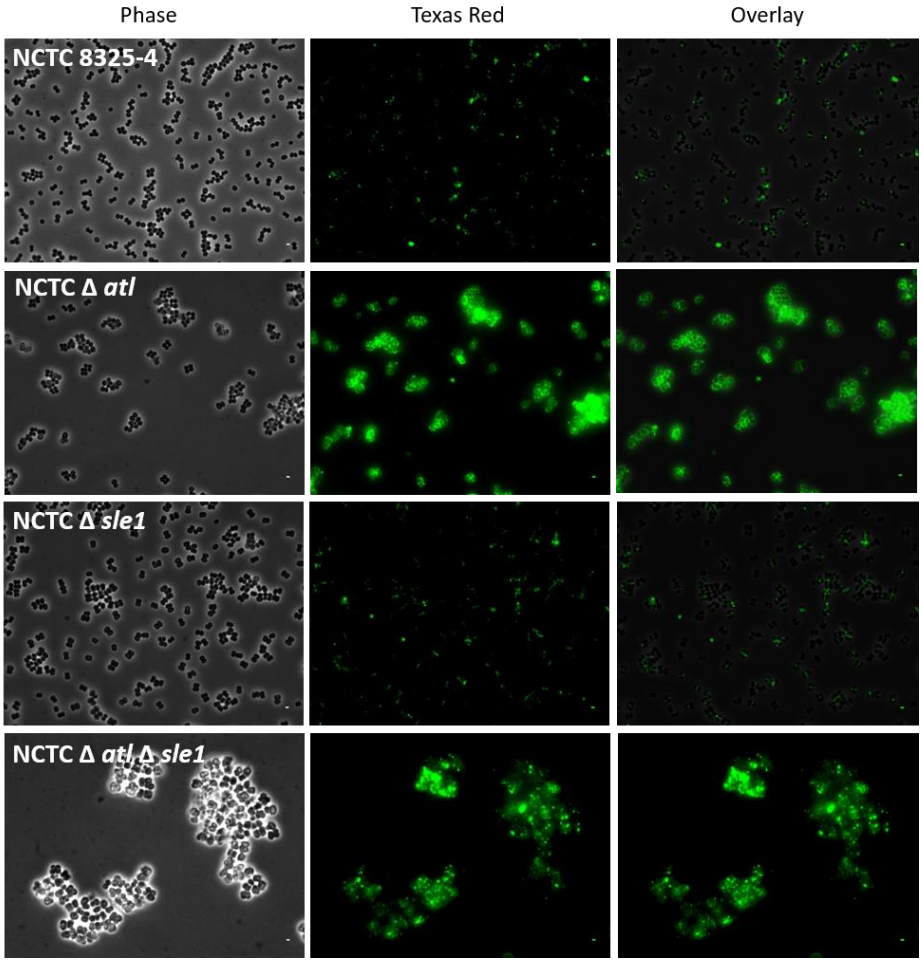
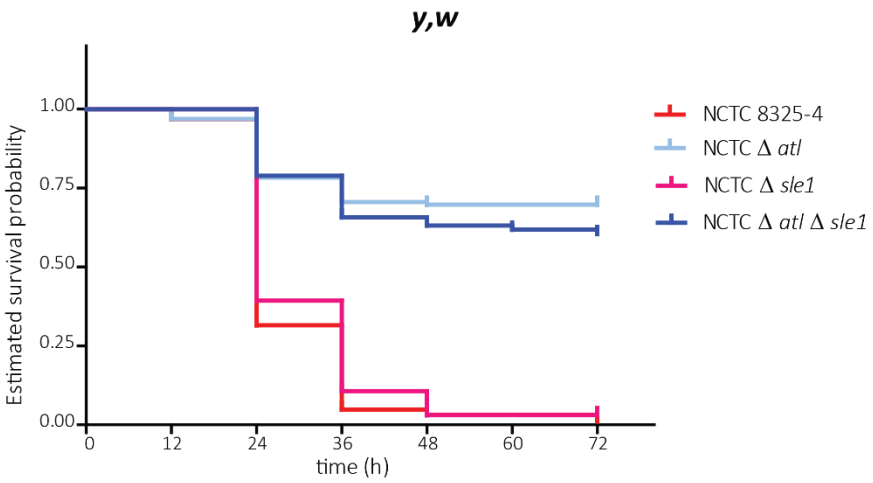


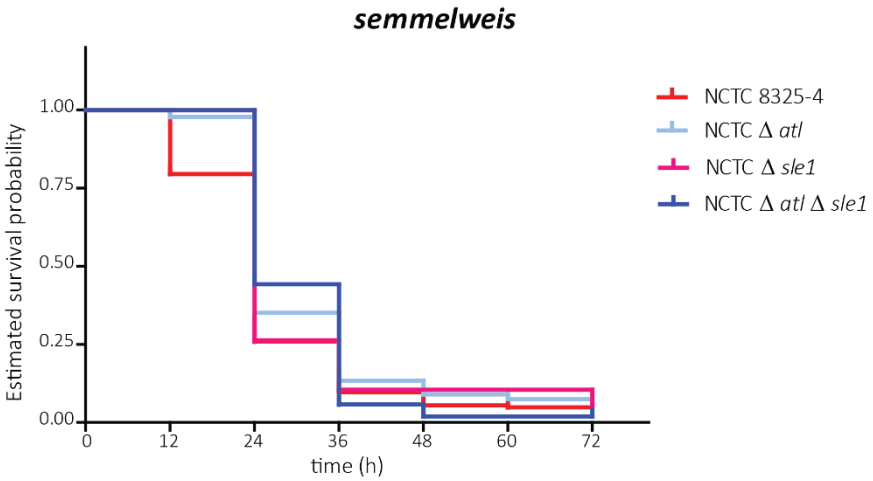
Figure 18. Co-precipitation of live cells with mCherry_PGRP-SA. mCherry_PGRP-SA is able to bind to the whole bacterial surface of the double mutant NCTC Δ *atl* Δ *sle1*, albeit it shows some accumulation of binding close at cell division sites. Scale intensities were adjusted to equalise NCTC Δ *atl* – mCherry_PGRP-SA.

A.



Statistical analysis *y,w*	NCTC Δ *atl*	NCTC Δ *sle1*	NCTC Δ *atl* Δ *sle1*
NCTC 8325-4	***	ns	***

B.

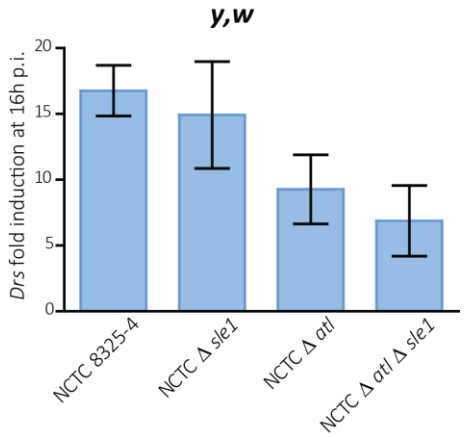


Statistical analysis	NCTC Δ *atl*	NCTC Δ *sle1*	NCTC Δ *atl* Δ *sle1*
NCTC 8325-4	**	ns	*

Figure 19. Survival curves of *y,w* and *semmelweis* flies by NCTC Δ *atl* Δ *sle1* and its parental strains. A. Survival curves of *y,w* flies. B. Survival curves of *semmelweis* flies. The survival curves of NCTC Δ *atl* and NCTC Δ *atl* Δ *sle1* were overlapping in both fly backgrounds and statistically less virulent than the parental strains.

Induced AMPs seem to have a secondary role upon infection with NCTC Δ *atl* and NCTC Δ *atl* Δ *sle1*.

Upon bacterial infection, recognition of Lys-type PGN by PGRP-SA and DAP-type PGN by PGRP-LC triggers either the TOLL or IMD pathways, respectively, which induce the expression of antimicrobial peptides (AMPs) [62], [64]–[67]. In order to address the role of TOLL-dependent AMPs against infection of NCTC Δ *sle1*, we determined the *drosomycin* transcript levels at 16h p.i. (Fig. 20). Infection with NCTC Δ *sle1*, did not show higher levels of *drosomycin* transcription. On the other hand, infection with both non-virulent mutants NCTC Δ *atl* and NCTC Δ *atl* Δ *sle1*, showed decreased levels of *drosomycin*. This may suggest that less virulent bacteria require less induction of AMPs probably due to an efficient clearance by the early immune responses of the Fly.



Statistical analysis	NCTC Δ *sle1*	NCTC Δ *atl*	NCTC Δ *atl* Δ *sle1*
NCTC 8325-4	ns	*	*
NCTC Δ *sle1*	N/A	ns	*
NCTC Δ *atl*	ns	N/A	ns
NCTC Δ *atl* Δ *sle1*	*	ns	N/A

Figure 20. Induction of *drosomycin* at 16h p.i. in *y,w* flies. *y,w* flies were infected in the same manner as for the determination of the survival curves. NCTC Δ *sle1* show a slight less induction of *drosomycin* than NCTC 8325-4, albeit not significant. Likewise, infection with the double mutant showed non-significant decreased levels compared to NCTC Δ *atl*. Clearly, the non-virulent mutants induce *drosomycin* to much lower levels than the virulent strains.

NCTC Δ *lytM* and NCTC Δ *atl* Δ *sle1* are impaired in haemolytic activity.

S. aureus is a major opportunistic pathogen that presents several virulence factors. I aimed to study whether haemolysins could affect the Host responses, in order to determine bacterial factors unrelated with PGN recognition that could help to explain the reduced virulence of NCTC Δ *lytM*, NCTC Δ *isaA* and NCTC Δ *02855*. In addition, the low levels of *drosomycin* in non-virulent mutants compared to virulent strains, allowed to surmise that bacterial factors impairing early immune responses could be important for virulence. Since the early immune responses rely on the role of haemocytes in phagocytosis and clearance, I enquired whether the *S. aureus* autolysins could be subverting the cellular responses.

Haemolytic assays in sheep blood, showed that NCTC Δ *atl*, albeit showing the same type of haemolytic activities, presents a different pattern of distribution with an extra external halo (Fig. 21A). As the response to NCTC Δ *atl* mutant is highly dependent on PGRP-SA (Fig. 13 and 19B) [1], it can be inferred that the haemolysins are not significantly contributing for loss of virulence.

The double mutant NCTC Δ *atl* Δ *sle1* presents β-haemolysin activity (Fig. 21A) but is compromised in the other haemolysins, similar to RN 4220 (Fig. 21C). This could in part account for the less activation of *drosomycin* compared to the single mutant NCTC Δ *atl* (Fig. 20). Likewise, NCTC Δ *lytM* solely presents β-haemolyses (Fig. 21B) which may contribute for a more reduced virulence than NCTC Δ *isaA* and NCTC Δ *02855* which show a wild-type haemolytic phenotype (Fig. 21B). On the other hand, the virulence of the *sle1* mutant (Fig. 9 and 10B), albeit binding of PGRP-SA (Fig. 7B), may reside in the fact that coupled with an insufficient PGN recognition to trigger a potent immune response, it retains the haemolytic ability identical to NCTC 8325-4 (Fig. 21A).

A.

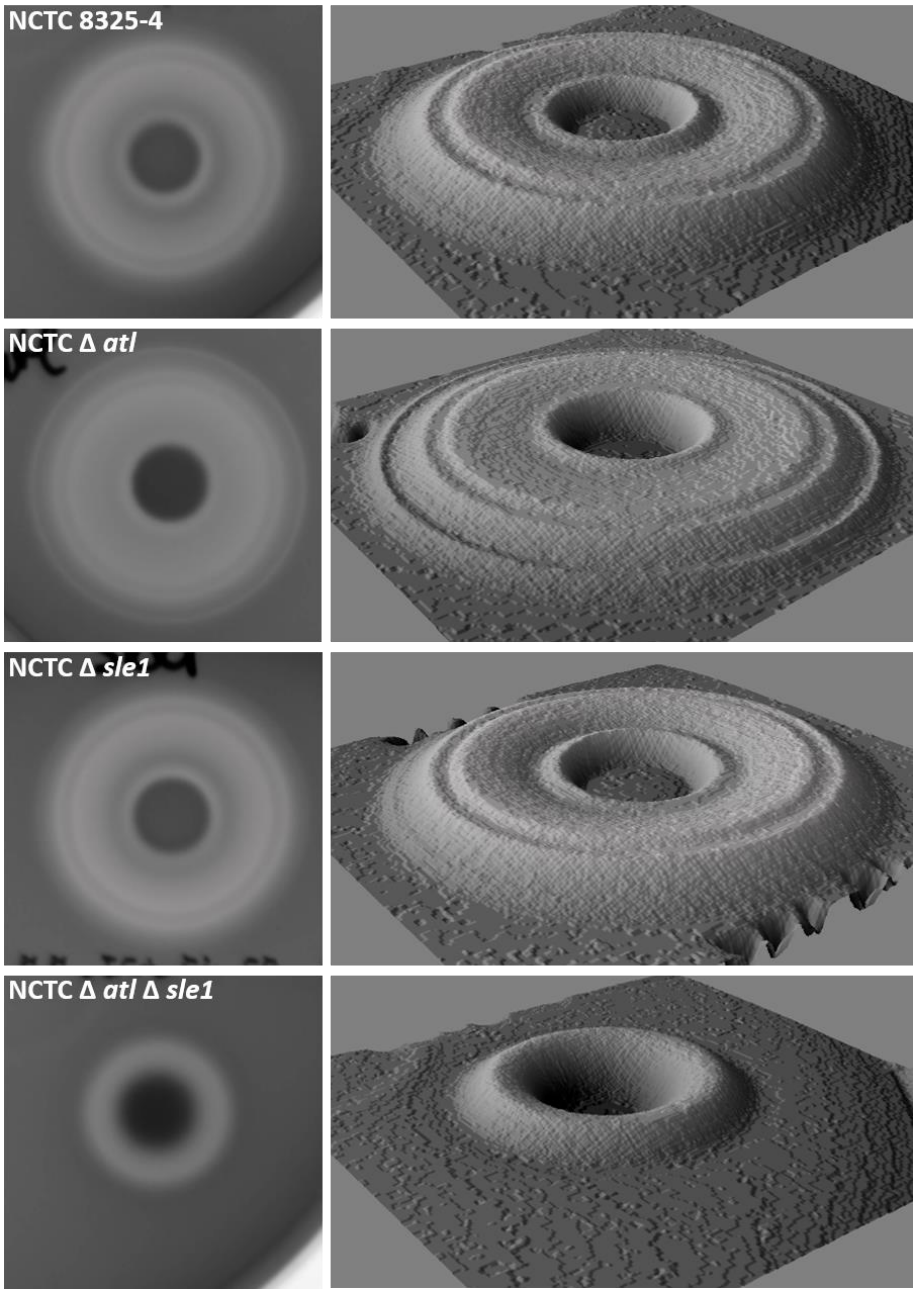


Figure 21. Haemolytic activity in sheep blood. It is presented the haemolytic profile of the strains used in this study. On the left column is it shown an image of the haemolytic activity and on the right column it is shown a 3D surface plot to help in the visualisation of the haemolytic halos. Briefly, overnight cultures were adjusted to the same optical density and inoculated in solid media supplemented with 5% (v/v) sheep blood.

B.

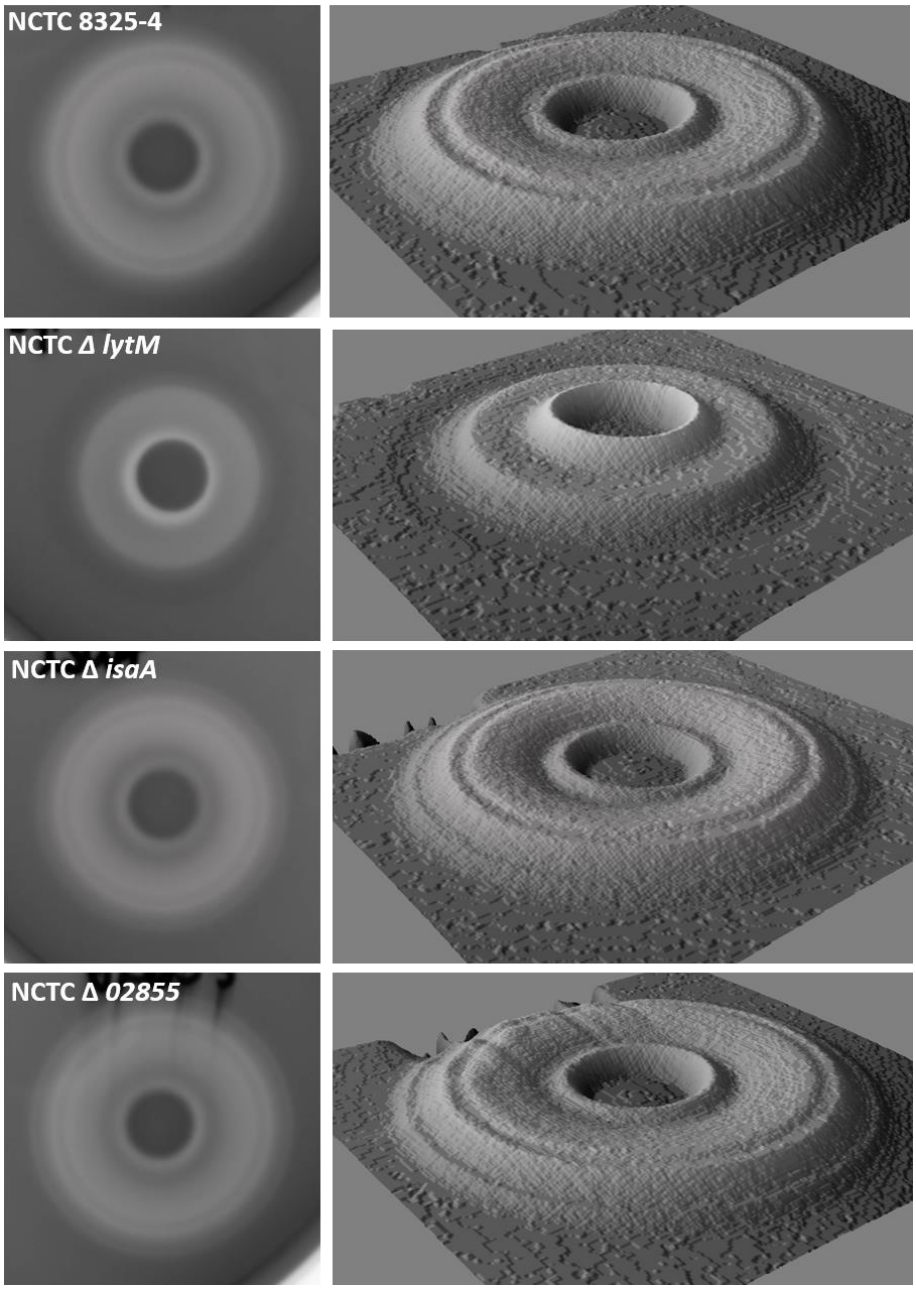


Figure 21. Haemolytic activity in sheep blood. The plates were incubated at 37ºC for 16h followed by incubation at 4ºC for 24h to better visualise and detect the activity of the β-haemolysin.

C.

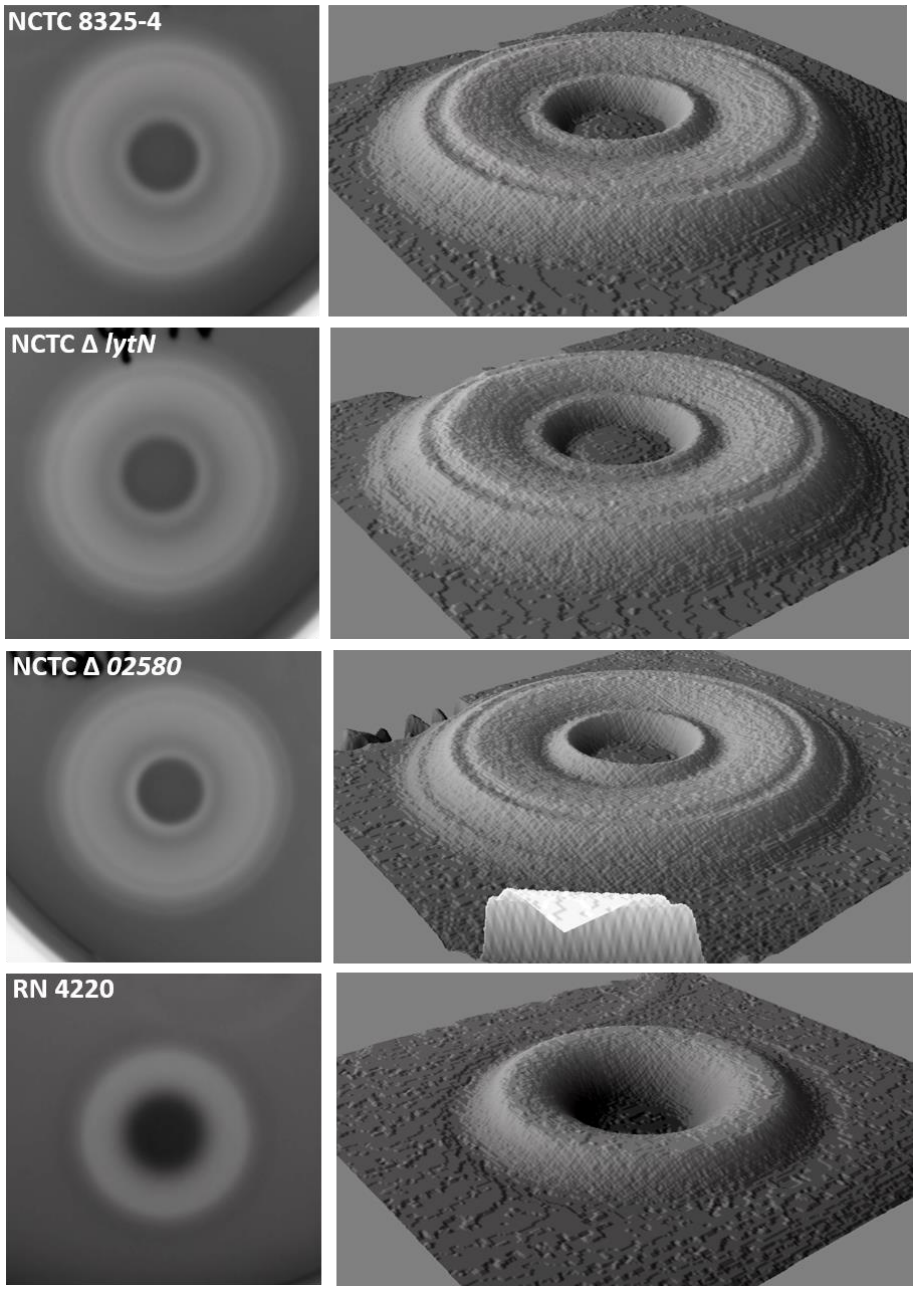


Figure 21. Haemolytic activity in sheep blood. RN 4220 is a laboratory strain impaired in the expression α and δ haemolysins, thus it can only be detected β-haemolyses [68].

Discussion

The structural and chemical properties of the PGN may affect and regulate the activity of autolysins.

The CW treatment with SDS releases components that are non-covalently linked to the CW, whilst it also denatures the proteins. Therefore, the treatment with SDS, which is commonly used to extract CW bound protein, may come with limitations on the detection of the activity of the enzymes. Nevertheless, this procedure successfully allowed the detection of different Atl forms (Fig. 5). We then enquired whether treatment with a muramidase, which would release bound proteins by PGN solubilisation, would allow the detection of different autolytic bands (Fig. 6).

The improved lytic activity of GL with *M. luteus* cells rather than with *S. aureus* cells (Fig. 5), has been proposed to be due to a pre-requisite of AM digestion prior to GL digestion [22]. However, it also could be the case that the GL activity is controlled by modifications in the glycan strands that are not present in the *M. luteus* PGN [69]. This could be a mechanism similar to the *O*-acetylation of Mur*N*Ac which regulates the activity of the lytic transglycosylases in Gram-negative bacteria [70]–[72]. In addition, it may also be due to the different PGN structures of the *S. aureus* and *M. luteus* PGN, such as the degree of cross-linking, length of the glycan chains, differences on the peptide moiety and/or the different CW compositions, such as the wall teichoic acids and the teichuronic acids [69].

As the lytic activity of Sle1 could only be detected with *S. aureus* cells as substrate (Fig. 5), either the Sle1 activity is specific for this PGN or it has a substantial optimal activity in this substrate. This suggests that Sle1 may require different PGN chemical properties than AM in order to be active. Indeed, the amidase activity of these proteins is attributed by different domains – AM possesses a PGRP domain and Sle1 a CHAP domain.

Overall, the results suggest that different autolysins are optimal to PGNs with specific characteristics/modifications. This may be a mechanism to control and regulate the lytic activity.

Treatment of the CWs with mutanolysin (M1), only revealed processed forms of Atl in an *S. aureus* substrate (Fig. 6). This may be an indication that high molecular weight forms of Atl do not covalently bind to the PGN and/or that the activity of the smaller forms is susceptible to SDS extraction. On the other hand, it is possible that M1 does not penetrate well through the CW and has limited access to the PGN. In this scenario, it would be observed the release of Atl forms bound to only certain PGN chains or fragments. Interestingly, some of these Atl forms are not detected by SDS extraction. Overall, the results suggest that Atl may present more processed forms than the ones that have already been identified.

Sle1 was not detected upon M1 treatment, in contrast to the AM form of Atl. It can be inferred that perhaps AM and Sle1 may localise temporally in different places of the cell or that Sle1 requires full denaturation-renaturation process in order to be active in a zymogram. If the SDS present in the loading buffer is not sufficient to fully denature the protein, it might be the case that Sle1 is not able to restore the activity. If the protein folds in a way that incorporates PGN molecules, the recovered Sle1 present in the M1 extracts could be too "close" to be able to interact with new PGN fragments, hence it would require a full denaturation-renaturation cycle. To address this hypothesis, I performed an SDS treatment to the M1 extracts prior performing the zymograms, and still did not detect Sle1 activity and the activity of the Atl enzymes was greatly reduced (data not shown).

PGRP-SA accesses surface exposed septal PGN fragments temporarily generated during cell division.

Cell division culminates with the splitting of the daughter cells. For cell splitting to occur, old PGN at the septum needs to be degraded. Autolysins have been shown to localise at the septum where PBPs also localise [73]. Atl, Sle1, LytM, LytN and IsaA have been shown to localise at the septum, albeit Atl, LytM and LytN are also found distributed over the whole cell surface [21], [33], [37], [41].

In order to prevent autolysis upon cell splitting, the process of degradation and insertion of new PGN material must be regulated in a spatial and temporal manner. Thus, the degradation and synthesis are coupled processes, which it is intuitively logical to assume that degradation happens (very shortly) before the insertion of new material. This implies that for a very short period of time it is possible the occurrence of some accumulation of PGN fragments both from old and new synthesised PGN fragments.

In the wild-type strain NCTC 8325-4, mCherry_PGRP-SA showed a very low binding to exponentially growing cells (Fig. 7). However, when it is detected, binding by mCherry_PGRP-SA is found at or close to the septum region. Therefore, it appears that PGRP-SA is able to recognise PGN at the septum prior to the recycling and generation of the old and new PGN chains. Accordingly, all the autolysins mutants present in this study showed binding at the septum. NCTC Δ *sle1* showed a higher defined binding of the septum than NCTC 8325-4 and the other mutants and NCTC Δ *atl* showed binding all over the cell surface, *i.e.* not exclusively at the septum region (Fig. 7, 8 and 18). Indeed, PGRP-SA only binds all over the cell surface when the PGN is surface exposed (*e.g.* the *tagO* mutant [61] and Fig. 4, Chapter III) or it is homogeneously accumulated at the cell surface (*atl* mutant [1] and Fig. 7, 8, 18).

The binding of PGRP-SA at the septum and cell splitting region can be an indirect evidence of the role of these proteins in cell growth and division. To a certain extent, it allows the inference of their cellular location. Atl is found to localise all around the cell surface and PGRP-SA binds all over the surface in an *atl* mutant. The stronger binding at the septum in the *sle1* mutant compared to the wild-type strain, is an indication of the Sle1 localisation at the cell division site, where it may assume a specific role for an efficient cell splitting.

Interestingly, the mutants that showed a more accentuated phenotype in impairment of cell division were NCTC Δ *atl* and NCTC Δ *sle1* which in turn are the mutants that presented either a better recognition by PGRP-SA or a more defined pattern of binding at the septum. This indicates that these are the mutants that present higher level of cell surfaced exposed PGN. In contrast to previous reports, NCTC Δ *lytN* mutant did not show visible compromised cell defects [33]. In accordance with previous reports, NCTC Δ *isaA* and NCTC Δ *lytM* also did not exhibited impairment in cell division or increased cell clustering [48], [58].

The activity of the autolysins influence the detection of PGN by PGRP-SA.

If PGRP-SA, in a wild-type bacterium, can almost exclusively only detect the PGN that is temporarily generated/exposed during cell division, then cells in a non-dividing stage or a population in a stage of a slower growth rate, should be more difficult to be recognised. According to the hypothesis presented above, recognition by PGRP-SA plays with the probability to access PGN fragments that slightly accumulate for very short periods of time in a specific sub-cellular region. Hence, if the growth rate of a population is lower, the probability of finding a dividing cell that presents a certain level of PGN accumulation is greatly reduced.

In liquid culture, bacteria show an exponential growth phase which is followed by a stationary growth phase. In this stage, the number of bacterial cells no longer increases [74]. During my experimental optimizations, I observed that the binding of mCherry_PGRP-SA is higher at early exponential phase, than at mid or late exponential phase (data not shown). When I incubated mCherry_PGRP-SA with cells in stationary growth phase, I observed a decrease in the binding of the protein (Fig. 8). This may substantially be due to the fact that the cells are not in a constant high rate of division, thus there is less PGN that is exposed (at the septum). In the case of the *atl* mutant cells in a stationary growth phase, although there is still a substantial accumulation of PGN fragments resultant from each cell division cycle, it is likely that these fragments are 1) present in less amounts and 2) old accumulated fragments from past division cycles have had time to be gradually cleaved by other autolysins. In accordance, at this growth stage, the mutants` cells show a less impaired cell division phenotype.

In the absence of Sle1, PGRP-SA binds to newly synthesised PGN fragments, accumulated at the septum, that are not incorporated into the PGN mesh.

There is a "predisposition" for the binding of PGRP-SA to occur at the septum region. However, the binding at the septum of NCTC Δ *sle1* cells is more evident than the binding to other mutants which have also been reported to be active at the septum, such as IsaA [51].

Filipe and colleagues have identified that the terminal Mur*N*Ac residue influences immunostimulation in *D. melanogaster* [75]. In an *isaA* mutant, it has been detected the overexpression of the other lytic transglycosylase, SceD [58]. Hence, it is likely that in NCTC Δ *isaA*, the

number of Mur*N*Ac residues in a form that is not easily recognised by PGRP-SA is maintained.

In collaboration, Filipe Lab and Pinho Lab[5] have shown that both Sle1 and LytM engage in the reshaping of the flat septum into a curved hemisphere upon cell division [44]. The *sle1* and *lytM* mutants show impairment in septum reshape which may be associated with a higher stiffness of the PGN in the absence of these autolysins [44]. If both mutants present a similar cell defect at the septum, but PGRP-SA is only able to bind better to NCTC Δ *sle1* (Fig. 7), it is an indication that the activity of Sle1 at the septum is impairing the recognition by PGRP-SA. In addition, it is also an indirect indication that Sle1 and LytM possess different roles in the process of septum re-shaping.

Sle1 and LytM can lead to the separation of two glycan strands from one another, albeit generating different PGN fragments (Fig. 1 and 3). Whereas the Sle1 amidase separates the glycans from the stem peptides, the LytM glycylglycine endopeptidase separates the link between the stem peptides of two glycan chains. This implies that septum reshape is dependent on the separation of the glycan strands, perhaps to 1) open up space for incorporation of new material and 2) to link old glycan strands with new synthesized PGN forms. The region where the meeting between the old and new CW with the join of new and old PGN occurs, may be a reason for the typical scars observed upon cell division [69]. This goes in accordance with the preferential localization of PBPs at the septum region, together with the localisation of the autolysins.

Sle1 possesses amidase activity which seems to be specific for a PGN with certain characteristics (Fig. 5 and first section of the Discussion). In addition, the phenotype of cell division defects cannot be rescued/compensated as efficiently as the removal of other autolysins can

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be (Fig. 7). This suggests that Sle1 may have a specific crucial role in the cell splitting. Accordingly, the phenotype of an *atl* mutant, the major autolysin, also cannot be compensated to wild-type levels (Fig. 7, 8 and 18). Since the *sle1* mutant has problems in reshaping of the septum, it means that it is a problem of PGN stiffness [44]. Therefore, it is likely that in the absence of Sle1, there is a certain moment in the splitting process that the cell is not able to introduce the new material due to lack of physical space. Hence, Sle1 has a fundamental role in opening up space for septum reshaping by allowing the insertion of new PGN material. Then, NCTC Δ *sle1* accumulates, specifically at the septum, fragments of newly synthesised PGN. PGRP-SA is able to recognise these PGN species that are not being incorporated within the polymer mesh. The crucial role of Sle1 in the aid of the insertion of new material, may be the reason for: 1) the impaired re-shaping of the septum in its absence; 2) its amidase activity which in turn is sensitive to certain PGN modifications and CW composition (Fig. 5 and 6, and first section of the Discussion).

LytN possesses both *N*-acetylmuramyl-L-alanine amidase and D-alanyl-glycyl endopeptidase activities (Fig. 3) [33]. It has been suggested that the latter activity of LytN may be equivalent to that of LytM, since the end result of hydrolysing either the D-alanine-glycine bond or the glycine-glycine bond, will lead to crosslinking relaxation with a polyglycine extremity [33]. Then, the absence of the LytM may be compensated by LytN and the other two putative glycylclycines (Fig. 4). Consequently, the level of PGN accumulation at the septum compared with the wild-type strain, is not sufficient to render a higher binding by PGRP-SA. Likewise, the absence of LytN may be to some extent compensated by the other amidases and glycylglycine endopeptidases, similar to the absence of the putative amidase 02855. The absence of 02580, a LYZ2 domain containing protein, is likely to be compensated by the GL activity of Atl.

In sum, PGRP-SA binds to newly synthesised PGN fragments accumulated at the septal cell surface, due to the non-incorporation of new material into the PGN mesh caused by the stiffness of the septal PGN that was not cleaved by the specific activity of Sle1.

Recognition of PGN is paramount for efficient antibacterial responses.

Infection of *y,w* flies identified NCTC Δ *atl*, NCTC Δ *lytM*, NCTC Δ *isaA* and NCTC Δ *02855* to be less virulent than the parental strain (Fig. 9). However, NCTC Δ *atl* is significantly less virulent than either NCTC Δ *lytM*, NCTC Δ *isaA* or NCTC Δ *02855* (Fig. 10). Whilst upon infection with NCTC 8325-4 the *y,w* flies succumb at 24h p.i., with either NCTC Δ *lytM*, NCTC Δ *isaA* or NCTC Δ *02855* there is a delay of 12h in the death of the flies. At 36h p.i. the probability of the Fly survival upon infection with these mutants and the wild-type strain is similar, ~20%. An *isaA* mutant had already been reported to be slightly less virulent in a mouse septic arthritis model, albeit not statistically significant [58]. In contrast, infection with NCTC Δ *atl* renders the flies a probability of survival of ~75% after 72h p.i. (Fig. 10). The reason for such reduced virulence of the *atl* mutant compared to the other less virulent mutants, is due to a better recognition by PGRP-SA. Accordingly, in a *semmelweis* background, the *atl* mutant is the most virulent mutant, close to the virulence levels of the parental strain (Fig. 11 and 13). On the other hand, no significant differences in the probability of survival of either *y,w* or *semmelweis* flies where detected for NCTC Δ *lytM*, NCTC Δ *isaA* and NCTC Δ *02855* (Fig. 11 and 14-16), similar to NCTC 8325-4 (Fig. 11 and 12). These results show that NCTC 8325-4 possesses pathogenicity levels that when the PGN is not recognised to a certain minimum levels, the *Drosophila* immune responses are unable to control the infection. Only when a certain amount of PGN is accessible to

recognition, can the Host counterattack the virulence of the bacteria. Hence, NCTC Δ *lytM*, NCTC Δ *isaA* and NCTC Δ *02855* are more virulent than NCTC Δ *atl* and the role of PGRP-SA cannot be detected. Likewise, NCTC Δ s*le1* is as virulent as the parental strain, albeit a better recognition of the septal PGN by PGRP-SA (Fig. 7, 9, 11, 17 and 19). The fact that the response to the *atl* mutant is highly dependent on PGRP-SA, proves that the reduced virulence has to do with a better PGN recognition and not due to the impairment on biofilm and adherence ability or susceptibility to other Host components.

The results suggest that 1) impairment of PGN detection by PGRP-SA is paramount since a bacterium that still retains virulence factors is not able to stablish an infection due to the recognition of its PGN (Fig. 21); 2) infection with bacteria that possess high levels of pathogenicity may mask the detection of the fundamental role of Host PGN receptors in the immune response; 3) impairment of PGN recognition, *i.e.* immune evasion mechanisms, may be the first line to protect the bacteria against the Host immune components, followed by the virulence factors, such as haemolysins, that help the bacteria to survive inside the Host (Fig. 21). The virulence factors may only be able to assume its role for as long as the bacteria remain undetectable by the Host. Furthermore, it may be that virulence factors might be able to compensate each other in their role on the subversion of the responses, but once the barriers that impair PGN recognition are compromised, the bacteria struggle to survive inside the Host.

PGN recognition by PGRP-SA may aid in the clearance by early immune responses.

The binding of PGRP-SA to the septum of NCTC Δ s*le1* cells (Fig. 7) is not sufficient to promote an efficient immune response (Fig. 9, 11 and

17). Nevertheless, we enquired whether the absence of Sle1 in an *atl* mutant background would contribute for loss of virulence. Regarding cell division, the double mutant NCTC Δ *atl* Δ s*le1* showed high impairment in the cell splitting corroborating previous reports [37] (Fig. 18). NCTC Δ *atl* Δ s*le1* is as virulent as the single NCTC Δ *atl* mutant (Fig. 19). Hence, the loss of virulence of the double mutant is almost exclusively due to the absence of Atl. Accordingly, the binding of mCherry_PGRP-SA to the double mutant cells did not seem to be higher than to the single *atl* mutant cells (Fig. 18).

We next wondered whether albeit not detected by monitoring the Host survival, the NCTC Δ s*le1* would induce higher levels of AMPs. We verified that the levels of *drosomycin* were similar between NCTC 8325-4 and NCTC Δ s*le1*, although the mutant showed slight lower levels of induction (Fig. 20). In contrast, the *drosomycin* levels were greatly reduced upon infection with either NCTC Δ *atl* or NCTC Δ *atl* Δ s*le1* (Fig. 20).

Induced late AMPs start to be generated once most of the bacteria has been cleared, as they assume protective roles and act to destroy the bacteria that escaped the immediate cellular responses [76]. Consequently, the high susceptibility of NCTC Δ *atl* and NCTC Δ *atl* Δ s*le1* to the Host responses (Fig. 19) coupled with the low levels of *drosomycin* (Fig. 20), is an indication that the majority of the bacteria were efficiently removed by cellular responses. Thus, there was little bacteria still present in circulation, translating into a low induction of the TOLL pathway (Fig. 1, Chapter III). Accordingly, the high levels of *drosomycin* upon infection with NCTC 8325-4 and NCTC Δ s*le1*, may be the result of a higher bacterial load in the haemolymph due to the failure of the cellular responses in controlling the bacterial load. In this scenario and accordingly to previous reports [76], the induced AMPs have major roles as clearing agents to mop up the bacteria in order to counterbalance the inability of haemocytes to clear the pathogens. This is an indirect indication that immediate early

responses are crucial against an *S. aureus* infection. In addition, since PGRP-SA is paramount against NCTC Δ *atl* infection, these early responses may be associated with the role of PGRP-SA recognition. It follows that, if PGRP-SA recognition is vital against infection and the immediate responses are involved against an *S. aureus* infection, then we are indirectly observing that the *atl* mutant is highly susceptible to cellular responses, which in turn are dependent on PGRP-SA. Therefore, the level of PGN recognised at the NCTC Δ *sle1* surface is not sufficient to promote a better clearance by haemocytes. Indeed, the Host Labs have previously reported that efficient responses against an *S. aureus* mutant without wall teichoic acids rely more on PGRP-SA rather than on induced AMPs [61]. This observation may seem contradictory, unless one realises that immediate responses are paramount for clearance of the bacteria and assume a TOLL-independent role of PGRP-SA (see Discussion of Chapter III and the contents of Chapter IV).

S. aureus haemolysins may compromise the clearance responses by haemocytes.

Since *S. aureus* is a major pathogen whose virulence factors seem to aid in the infection of the flies, we sought to determine what factors could be vital for the establishment of the infection when detection of PGN is not significantly compromised. As the data suggests that early immune responses are important for bacterial clearance, we surmise that these virulence factors are acting upstream of the late induced responses. Subsequently, it should be factors that might impact the role of haemocytes.

Haemolytic assays showed that NCTC Δ *lytM* and NCTC Δ *atl* Δ s*le1* are compromised in the haemolytic activity (Fig. 21). Whereas the slight reduced virulence of NCTC Δ *isaA* and NCTC Δ *02855* may to some extent

be due to a slight better binding of PGRP-SA at the septum (Fig. 7, 9 and 10), for NCTC Δ *lytM* it may be mostly due to the impairment in haemolysin activity. Accordingly, in the *lytM* mutant it was not observed such levels of mCherry_PGRP-SA recognition as for NCTC Δ *isaA* and NCTC Δ *02855* (Fig. 7). The reason for impairment of haemolytic activity may be due to the fact that the pentaglycines bridges are involved in the exposure of cell surface proteins [77]. In addition, the impairment of haemolytic activity of NCTC Δ *atl* Δ s*le1*, may account for the slight reduced *drosomycin* levels compared to NCTC Δ *atl* (Fig. 21). This suggests that the double mutant is being slightly better cleared than the single *atl* mutant. Nevertheless, it is evident that a better PGN recognition by PGRP-SA greatly aids in the clearance responses.

Conclusions

The role of autolysins appears to go beyond the maintenance of bacterial physiology. On one hand they are crucial for cell growth and proliferation which are essential for survival. On the other hand, their activity renders the bacteria more susceptible to Host recognition, since PGRP-SA (and perhaps other Host PGN receptors) are able to detect the PGN fragments generated during cell division.

The recognition of PGN is paramount against a bacterial infection in *D. melanogaster*. An *S. aureus atl* mutant is non-virulent in immune wild-type background flies, showing reduced levels of bacterial load in the course of infection [1]. Overall, the results presented in this chapter indicate that upon infection with pathogenic bacteria, the detection of a certain amount of PGN is sufficient to guarantee Host survival whilst clearing the bacteria, *i.e.* avoid an asymptomatic infection through establishment of the bacteria in a hidden ecological niche inside the Host.

It is then logical that the bacteria respond through evasion mechanisms that impair PGN recognition. Accordingly, the data presented in this chapter together with the stereo-hindrance of PGN by wall teichoic acids [61], show that as long as the bacteria can avoid PGN recognition, they successfully establish an infection. This suggests that the mechanisms of virulence are primordially dependent on the evasion of PGN detection, *i.e.* on the survival of the bacteria. PGN detection through binding of Host receptors is likely to impair proper growth and division. When impairment of PGN recognition is compromised, the Host successfully controls the infection and thus the virulence factors assume a secondary role. They are mostly efficient for as long as the PGN is protected from recognition.

In the absence of the major autolysin Atl, there is accumulation of PGN fragments around the cell surface which are exposed and hence accessible for PGRP-SA recognition [1]. The results here presented suggest

that the PGN recognition is aiding in the clearance of the bacteria. Early immune responses are the first line against infection. They rely mainly on the function of haemocytes as macrophages, which internalise and clear the bacterial cells. The late immune responses follow the cellular responses. They rely mainly on the induced AMPs that are triggered upon PGN recognition. Since infection with the non-virulent *atl* mutant induces lower levels of *drosomycin* than the parental virulent strain, the AMPs appear to play a secondary role in this infection. As their primary function is to clear bacteria that escaped the early immune responses, this suggests that the *atl* mutant is being better cleared through the recognition of PGN by PGRP-SA. In sum, the data suggests that upon an *S. aureus* infection the early responses are determinant for bacterial clearance and that PGRP-SA may be involved in these early responses. In addition, as pathogenic bacteria, *S. aureus* presents virulence factors that act early on the infection course, most likely to subvert the early immune responses. Again, if detection of PGN is enough to promote bacterial clearance, then the PGN recognition is of uttermost importance for Host survival and thus bacteria present mechanisms to protect it from recognition. The importance of the protection of PGN detection may be a primordial survival instinct of the cell. A PGN with Host receptors bound to it compromises the growth and cell division, thus survival. This in part explains why the recognition of PGN from the Host side is so important and it seems to trigger both cellular (clearance) and humoral (induced AMPs) responses.

Regarding the activity of the autolysins in the bacterial physiology, there seems to be an interplay between the PGN chemical and/or structural composition and autolytic activity. For instance Sle1, a major enzyme for efficient cell splitting, appears to be specific for a PGN that possesses certain (unknown) characteristics. In the absence of Sle1, there is accumulation of newly synthesised PGN that does not have space to be incorporated into the PGN mesh. Although PGRP-SA is able to recognise

these PGN fragments, the amount of detected PGN is not sufficient for the Host to trigger efficient responses against the pathogen. Thus, although Sle1 plays a specific crucial role in cell splitting, the other autolysins can control the accumulation of PGN at the septum in this mutant to a certain extent. Perhaps the autolysins can cleave the new PGN fragments that cannot be inserted to give time for other autolysins to act on the PGN stiffness, thus allowing a delayed septum reshaping. In addition, it can be assumed that because the new PGN is exposed, it is a PGN that has not been modified by post-incorporation modifications. This "naked" PGN that has not been found in the polymeric PGN [78] may be also a better substrate than a PGN that has suffered modifications. For instance, as revised in the previous Chapter, some Lysozymes are sensitive to modifications of the glycan chains. Lastly, it is likely that upon cell division, the amount of cleaved PGN is equivalent to the amount of new incorporated PGN. Since Sle1 seems optimal for a particular sub-type of PGN, this is an indication that septal PGN has characteristics that may not be found in other subcellular regions. Also, it is possible that an accurate quantification of PGRP-SA binding, allows to extrapolate the amount of new and old PGN that is inserted or removed during a cell division cycle.

It is likely that the coordination of the temporal and spatial activity of the autolysins is a transversal bacterial mechanism to guarantee the survival of the cell to avoid both lysis and the detection of PGN by Host components. One of the biological reasons for the observed extremely fast splitting of the daughter cells and septum re-shaping [44] may be to avoid the accumulation of certain levels of PGN at the septum that are accessible to Host recognition and thus endanger the survival of the bacteria. I propose that the sum of the activity of all autolysins is key to impair the detection of PGN during cell division, although the major autolysin Atl presents a more relevant role.

Material and Methods

Bacterial strains and growth conditions.

Table 1 presents the *S. aureus* strains used in this study. *S. aureus* strains were grown either in Tryptic Soy broth (TSB; Difco) or in Tryptic Soy agar (TSA; Difco), supplemented when appropriate: Erythromycin (Sigma-Aldrich, Germany) at 10 μg/ml, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal; Apollo Scientific, UK) at final concentration at 100 μg/ml. *E. coli* strains DC10B and BL21(DE3) were grown either in Luria–Bertani broth (LB; Difco, France) or in Luria–Bertani agar (LA; Difco), supplemented with 100 μg/mL of Ampicillin (Sigma-Aldrich). All cultures were grown at 37ºC with aeration, except when stated otherwise.

Table 1. List of strains used in this study, plasmids and primers for cloning.

Strains	Plasmids		Primers
NCTC 8325-4	N/A		N/A
NCTC Δ *atl*	N/A		N/A
NCTC Δ *sle1*	pMAD00427	P1_00427	CATGCCATGGGCAGTAGATGCACAACAAACTG
		P2_00427	CATTATATATTTATATACGTAAGACTTTATTTAAAATCCTCCTCTTGCTTAAC
		P3_00427	GTTAAGCAAGAGGAGGATTTTAAATAAAGTCTTACGTATATAAATATATAATG
		P4_00427	TGGAGATCTCAGCGCGTGTACTTGTGATTC
NCTC Δ *lytN*	pMAD01219	P1_01219	CATGCCATGGCATGTTGGTGTAGTATCTCGTTCAGGTAC
		P2_01219	CATATTTCAATGTCCTGCCTTCGATTTAAATTAGATGAAACACTCCTTACAATAATATAC
		P3_01219	GTATATTATTGTAAGGAGTGTTTCATCTAATTTAAATCGAAGGCAGGACATTGAAATATG
		P4_01219	TGGAGATCTCTTTATATATTTCTTGCATTC
NCTC Δ *02855*	pMAD02855	P1_02855	CATGCCATGGGTTGATGAATCTTTGACTG
		P2_02855	AAAATTAACTTGGGAGATAAAATAATCAAAAAACTTCTATCACG
		P3_02855	CGTGATAGAAGTTTTTTGATTATTTTATCTCCCAAGTTAATTTT
		P4_02855	TGGAGATCTGTATATTAATCGAGGTGAGG
NCTC Δ *02580*	pMAD02580	P1_02580	CATGCCATGGCCGCGAAATGTATGGTAAC
		P2_02580	CTTTTGATGAGGCTGTTTCATAAGACATCGGTGCTTAAAGGAGC
		P3_02580	GCTCCTTTAAGCACCGATGTCTTATGAAACAGCCTCATCAAAAG
		P4_02580	TGGAGATCTGAAAGATGACGACATGAACCG
NCTC Δ *lytM*	pMAD00248	P1_00248	CATGCCATGGGCAATGAAGCAGGTACATTTG
		P2_00248	GCAACTTGGGATTTTCTGTATTAGTATAAAACATCCTCCATTAAAG
		P3_00248	CTTTAATGGAGGATGTTTTATACTAATACAGAAAATCCCAAGTTGC
		P4_00248	TGGAGATCTGGAGCGTAACTGATGATAG
NCTC Δ *isaA*	pMAD02889	P1_02889	CATG CCATGG CTACTTGGTAAATCTCCTCGCTTAATTTC
		P2_02889	CATAAACTTACAATTATTACTGGAGGATTTTTTACTTAATTCCTTCATAATAAATAAAAGTAATGTTTAGAC
		P3_02889	GTCTAAACATTACTTTTATTTATTATGAAGGAATTAAGTAAAAAATCCTCCAGTAATAATTGTAAGTTTATG
		P4_02889	TGGAGATCTGTAGTAGCGTTCTTTGTTGTACCATTTG
NCTC Δ *atl* Δ *sle1*			pMAD00427 was transduced into NCTC *Δ atl* cells

Fly Strains.

All flies stocks were grown on standard cornmeal-agar medium at 25ºC. Isogenic Drosophila Bloomington #6599 (*y,w*) flies were used as

wild-type flies. *semmelweis* flies were used as a *PGRP-SA* mutant background [62].

Construction of the bacterial mutants.

Table 1 presents the constructed *S. aureus* null mutants strains, plasmids and primers used. The upstream and downstream regions of the genes to be deleted were amplified using pair of primers denominated as P1/P2 and P3/P4, respectively. The resulting PCR fragments containing the upstream and downstream regions of each gene were joined by overlap PCR using the pairs of primers P1/P4. The PCR product was digested with NcoI (sequence in P1 primers) and BglII (sequence in P4 primers) (Fermentas) and cloned into the pMAD vector [79] which was propagated in *E. coli* DC10B and the inserts were sequenced. The plasmids were then electroporated into *S. aureus* RN4220 strain at 30ºC, using Erythromycin and X-gal selection, and transduced into NCTC8325-4 using phage 80a [80]. The integration of the plasmids into the chromosome and their excision was done as previously described [81]. Gene deletions were confirmed by sequencing of the amplified fragments with P1/P4 pair of primers.

Zymography assay.

The zymograms were performed as described in Vaz and Filipe, 2015 [69]. For crude autolytic extract with mutanolysin, the same procedure was followed except that instead of incubation with 4% (w/v) SDS, the cells were incubated with 0.2 mg/mL of mutanolysin.

Purification of mCherry_PGRP-SA.

The recombinant protein mCherry_PGRP-SA was purified using a protocol adapted from previous reports [1], [61]. *E. coli* BL21(DE3)

competent cells were transformed with pET21a derivatives carrying mCherry_PGRP-SA. Briefly, different batch cultures in LB supplemented with 100 μg/mL of Ampicillin (Sigma-Aldrich) were grown at 25ºC, 120 rpm. When the cultures reached an OD_{600nm} ~0.5, they were induced with addition of IPTG (Apollo Scientific, UK) at 1 mM final concentration in the same growth conditions for 16-18 h. The cells were harvested and washed once in Equilibration Buffer (50 mM Na_2PO_4 pH 7.4; 300 mM NaCl). The pellets were resuspended again in Equilibration Buffer and lysis was carried out by French Press at 1000 psi. The lysate was centrifuged at 20070 x *g* for 20 min at 4ºC. Interestingly, the cytoplasmic proteins, *i.e.* the proteins that are left on the supernatant after lysate centrifugation, proved to not be functional (could not bind neither to live cells nor to PGN) and they did not bind avidly to the resin, thus the yield of the cytoplasmic fractions were very low. Only the protein in inclusions bodies (the pellets of the lysates), after denaturation-renaturation steps, revealed to be functional. Perhaps the protein that is free in the cytoplasm binds to the *E. coli* PGN upon lysis of the cells and cannot be subsequently renatured into its proper structure. Therefore, the supernatants were discarded and the pellets were kept suspended in 20 mL of Resuspension Buffer (20 mM Na_2PO_4 pH 7.4; 500 mM NaCl; 8 M urea) and left shaking on a rocker in a cold room for ~60 hours until they appeared homogeneous. The solubilised pellets were diluted down to 4 M Urea by the addition of Dilution Buffer (20 mM Na_2PO_4 pH 7.4; 500 mM NaCl) and left again homogenising at 4ºC for a further 24h. The homogenates were centrifuged and the supernatant was mixed with TALON® Metal Affinity Resin (Clontech Laboratories, USA) (previously washed with Wash Solution) and incubated for 1 h on the rocker at 4ºC. The Protein-Talon resin complexes were recovered by centrifugation (supernatants kept on a falcon tube on ice) and washed 4 times with Wash Solution (50 mM Na_2PO_4 pH 7.4; 300 mM NaCl; 4 M Urea), once with 5mM Imidazole Wash solution and once

with 10mM Imidazole Wash Solution. Proteins were eluted with Elution Buffer (50 mM Na_2PO_4 pH 7.4; 300 mM NaCl; 150 mM Imidazole) for 1 hr at 4ºC on rocker. The free-protein Talon resin was spun down by centrifugation and the supernatants (the pure proteins) were collected into a fresh falcon tube and kept on ice. The collected supernatants of the first Protein-resin spun down, were incubated again with the free-protein Talon resins (previously re-washed with Wash Solution) and the procedure was repeated. The supernatants from the two elution batches were combined and dialysed overnight in a 10K MWCO SnakeSkin™ Dialysis Tubing (ThermoFisher Scientific, USA) at 4ºC against 5 L PBS 1X pH 6.0, then 6-8h against 2 L PBS 1X pH 6.0 made fresh and finally overnight in 2 L PBS 1X pH 6.0 also made fresh. The proteins were recovered from the dialysis` bags and centrifuged to remove any residues of the resin. The proteins were quantified using a Nano-drop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, NC). The purity was accessed by SDS-PAGE [82] and visualised with Coomassie Blue followed by Silver Staining (both home-made solutions). Aliquots of the proteins were kept at 4ºC for immediate and/or constant use or they were snap frozen in liquid nitrogen and stored at -80ºC.

Co-precipitation assays of live cells with mCherry_PGRP-SA.

The bacterial binding assays are qualitative and were performed with the fluorescent recombinant protein mCherry_PGRP-SA for microscopy visualisation.

Bacteria were grown to OD_{600nm} ~0.5 or an O/N culture for stationary phase experiments. 1 mL aliquot was centrifuged for 5 min at RT, 16.1 x1000 *g*. The cells were washed with 500 μL of PBS 1x pH 6.0 and centrifuged again. They were resuspended in 0.3 mg/mL final concentration of mCherry_PGRP-SA in a 200 μL reaction volume, filling up the volume with PBS 1x pH 6.0 and incubated at RT for 5 min without

shaking. The "cells-mCherry_PGRP-SA" complexes were harvested for 5 min at RT, 7.5 x1000 *g*. The pellets were washed twice with 200 μL of PBS 1x pH 6.0. Finally, the pellets were resuspended in the left over volume of the washings and 2 μL was loaded on 1.2% (w/v) Agarose- PBS 1x pH 6.0 slides. Images were acquired in a Zeiss Axio ObserverZ1 microscope equipped with a Photometrics CoolSNAP HQ2 camera (Roper Scientific using Metamorph software, Meta Imaging series 7.5) and analysed using the ImageJ software.

Survival curves.

Survival assays were performed as previously described [1]. Bacterial cultures were grown overnight, washed and resuspended in PBS (Phosphate-Saline Buffer). The OD_{600nm} was adjusted to ~0.350 with PBS using. 32.2 nL of bacterial suspension was injected in thorax of 3-5 day old flies using a nanoinjector (Nanoject II; Drummond Scientific, Broomall, PA). The infected flies were kept at 30ºC and monitored for 72h every 12h. Estimated survival curves were analysed using the Log-rank (Mantel-Cox) test to determine statistical significance between the curves. The number of flies infected were as follows (in the order *y,w* and *semmelweis* flies, when applicable): NCTC 8325-4 – 187, 166; NCTC Δ *atl* – 129, 134; NCTC Δ *sle1* – 70, 76; NCTC Δ *lytN* – 38; NCTC Δ *02855* – 38, 58; NCTC Δ *02580* – 59; NCTC Δ *lytM* – 37, 40; NCTC Δ *isaA* – 100, 79; NCTC Δ *atl* Δ *sle1* – 76, 52. All data were plotted and analysed using GraphPad Prism 5 (GraphPad Software, Inc.).

Quantification of *drosomycin* levels.

Female *y,w* flies were infected as conducted for the survival assays. For quantification of *drosomycin* transcript levels, flies were collected at 0h p.i. and at 16h p.i.. PBS was used as a control for the induction of

drosomycin upon injection. The procedure was conducted as previously described [1]. Briefly, the flies (n = 6/sample) were homogenised and the total RNA was extracted using the "Total RNA Purification Plus Kit" (Norgen, Canada) according to the manufacturers' instructions. The RNAs were quantified using a Nano-drop ND-1000 spectrophotometer (Thermo Scientific). 500 ng of total RNA was used to produce cDNA using "Maxima First Strand cDNA Synthesis Kit" (Thermo Scientific) in a T100 Thermal Cycler (Bio-Rad) and stored at −20°C. The housekeeping gene *tbp* (*TATA-box-binding protein*) [83] was used as a control to normalize the transcript levels of *drosomycin* both at 0h and 16h p.i.. *drosomycin* and *tbp* levels were measured using the pair primers Drs(+)/Drs(−) and Tbp(+)/Tbp(−) [1]. qPCR reactions were performed using the SensiFast SYBR No-ROX Kit (Bioline, UK) according to the manufacturer's instructions, in a Rotor-Gene Q real-time PCR cycler with a 72-well rotor (Qiagen). Three biological repeats were performed per time point. Gene expression was calculated on the basis of the comparative threshold cycle (CT) value [84]. The *drosomycin* CT values at 16h p.i. were normalised against *tbp*/PBS injection/0h p.i..

Haemolytic assays.

O/N bacterial cultures were adjusted to the same OD_{600nm} ~2.0. 3 µL of culture was put onto TSA supplemented with 5% (v/v) sheep blood (Difco, France). The plates were incubated with the lid facing upwards at 37ºC for 16h followed by incubation at 4ºC for 24h before image acquisition using a Gel Doc™ EZ Gel (Bio-Rad, USA). 3D surface plots were performed using ImageJ software.

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CHAPTER III

PGRP-SA and PGRP-LC recognise both Lys and DAP residues of the peptidoglycan *in vivo*

Contributions to this Chapter:

Filipa Vaz[1,2] designed the experiments, analysed the data and performed all of the following experimental procedures: purification and quantification of the proteins and peptidoglycans, co-precipitation assays, constructed the mutant flies, all the *in vivo* studies on survivals, CFUs and *ex vivo* studies on haemocytes.

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Gonçalo Covas[2] participated in the optimization of the peptidoglycan quantification method, developed the Hemo buffer and constructed the recombinant protein mCherry_PGRP-LC.

Richard Parton[1] helped in the microscopy imaging of the mCherry_PGRPs binding to live cells and in the development of the time-lapse microscopy technique.

Sergio Filipe[2] **and Petros Ligoxygakis**[1] designed the experiments and analysed the data.

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Summary

In this Chapter I present data that whilst contradicting the current model of bacterial recognition in *Drosophila melanogaster*, it also helps to clarify some literature reports. Some reports were not entirely in accordance and others although not conflicting, were not in total communion with the model.

According to Janeway, as pattern recognition receptors, PGRPs should present affinity to several types of peptidoglycan (PGN). Instead, the current model is based on the specificity of PGRP-SA and PGRP-LC towards Lys- vs. DAP- type PGN, respectively. Why would two major immune receptors be limited in the detection of PGN due to the discrimination of a single molecule? I performed *in vitro* studies of PGRP-SA and PGRP-LC binding to two Gram-positive bacteria that present a Lys – *Staphylococcus aureus* – and a DAP – *Bacillus subtilis* – residue in their PGN. Subsequently, I addressed the biological role of the observed *in vitro* bindings through survival assays by infection of flies mutated in *PGRP-SA*, *PGRP-LC* and both *PGRP-SA/PGRP-LC*. In addition, I enquired whether an exposed PGN at the cell surface, would turn the bacteria more susceptible to recognition by these PGRPs.

I propose that the wall teichoic acids (WTA) and the outer membrane of Gram-positive and Gram-negative bacteria, respectively, are conserved bacterial immune evasion strategies towards the recognition by host PGN receptors. In particular, WTA impair recognition of Lys- and DAP- type PGN by both PGRP-SA and PGRP-LC, which do not discriminate between these two residues. It is the access to the PGN through the cell wall and not the specificity of the PGRPs that dictates the recognition and downstream triggering of the immune responses.

Introduction

In *Drosophila*, according to the current model of bacterial recognition and Host response, the Peptidoglycan Recognition Proteins (PGRPs) possess discriminatory ability between DAP- and Lys- type peptidoglycan (PGN). It is thought that PGRP-SA, the main receptor of the TOLL pathway, *in vivo* binds exclusively to Lys-type Gram-positive bacteria, such as *Staphylococcus aureus* (Fig. 1) [1], [2]. Likewise, it is thought that PGRP-LC, the main receptor of the IMD pathway, specifically binds to DAP-type bacteria – Gram-negative and Gram-positive rod shape bacteria, such as *Escherichia coli* and *Bacillus subtilis*, respectively (Fig. 2) [3]–[5]. The observed binding specificity of these two PGRPs, have been presented as the reason for the *in vivo* observation of the differential activation of the pathways [6].

PGRPs – the conserved innate immune receptors in Animals

In 1996, Yoshida and colleagues identified in *Bombyx mori* that the recognition of PGN and fungi β-1,3 glucan happened through distinctive specific receptors that converge to activate the prophenoloxidase cascade (PPO) cascade [7]. A haemolymph protein with high affinity for PGN, devoid of lytic activity thus excluded to be a lysozyme protein, was identified as the bacterial receptor. Therefore, they named it Peptidoglycan Recognition Protein (PGRP) and soon after proteins with such features started to be identified in other animals. Similar to the PPO activation in *B. mori*, 4 years later it was clear that in *D. melanogaster* both Fungi and Bacteria activate the same cascade, the TOLL pathway, albeit through different receptors [1]. This happens through the recognition of PGN of Gram-positive bacteria by PGRP-SA. It is now known that PGRPs

are a family of conserved innate immune receptors present in almost all animals from Invertebrates (mollusks, echinoderms and insects) to Vertebrates (mammals including Humans) [8]. They have not been identified in Plants and lower metazoa, including nematodes such as *Caenorhabditis elegans*.

The PGRPs contain a C-terminal conserved homologous domain, the PGRP domain. It is responsible for the PGN-binding ability, it is about 165 amino acids long, it shares ~30% sequence similarity with the bacteriophage T7 lysozyme [9] and structural homology to the peptidoglycan-binding type 2 amidase domain [8], [10]–[13]. In fact, some PGRPs can only be structurally differentiated from type 2 amidases by the presence of an N-terminal fragment, absent in the amidases [14]. This 30-50 amino acids terminus is known as "PGRP-specific fragment" and it is the region with highest sequence and conformational variability among all PGRPs. The function remains unknown but as it forms a hydrophobic groove opposite to the PGN-binding pocket, it is presumed to serve as a docking site for effector and signalling molecules [14], such as in the case of PGRP-SA – GNBP1 (Fig. 6) [15]–[17]. Almost all PGRPs have two closely spaced conserved cysteines in the middle of the domain that form a disulphide bond, which is needed for the integrity and activity of PGRPs [1].

PGRPs are classified according to function, cellular localization and transcript length. Regarding transcript length there are three groups: short (S), intermediate (I) (exclusive to mammals) and long (L). It is the classification of the transcript length that was used as a nomenclature for PGRPs. Hence, PGRP-S, PGRP-I and PGRP-L. For Invertebrate PGRPs this nomenclature still stands, but the designation of the Vertebrate PGRPs was replaced to PGLYRP by the Human Genome Organization Gene Nomenclature Committee: PGLYRP-1, PGLYRP-2, PGLYRP-3 and PGLYRP-4, equivalent to the PGRP-S, PGRP-L, PGRP-Iα and PGRP-Iβ, respectively.

The short PGRPs, PGRP-S and PGLYRP-1, are about 200 amino acids long and 18-20 kDa size, have a signal peptide and only one PGRP domain. In contrast, most PGRPs –L and –I, are at least twice as large and may have more than one PGRP domain and undergo alternative splicing generating different isoforms.

Functionally, phylogenetic studies divide PGRPs into two categories: the ones that retained the amidase activity – the catalytic PGRPs – and the ones that lost this activity. Non-amidase Insect PGRPs are involved in the trigger of signal transduction pathways and proteolytic cascades. Non-amidase Mammalian PGRPs are bactericidal and signalling capabilities are not known. They are sub-categorize into two branches regarding tissue expression: PGLYRP-1, present in phagocytic granules [18] and PGLYRP-3 and PGLYRP-4 that are present on skin and mucous membranes [19].

Regarding the sub-cellular compartment, PGRPs can be secreted, transmembrane or cytoplasmic. Nevertheless, the same PGRP protein can show variant forms in all of these compartments. For example, PGRP-LE lacks a signal peptide but possesses intracellular and extracellular variants (Fig. 2). All mammalian PGLYRPs are both intracellular and secreted.

The TOLL pathway in *Drosophila* Immunity

The components of the TOLL pathway were initially identified as being involved in early embryonic development in *D. melanogaster*, particularly in the Dorso-Ventral axis [20]. The findings of the genetic control during the early developmental stage, rendered C. Nusslein-Volhard, E. F. Wieschaus and E. B. Lewis the Nobel Prize in Medicine in 1995. With the unravelling of these genes, it became known the *Drosophila* NF-kB homolog, Dorsal [21] (Fig. 1 and Fig. 5 of Chapter I). Although mammalian TLRs seem to have no role in development [22][21]–[23], in

Drosophila they are involved both in immunity [24] and developmental processes [21]–[23]. The discovery of the Toll receptor (Toll-1) [25] and subsequently the function of the TOLL pathway in antifungal response in *Drosophila* [24] was again vital to stablish the similarities and conservation of innate immune mechanisms in invertebrates and mammals. The discovery of the *toll* in innate immunity and the mammalian TLRs, earned J. Hoffmann and B. Beutler the Nobel Prize in Physiology or Medicine in 2011.

There are three extracellular cascades that trigger the TOLL pathway: Persephone (PSH) which is directly activated by virulence factors of Fungi and Gram-positive bacteria [26], [27]; the Gram-negative binding protein-3 (GNBP3) that recognizes β-glucans present in the Fungal cell wall and the PGRP-SA that recognises PGN. Next it is described the triggering of the TOLL pathway by bacteria through recognition of PGN (Fig. 1).

Extracellular medium – recognition of the pathogens:

1. Recognition of PGN by PGRP-SA.

Lys-type PGN is mainly recognized by PGRP-SA which stablishes a complex with GNBP1. Also, PGRP-SD is a receptor with partial redundancy to the PGRP-SA–GNBP1 complex [28], [29]. It appears that PGRP-SD can also recognize DAP-type PGN from Gram-negative bacteria [30]. GNBP1 showed endomuramidase activity and in low cross-linked Lys-type PGN, it was proposed to produce new glycan reducing ends that are presented to PGRP-SA [29], [31]. In contrast, it has been suggested that full-length GNBP1 has no enzymatic activity [32], but the functionality of the recombinant GNBP1 was not determined.

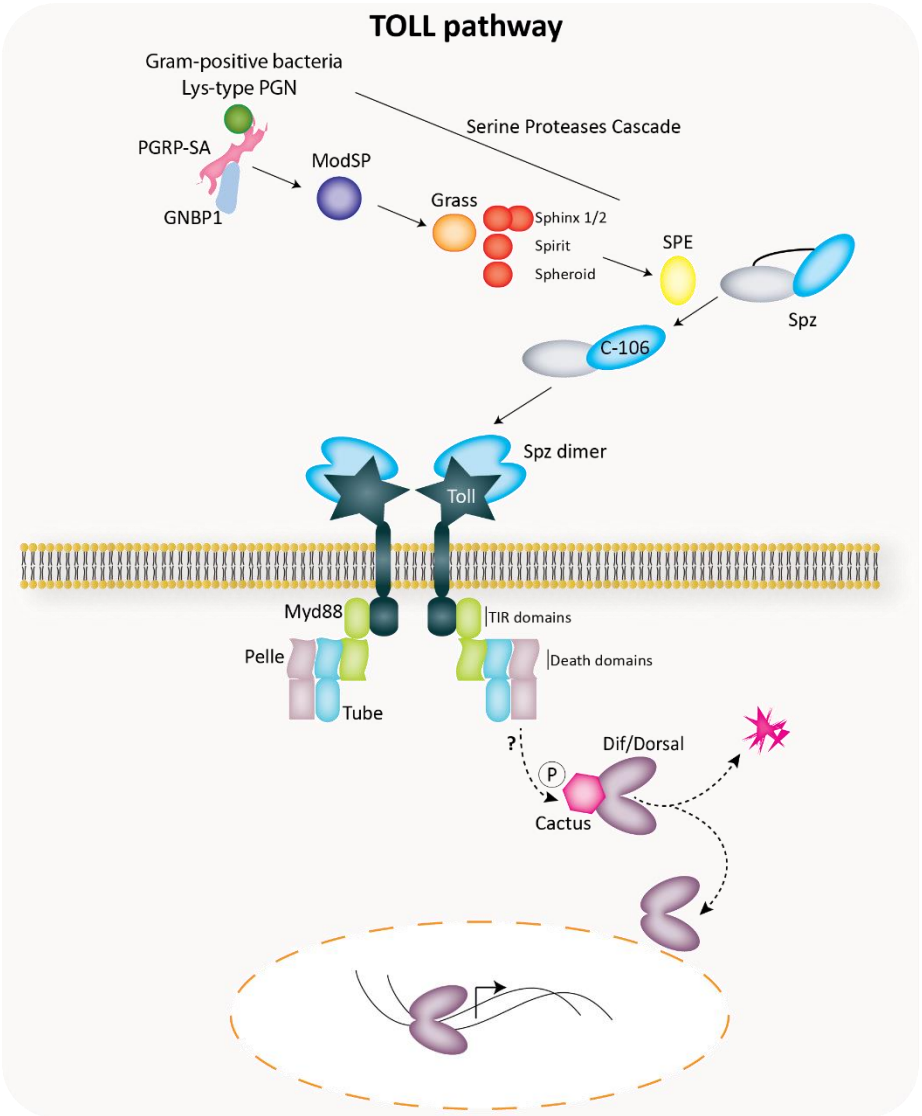


Figure 1. Schematic summary of the activation of the TOLL pathway by PGN recognition. Unlike its mammalian counterpart, the *Drosophila* Toll receptor does not interact directly with the PAMP, rather the PAMP is recognised by secreted receptors. PGRP-SA is the main receptor for bacterial recognition of the TOLL pathway. In contrast to IMD, the intracellular steps of the TOLL cascade are yet to be better understood.

2. Activation of the modular serine protease ModSP.

The complex PGN – PGRP-SA – GNBP1 activates ModSP, a modular Serine protease conserved in insect immune reactions. ModSP is the first non-receptor of the cascade. It integrates the signals both from the PGN recognition by PGRP-SA and from GNBP3 sensing of Fungi [32].

3. Activation of Grass and serine proteases.

The ModSP serine protease activity acts upon Grass which activates a set of 4 serine proteases: Sphinx1, Sphinx2, Spirit and Spheroid [26], [32], [33].

4. Activation of the Spatzle processing enzyme (SPE).

The activity of the serine proteases activates SPE so that it will cleave its substrate, the full length Spatzle [34], which in turn will interact with the Toll (Toll-1) receptor. SPE is the point where pathogen recognition information is integrated via the three different recognition pathways that can activate the TOLL cascade: PSH cascade together with the Fungal and Bacterial recognition that already converged in the upstream step at ModSP.

5. Activation of Spatzle.

Spatzle (Spz) is synthesized and secreted as an inactive precursor consisting of a pro-domain and a C-terminal region (C-106). In non-signalling conditions, the pro-domain masks the hydrophobic C-106 region. SPE cleavage of the pro-domain of Spz, induces conformational changes and exposes the C-106 regions that are critical for binding to Toll [35]. Still, the pro-domain remains associated with the C-terminus until binding to Toll [36].

Extracellular-membrane interface – activation of the Toll receptor:

6. Binding of Spz to a dimeric Toll receptor.

The binding of 2 Spz dimers via C-106 to the extracellular N-terminus of 2 Toll receptors, triggers a conformational change in the Tolls

that activate intracellular downstream signalling. Unlike mammalian TLRs, Toll receptor does not interact directly with PAMPs [37].

Intracellular-cytoplasmic medium – activation of the non-active TFs:

7. Formation of heterotrimeric complexes Toll-adaptor proteins.

Endocytosis is paramount for efficient Toll signalling [38]. Upon activation of the Toll, there is recruitment of the adaptor proteins: Myd88, Tube and the kinase Pelle. Thus, it is formed a heterotrimeric complex between the TIR intracellular domains of Toll and the death domains (DD) of the adaptor proteins, where Tube is flanked by Myd88 and Pelle [39]–[41] [41]–[43]. As such, MyD88 and Pelle do not come into contact with each other. Instead, two distinct DD of Tube separately bind MyD88 and Pelle [41].

8. Activation of Cactus.

From the oligomeric Toll-MyD88-Tube-Pelle complex, the signal proceeds to the phosphorylation and degradation of the IkB Cactus. The nuclear translocation of both Dorsal and Dif (Dorsal-related immunity factor) requires Cactus degradation [44] which is thought to happen through phosphorylation by Pelle [45].

9. Activation of Dorsal and Dif.

After Cactus degradation, it is thought that Dorsal (and/or Dif) interacts with Pelle, Tube, and Cactus [46]–[48] and upon activation translocate to the nucleus.

Intracellular-nucleus compartment – expression of the AMPs:

10. Induction of the expression of AMPs.

Dorsal and Dif bind to the NF-kB sequences present in the promoter region of the Toll-dependent antimicrobial peptides (AMPs), thus inducing their transcription [49]. The antifungal peptide Drosomycin seems to be the major AMP of the Toll humoral response. Dif was identified

in Drosophila as a dorsal-related immune responsive gene that does not participate in Dorso-Ventral patterning. Dif mediates TOLL-dependent AMPs expression both in larvae and adults [50], [51] whereas Dorsal seems be active only in larvae [69].

The IMD pathway in systemic responses

The IMD pathway has major roles in gut immunity. In contrast to TOLL, IMD is exclusively triggered by PGN via both extracellular and intracellular sensing, particularly by the DAP-type PGN of Gram-negative bacteria and Gram-positive *Bacilli* (Fig. 8) [113].

Extracellular-membrane interface – recognition of DAP-type bacteria:

1. PGRP-LC and PGRP-LE recognize DAP-type PGN.

DAP-type PGN is mainly recognised by the isoforms of the transmembrane type II receptor PGRP-LC through their extracellular PGRP domains [5]. This recognition can happen with the aid of extracellular PGRP-LE composed only by the PGRP domain [52]. In addition, PGRP-LE is also involved in intracellular recognition of DAP-type PGN. More recently, it has been shown that PGRP-SD activates the IMD pathway and it presents PGN to PGRP-LC [53].

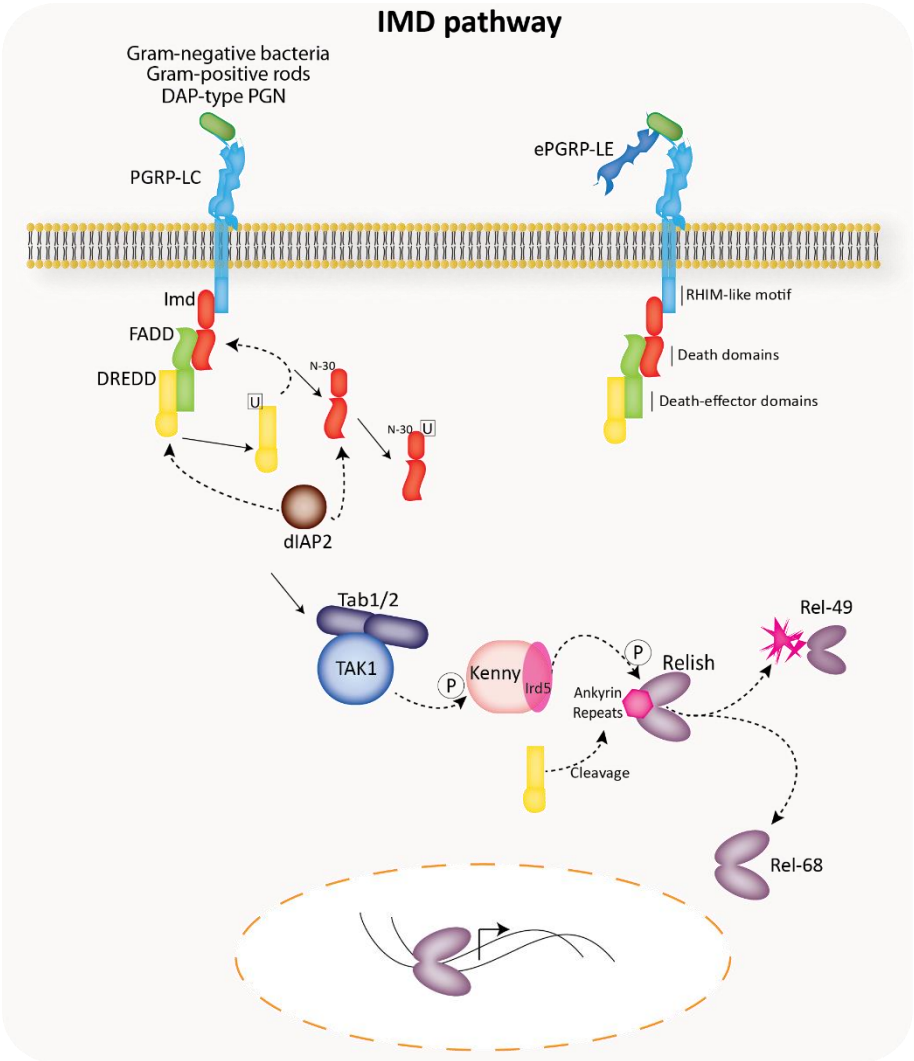


Figure 2. Schematic summary of the activation of the IMD pathway. PGRP-LC is the main receptor of the IMD pathway, which unlike PGRP-SA is a transmembrane protein. PGRP-LE functions as an extracellular receptor and interacts with PGRP-LC to trigger the cascade.

Intracellular-cytoplasmic medium – activation of the non-active TFs:

2. Recruitment of the cytoplasmic protein Imd.

Intracellular signalling happens through the RHIM-like motifs[3] found both in PGRP-LC and -LE [52], [54] that mediate binding to the receptor Death domain containing protein Imd, homologous to mammalian RIP1 (Fig. 5, Chapter I) [55]. Subsequently, Imd associates with *Drosophila* FADD[4] via a homotypic death-domain interaction [56]. FADD then recruits and interacts via the DD with the homologue of mammalian caspase-8, DREDD[5] [57]–[59].

3. Activation of Imd.

DREDD is present in the cytoplasm in its non-active form. Activation of DREDD happens through ubiquitination by the dIAP-2[6]. Ubiquitinated DREDD cleaves and removes a fragment of 30 amino acids at the N`-terminal of Imd. This cleavage exposes a binding site in Imd for dIAP-2 [60]. Subsequently, the cleaved form of Imd is the substrate for dIAP-2 ubiquitination through its RING domain[7]. The 30 amino acid free ubiquitinated Imd is the scaffold for the recruitment of the next downstream components [61].

4. Activation of Relish.

In the last steps of the cytoplasmic cascade, upon activation of Imd, there is the recruitment of TAK1[8] and its adaptor TAB2[9] [62]. It is thought that TAK1 triggers activation of the IKK complex (Kenny and Ird5). Activated IKK will then phosphorylate the NF-kB transcription factors Relish [63]. Phosphorylation of Relish is key to enhance/activate its role as

[3] Receptor-interacting protein (RIP) homotypic interaction domain.
[4] The Drosophila homologue of FAS-associated death-domain protein.
[5] Apical caspase death-related Ced-3/Nedd2-like protein.
[6] Drosophila Inhibitor of apoptosis-2.
[7] Zinc finger domain involved in mediating protein-protein interactions.
[8] Transforming growth factor -β (TGFβ)- activated kinase 1.
[9] TAK1-associated binding protein 2.

a transcription factors [63]. However, the full-length Relish does not translocate to the nucleus. Independently of IKK phosphorylation, Relish must be cleaved into two forms by DREDD. Thus, DREDD cleaves Relish at multiple N-terminal sites splitting into Rel-49 ankyrin repeat fragment that remains cytoplasmic and Rel-68 where the NF-kB part resides [63][64].

Intracellular-nucleus compartment – expression of the AMPs:

5. Induction the expression of AMPs by Rel-68.

Phosphorylated Rel-68 translocate into the nucleus, binds to the DNA NF-kB sequences and induces the expression of the AMPs. Diptericin is the main AMP that is expressed through the IMD pathway.

The bacterial recognition model

In 1997, Lemaitre and colleagues [6] published a study regarding the induction of AMPs upon infection of adult flies with live cells of both Gram-positive and Gram-negative bacteria. They observed a preferential induction of the IMD-dependent AMPs with Gram-negative bacteria and DAP-type PGN Gram-positive *Bacilli*. The Toll-dependent AMPs showed a higher expression with Lys-type PGN Gram-positive bacteria.

In 2001, Michel and colleagues [1] showed that the activation of the TOLL pathway by bacteria and fungi happens through distinct recognition processes, whereby bacterial recognition is dependent on the haemolymph circulating protein PGRP-SA. PGRP-SA *semmelweis* mutant flies showed to be highly susceptible to infection by Gram-positive bacteria DAP-type PGN *Bacillus megaterium* and Lys-type PGN *Streptococcus faecalis*. In contrast, upon infection by Gram-negative bacteria, these mutants were not susceptible to *E. coli* and only a small percent of the population succumbed with *Erwinia carotovora carotovora*.

A year later in 2002, three publications reported the role of PGRP-LC as a pattern recognition receptor of the IMD pathway that recognises Gram-negative bacteria. Ramet and colleagues [3] identified PGRP-LC as a cell surface receptor specifically involved in Gram-negative bacteria phagocytosis and induction of AMP synthesis. Gottar and colleagues [4], identified PGRP-LC as a component of the IMD pathway acting upstream of Imd, whose mutant flies showed reduced survival to Gram-negative infections but not to Gram-positive or fungal infections. Choe *et al*. 2002 [5], identified PGRP-LC (PGRP-LCx) as an absolutely required receptor for the induction of AMPs mediated by Relish. However, conflicting to some extent with the previous reports, TOLL-dependent AMPs also failed to be expressed to normal levels in the PGRP-LCx mutants when infected with *E. coli*. In addition, these mutants, in contrast to the wild-type flies, 6h post-infection (p.i.), showed no detectable induction of *Diptericin* with the Gram-positives *Micrococcus luteus* and *Bacillus subtilis* and the Gram-negative *Enterobacter cloacae*.

In 2003, Leulier and colleagues [65] reported that DAP-type PGN is the most potent inducer of IMD and Lys-type PGN of the TOLL pathway. Consequently, the authors proposed that the ability of *Drosophila* to discriminate between Gram-positive and Gram-negative bacteria relies in the recognition of specific forms of PGN regarding the residue at the third position of the stem peptide.

Finally in 2004, four publications helped to finally shape and support the current model of DAP- vs. Lys- type PGN discrimination by PGRP-LC and PGRP-SA and that this discriminatory ability is the reason behind the observed preferential activation of IMD vs. TOLL pathways for Gram-negative and Gram-positive DAP-type PGN bacteria vs. Gram-positive Lys-type PGN bacteria, respectively. Stenbak and colleagues [66], reported that the two structural features of Gram-negative PGN – the presence of DAP and the 1,6 - anhydro form of the terminal *N*-acetylmuramic acid (Mur*N*Ac)

residue of the glycan chain – allow PGN discrimination between Gram-negative and Gram-positive bacteria. Moreover, they concluded that the IMD pathway is activated by Gram-negative PGN but not by Lys-type PGN. Kaneko *et al.* 2004 [67] corroborated these results as they concluded that the stem peptide of the Gram-negative PGN is sufficient to activate the IMD pathway. Moreover, they also observed that the recognition of monomeric PGN requires different PGRP-LC isoforms, as they showed that upon recognition of tracheal cytotoxin (TCT), PGRP-LCx interacts with PGRP-LCa. Finally, two crystal structures of PGRP-SA were published. Chang and colleagues [2] showed PGN co-precipitation assays using 20 mM Tris-HCl pH 7.8; 300 mM NaCl buffer with recombinant PGRP-SA expressed in insect cell lines. They observed that the rPGRP-SA bound to the DAP-type PGN of the Gram-negative bacteria *E. coli* and *Pseudomonas aeruginosa*. These results suggest that Michel and colleagues observation of a PGRP-SA-independent response upon infection with whole live *E. coli* cells is not related to the inability of PGRP-SA to bind to DAP-type PGN [4]. In addition, they observed that rPGRP-SA bound to Lys-type PGN bacteria *M. luteus* and *S. faecalis* but binding to the Gram-positive amidated DAP-type PGN *B. subtilis* and non-amidated DAP-type PGN *B. megaterium* was undetectable. Overall, these results corroborate Leulier *et al.* 2003 [65]. As for the presented rPGRP-SA crystal structure, it was analysed by superimposition with the PGRP-LB structure [14]. They concluded that PGRP-SA possesses a weak hydrolytic activity towards DAP-type PGN of *E. coli* characterised as an L,D-carboxypeptidase. Reiser and colleagues [68] also proposed a PGRP-SA crystal structure. They did not predict an L,D-carboxypeptidase activity, however they suggest that PGRP-SA might possess a lytic activity belonging to the serine proteases family.

In 2005, Filipe and colleagues [31] identified that the minimal structure needed to activate the TOLL pathway is a muropeptide dimer with a free reducing end of the Mur*N*Ac residue. Similar to Leulier *et al.*

2003 [65] for the induction of the IMD pathway by DAP-type PGN, muramidase treated PGN from *M. luteus* and *S. aureus* did not induce the TOLL pathway.

Interestingly, Mellroth *et al.* 2005 [69] provided very conflicting results from *in vitro* PGN-PGRP co-precipitation assays. They showed that PGRP-LCx and also PGRP-SA to some extent, were promiscuous to DAP-, Ornithine- and Lys-type PGNs. Nevertheless, in 2006, more support to the model of PGN discrimination by PGRPs was added by Chang *et al.* 2006 [70] and Swaminathan *et al.* 2006 [71]. Chang and colleagues [70] published the crystal structure of the complex TCT-PGRP-LCx-PGRP-LCa. This report was again vital to corroborate the current model of bacterial recognition by PGRPs as it reported again the specificity of PGRP-LC for the DAP residues. Swaminathan *et al.* 2006 [71], by using PGN analogues and TCT determined the binding constants of Human and Drosophila PGRPs. They concluded that PGRPs use dual strategies to discriminate PGN: 1) by the composition of PGN stem peptide and 2) by the composition of peptide cross-bridge.

Finally, in 2008 Leone and colleagues [30] published the crystal structure of rPGRP-SD and again favoured the model of DAP- vs. Lys- type PGN discrimination by PGRPs. The pull down assays failed to detect binding of PGRP-SA to DAP-type PGN of *B. subtilis* corroborating Chang *et al*. 2004 [2].

A “small step” towards a new bacterial recognition model

Although the model of bacterial recognition is well stablished, there are still some conflicting results in the literature. As a PRR, a major PGRP should, theoretically, be able to identify a specific bacterial component in a broad range of different bacteria. Thus, the model of PGRPs specificity and discrimination of the stem peptide, the most variable region

of the PGN molecule, conflicts with this idea.

In order to understand the specificities of PGRP-SA and PGRP-LC to the PGN, I worked with *S. aureus* and *B. subtilis* as bacterial models. As Gram-positive bacteria they present a similar cell wall (CW) composition whilst harbouring distinct PGN types regarding the peptide moiety, Lys- and amiDAP (amidated DAP)- type PGN, respectively.

Bacteria present numerous strategies that allow them to survive inside the host, to stablish an infection and cause disease. The microbial cell surface is the interface between the Host and the pathogen, thus it is where we find major virulence components and factors of immune evasion. The virulence factors are compounds produced by the bacteria that aid directly to their pathogenicity and generally cause damage or alteration of the normal physiology and function of the host cells. The microbial factors of immune evasion involve strategies that subvert the role of immune components. As such, these are generally components that hide the pathogen from immune receptors, harvest the function of an immune cell or signalling cascade and/or also cause destruction of the immune cells. These strategies are commonly shared by bacteria and the effect is transversal to different Hosts. Accordingly, the Host groups have identified two different bacterial CW factors that are immune evasion mechanisms: the Wall Teichoic Acids (WTA) [72] and the major autolysin Atl [73]. Both through different mechanisms, impair the recognition of PGN by PGRP-SA. The absence of these components translates into loss of virulence by the bacteria. Genetic knock-out mutants of WTA in *S. aureus* and *B. subtilis* [74], [75], have the PGN exposed at the cell surface (Fig. 3).

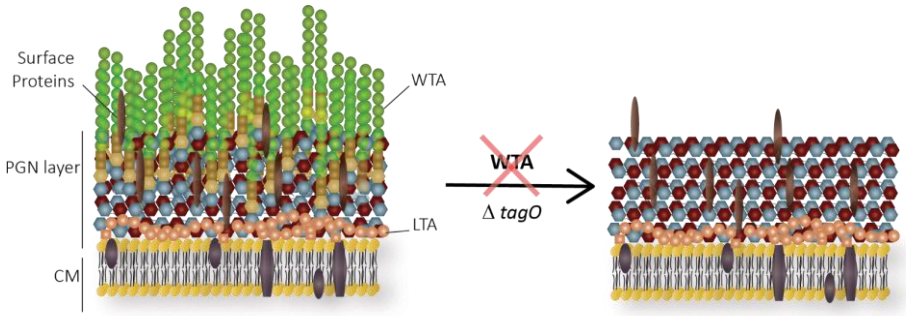


Figure 3. Schematic representation of the cell wall of a Gram-positive wild-type vs. a WTA mutant bacteria. The *tagO* gene encodes the first enzyme of the biosynthetic pathway of the WTA. Thus, in the genetic knock-down mutant strains *S. aureus* Δ *tagO* (NCTC 8325-4 Δ *tagO*) [74] and *B. subtilis* Δ *tagO* (EB6 Δ *tagO*) [75], the PGN is substantially more exposed and directly accessible/vulnerable to the Host immune receptors.

Using these bacterial mutants, I studied the recognition of PGRP-SA and PGRP-LC and the effect of these polymers in shielding the different PGNs from both PGRPs. To study the binding abilities of the PGRPs, recombinant non- and fluorescently- tagged PGRP-SA and the PGRP ectodomain of PGRP-LCx were used in co-precipitation assays. These assays were performed using either live bacterial cells or purified PGN, which was quantified and adjusted to the same number of moles of Mur*N*Ac. Moreover, the assays were conducted in the Haemolymph buffer (Hemo buffer, Materials and Methods section), a buffer with a composition is closer to the haemolymph than the buffers regularly used in *in vitro* experiments. To address the biological significance of the *in vitro* data, I constructed PGRP-SA, PGRP-LC and PGRP-SA/PGRP-LC mutant flies in a similar genetic background and followed their survival upon septic infection.

Results

PGRP -SA and -LC bind to live cells of both WTA mutants.

As previously reported, mCherry_PGRP-SA binding to *S. aureus* cells is only detected when WTA are absent (Fig. 4A) [72]. Unexpectedly, it was observed that mCherry_PGRP-SA can bind to the *B. subtilis* Δ *tagO* mutant cells. Also contradicting most of the literature, mCherry_PGRP-LC is able to bind to *S. aureus* Δ *tagO* cells (Fig 4B).

When carefully looked at, although the PGRPs bind to the whole surface of the Δ *tagO* mutants, it appears that some regions present more bound PGRP than others. This may be due to a higher concentration of PGN in those regions and/or the presence of forms of PGN that are more easily bound by the PGRPs.

Figure 5 shows the expression of a fluorescently tagged version of the native form of PGRP-LCx [76] expressed in haemocytes infected with bacteria. These results corroborate the results of the *in vitro* assay with mCherry_PGRP-LC. In addition, GFP-PGRP-LCx can bind to both wild-type and mutant cells of both *S. aureus* and *B. subtilis*.

The data suggest that PGRP-SA and PGRP-LC are promiscuous towards the third residue of the PGN peptide moiety. Although the binding of PGRP-SA to the DAP-type PGN has been reported, it has not been detected for the *B. subtilis* PGN. Thus, although the current model does reckon some promiscuity of PGRP-SA, it imposes that this binding is not important *in vivo* for the host survival and that the modification of the DAP residue into amiDAP, affects the stability of the binding of the protein to the PGN [2], [30], [68]. Regarding PGRP-LC, it is not considerate to be as promiscuous as PGRP-SA, although *in vitro* assays have shown such promiscuity [69].

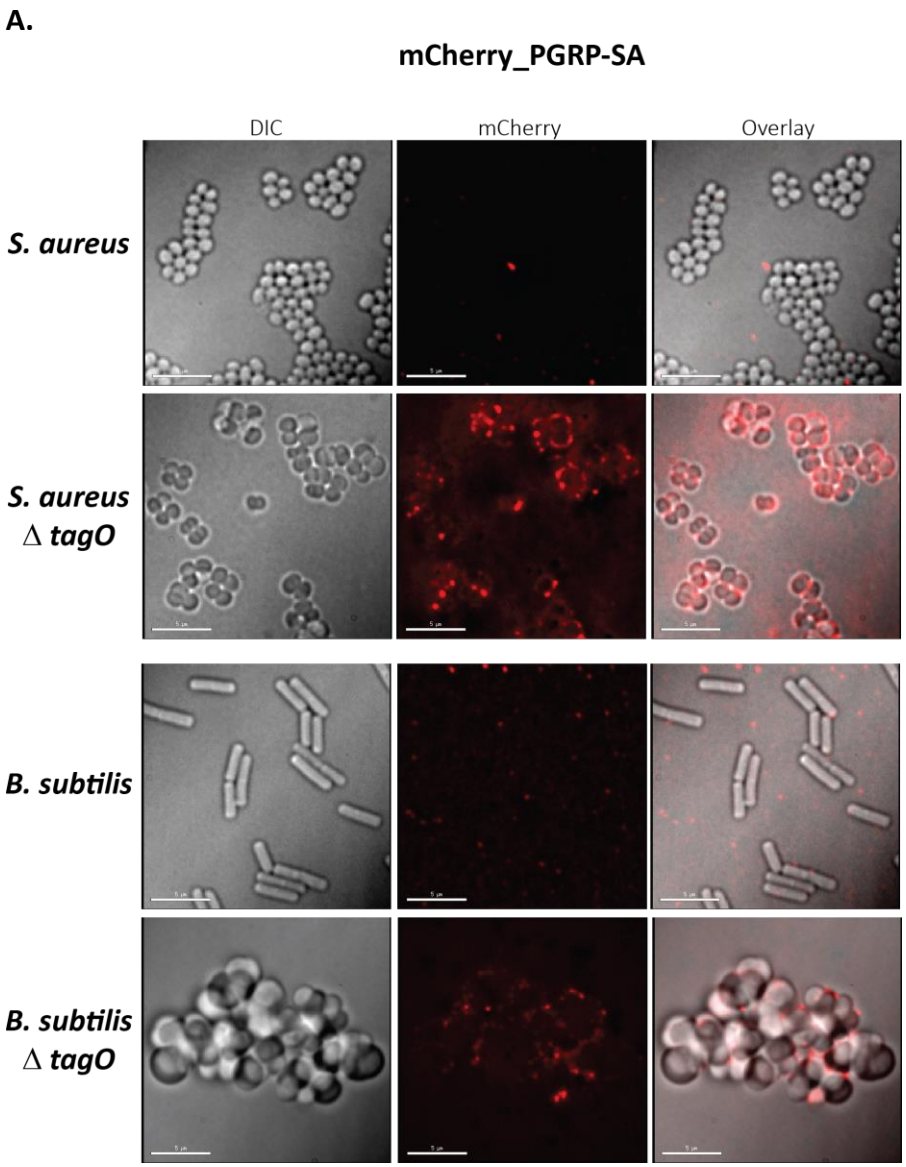


Figure 4. Co-precipitation of live cells with mCherry_PGRP-SA and mCherry_PGRP-LC. Cultures in early exponential phase of the parental and Δ *tagO* mutants of both *S. aureus* and *B. subtilis* were incubated in Hemo Buffer with equal concentrations of either mCherry_PGRP-SA (Panel A) or mCherry_PGRP-LC (Panel B).

B.

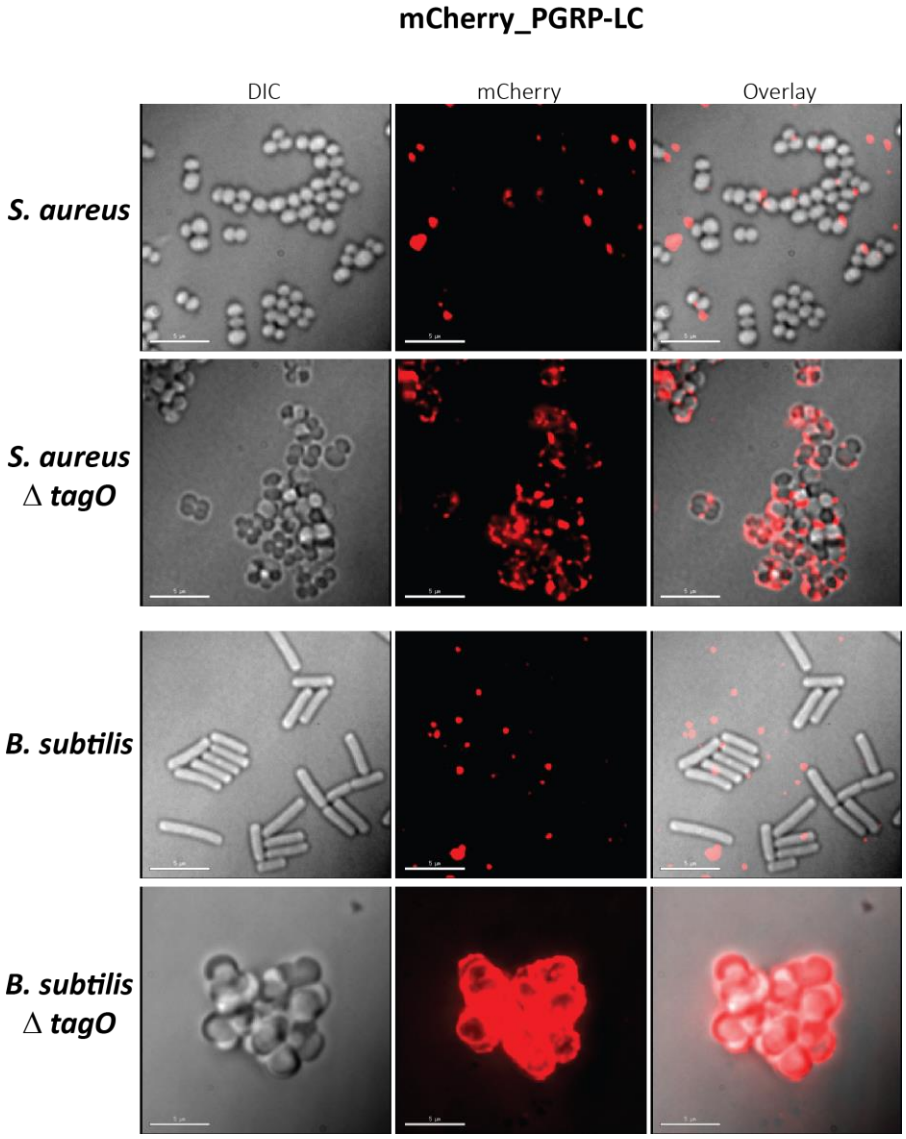


Figure 4. Co-precipitation of live cells with mCherry_PGRP-SA and mCherry_PGRP-LC. The protein bound to the cells was pulled down with them and the binding was visualised by microscopy. Because of the clustering of the Δ *tagO* mutants, it was not possible to properly quantify the signals. Scale intensities were adjusted to equalise *S. aureus* Δ *tagO* – mCherry_PGRP-SA.

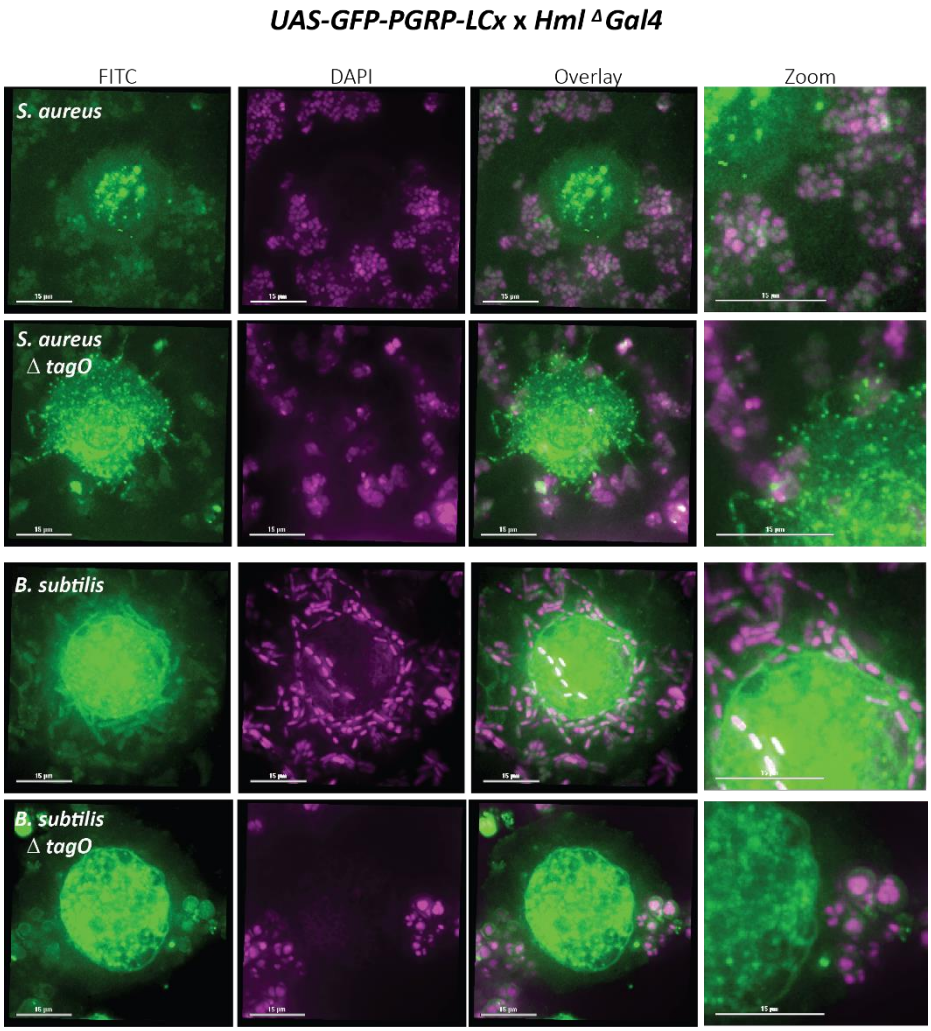
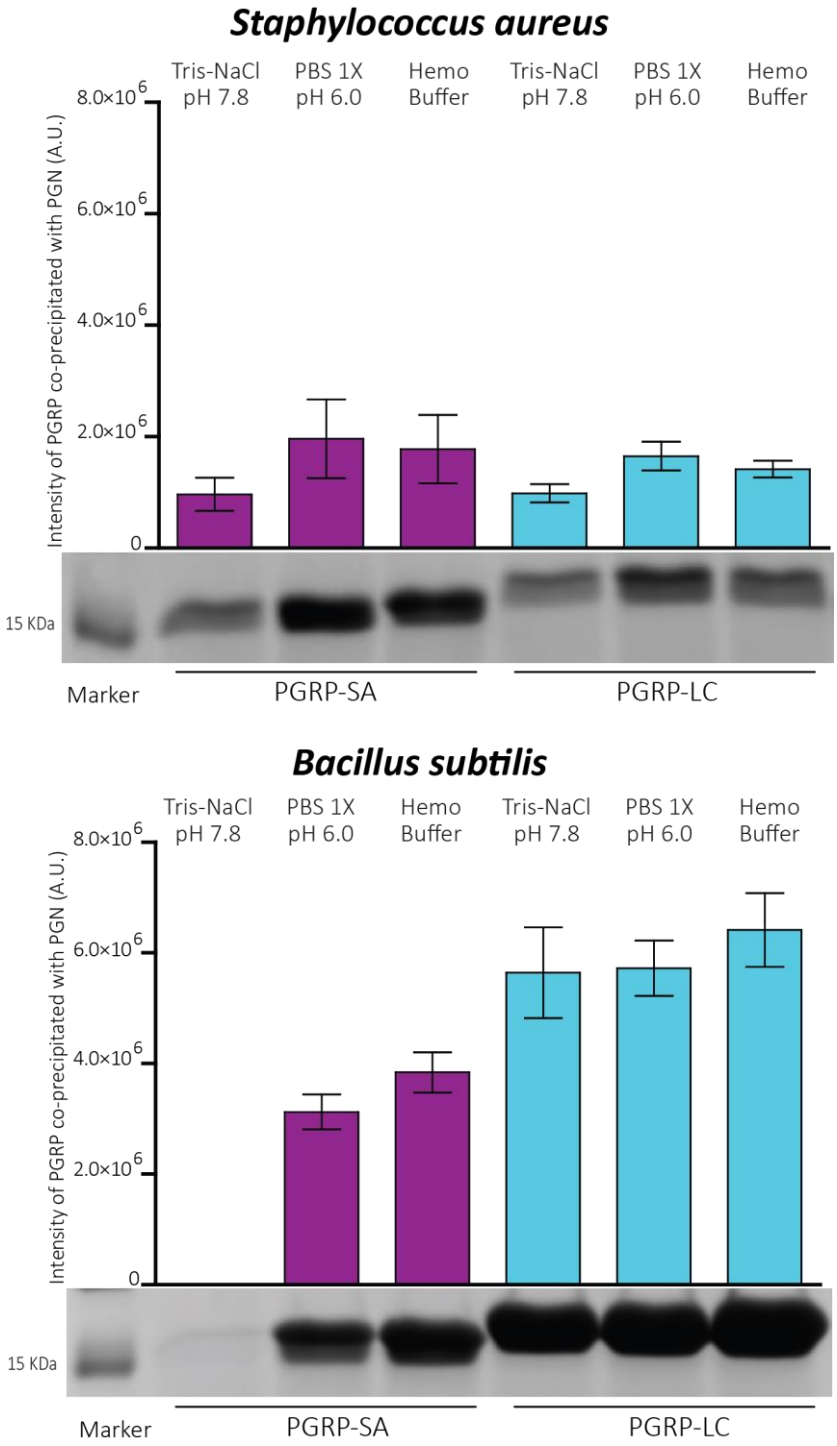


Figure 5. GFP-PGRP-LCx binding to live bacterial cells. Haemocytes expressing *GFP-PGRP-LCx* were extracted from third-instar larvae and kept in Schneider media supplemented with Fetal Calf Serum. After adherence to the glass slide, they were infected with the bacteria. It is presented snapshots of the interaction of the haemocytes and the bacteria, that was monitored for 12h every 15min acquisition. GFP-PGRP-LCx can be found at the lamellipodia, at the cytoplasm and in the outside environment.

PGRP-SA and PGRP-LC bind to both Lys- and DAP- type PGN.

The PGN from the wild-type and mutant Δ *tagO* cells of both *S. aureus* and *B. subtilis* was purified and quantified to conduct co-precipitation assays with non-fluorescent forms of the rPGRPs. The assays using the very non-physiological Tris-NaCl buffer showed no detection of rPGRP-SA binding to *B. subtilis* PGN, as previously reported [2], [30] (Fig. 6A). As the buffer compositions got more similar to the physiological conditions – PBS 1X pH 6.0 and Hemo Buffer – not only did the PGRPs show a higher affinity for the substrate but only then it was detected binding of rPGRP-SA to the amiDAP-PGN of *B. subtilis*. Under conditions similar to the haemolymph, rPGRP-SA and rPGRP-LC showed similar binding to the *S. aureus* Lys-type PGN (Fig. 6B). Regarding the binding to the *B. subtilis* DAP-type PGN, co-precipitation of rPGRP-SA-PGN occurs at ca. $4x10^6$ while rPGRP-LC-PGN at $6x10^6$. This higher binding of PGRP-LC might not be due to a preference to the DAP-residue towards Lys. Instead, it may have to do with the inherent differences of the glycan chains as it has been shown that the Mur*N*Ac residue seems to be paramount for PGRPs recognition and trigger of the immune cascades [65], [66]. Accordingly to what the Host groups had previously observed [72], a decreased cross-linking in *S. aureus* Δ *tagO* did not affect the binding affinity to rPGRP-SA (nor to rPGRP-LC) (Fig. 6B). In fact, the *S. aureus* Δ *tagO* PGN appears to have different physical-chemical characteristics: this PGN presents itself as a suspension of several insoluble small PGN clusters that do not compact as tightly as the other PGN suspensions. Thus it is harder to settle down, it forms a not so well compact pellet and as a consequence it is easier to be (accidently) aspirated with the supernatant throughout the procedure.

A.


Staphylococcus aureus
Tris-NaCl pH 7.8
PBS 1X pH 6.0
Hemo Buffer
Tris-NaCl pH 7.8
PBS 1X pH 6.0
Hemo Buffer
Intensity of PGRP co-precipitated with PGN (A.U.)
8.0×10^6
6.0×10^6
4.0×10^6
2.0×10^6
0
15 KDa
Marker
PGRP-SA
PGRP-LC
Bacillus subtilis
Tris-NaCl pH 7.8
PBS 1X pH 6.0
Hemo Buffer
Tris-NaCl pH 7.8
PBS 1X pH 6.0
Hemo Buffer
Intensity of PGRP co-precipitated with PGN (A.U.)
8.0×10^6
6.0×10^6
4.0×10^6
2.0×10^6
0
15 KDa
Marker
PGRP-SA
PGRP-LC

B.

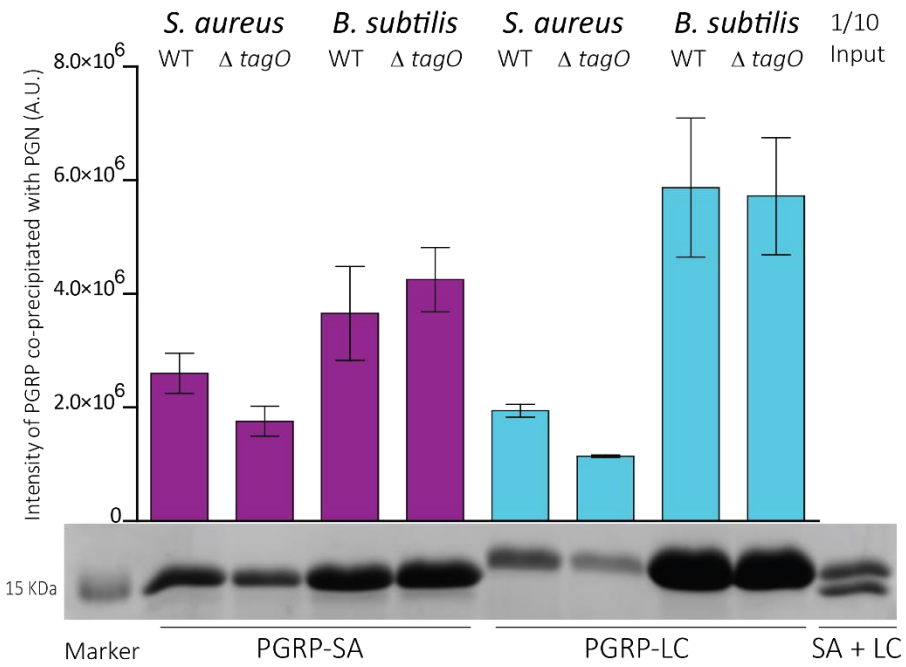


Figure 6. Co-precipitation of PGN with rPGRP-SA and rPGRP-LC. PGN suspensions previously adjusted to the same number of moles of Mur*N*Ac were incubated with equal concentrations of either rPGRP-SA or rPGRP-LC. PGN is insoluble in an aqueous solution hence the amount of protein that binds is pulled down with it. Each assay was repeated in two different days and the bars represent the mean with standard deviation. Panel A. shows the rPGRPs binding to the wild-type *S. aureus* and *B. subtilis* PGNs, using different buffers. The results from the Tris-NaCl buffer corroborate the previously reported non-binding affinity of PGRP-SA to *B. subtilis* PGN [2], [30]. However, when approaching more biological conditions the binding affinity for the PGNs increased and it was detected the binding of PGRP-SA to *B. subtilis*. Panel B. shows pull down assays of the rPGRPs in Hemo Buffer with PGNs from the parental and the Δ *tagO* strains. There seems to be no preference for a Δ *tagO*-PGN compared to the respective wild-type PGN.

PGRP -SA and -LC are important against infection by Lys- and DAP- type PGN bacteria in the Host.

In order to address the importance of PGRP-SA and PGRP-LC against infection by *S. aureus* and *B. subtilis*, I constructed a double mutant deficient in both proteins. The *semmelweis* flies express a non-functional form of PGRP-SA and are highly susceptible to infection by Gram-positive bacteria [1]. PGRP-SA^{seml} possesses a single substitution of the cysteine (Cys) at the position 54 (of the protein without the signal peptide) by a tyrosine. As a consequence, there is disruption of the disulphide bridge occurring between Cys48 and Cys54 [1], [2]. *PGRP-LC*$^{\Delta E12}$ are flies that do not express PGRP-LC due to a deletion of the entire coding region and are compromised against infection by Gram-negative bacteria [4]. The *semmelweis* flies and the *PGRP-LC*$^{\Delta E12}$ flies were used to construct a double mutant *semmelweis*/*PGRP-LC*$^{\Delta E12}$ and the correspondent single *semmelweis* and *PGRP-LC*$^{\Delta E12}$ mutants. To further assess the importance of these receptors, the heterozygous flies of each mutant were also used for *in vivo* studies as controls, since they share a more similar genetic background than any wild-type Fly.

Cultures in the stationary growth phase were injected in the flies that were kept at 25ºC and monitored for 72h, every 12h. Infection with *S. aureus* and *B. subtilis* wild-type bacteria indeed confirm the importance of PGRP-SA and PGRP-LC, respectively (Fig. 7 and Fig. 9). However, contradicting the previous studies, it was observed that PGRP-SA and PGRP-LC are both important upon infection by both bacteria. For any of the four bacteria injected, the homozygous double mutant *semmelweis*/*PGRP-LC*$^{\Delta E12}$ was more susceptible than all the other mutant backgrounds tested (Fig. 7-11).

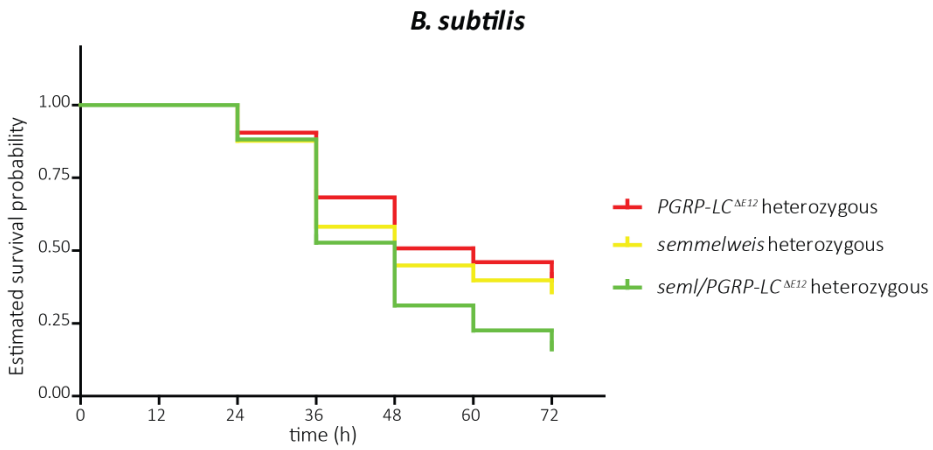
Figure 7 presents the survival data and CFUs/Fly upon infection with *B. subtilis* wild-type cells. The heterozygous double mutant was more

susceptible than the heterozygous single mutants (Fig. 7A). Similarly, the homozygous double mutant was more susceptible than the single mutants (Fig. 7B) and the double heterozygote (Fig. 7C). The *B. subtilis* Δ *tagO* mutant showed drastic loss of virulence since only the homozygote double mutant was susceptible to infection (Fig. 8). Figures 9 and 10 show the survival curves for *S. aureus* and *S. aureus* Δ *tagO*, respectively. Again, the double mutant is more susceptible to infection than any of the other mutant flies.

Regarding the importance of PGRP-LC, upon infection with both *B. subtilis* and *S. aureus*, it was not observed significant differences between the heterozygous and homozygous *PGRP-LC*$^{\Delta E12}$ flies (Fig. 7C and 9C). However, when the WTA are absent the role of PGRP-LC was evident. The *PGRP-LC*$^{\Delta E12}$ homozygous flies were more susceptible to *S. aureus* Δ *tagO* than the correspondent heterozygous flies (Fig. 10C).

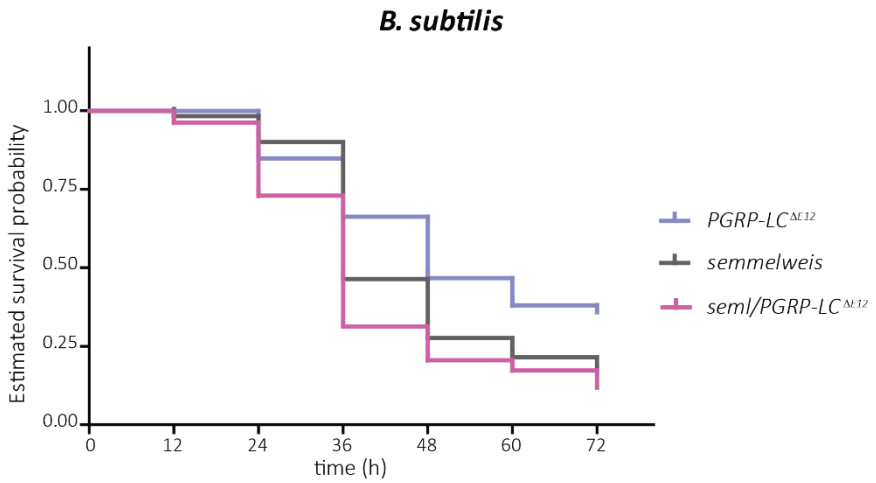
The role of PGRP-SA is evident upon infection with either *B. subtilis* and *S. aureus* parental strains, since the lack of the two copies of the *PGRP-SA*^{WT} gene proved to be more susceptible than the presence of only one copy (Fig. 7C and 9C).

A.



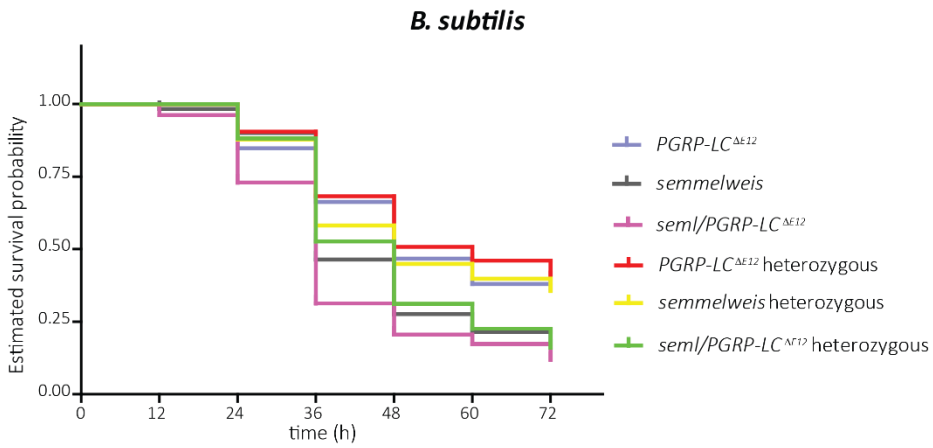
Heterozygous		
	seml vs. *PGRP-LC*$^{\Delta E12}$	ns
	seml vs. *seml/PGRP-LC*$^{\Delta E12}$	*
	PGRP-LC$^{\Delta E12}$ vs. *seml/PGRP-LC*$^{\Delta E12}$	**

B.



Homozygous		
	seml vs. *PGRP-LC*$^{\Delta E12}$	***
	seml vs. *seml/PGRP-LC*$^{\Delta E12}$	**
	PGRP-LC$^{\Delta E12}$ vs. *seml/PGRP-LC*$^{\Delta E12}$	***

C.



Homozygous	*seml*	**
vs.	*PGRP-LC* $^{\Delta E12}$	ns
Heterozygous	*seml/PGRP-LC* $^{\Delta E12}$	*

D.

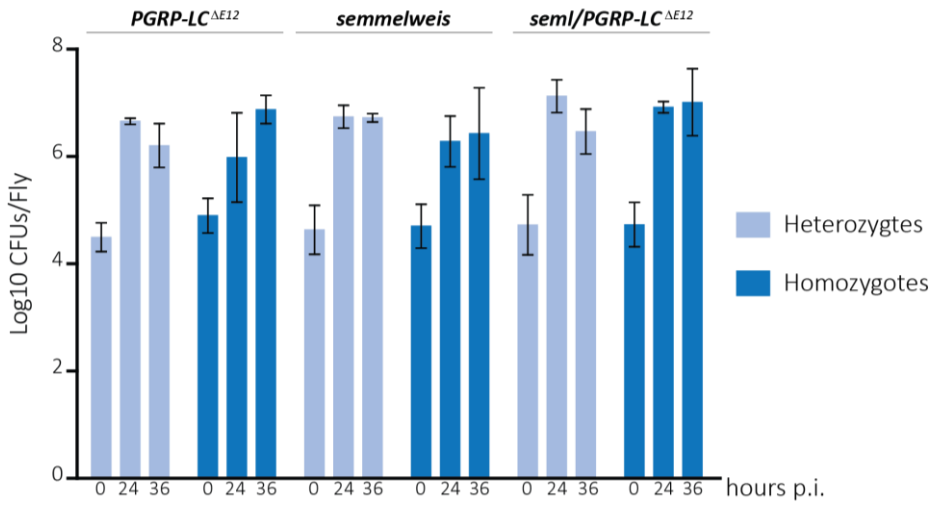
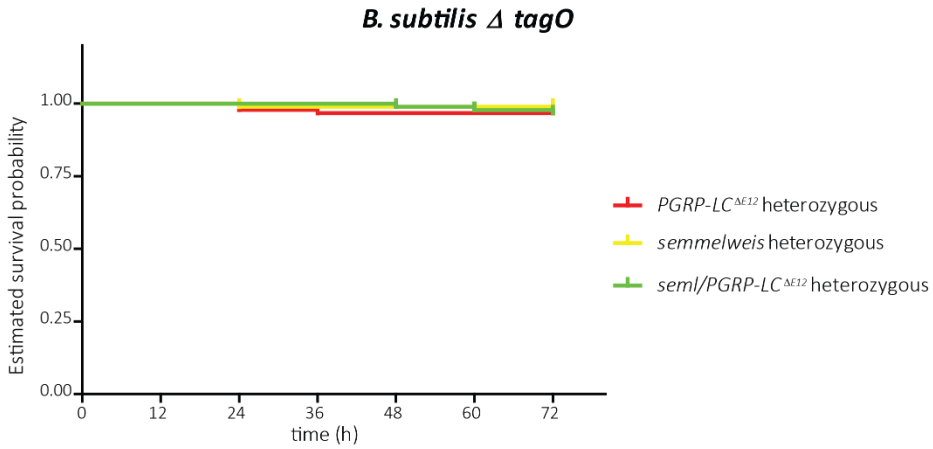


Figure 7. Survival data for *B. subtilis*. Panel A. Survival curves of the heterozygotes. The heterozygous double mutant was more susceptible than the heterozygotes *semmelweis* and *PGRP-LC*$^{\Delta E12}$ that were indistinguishable. Panel B. Survival curves of the homozygotes. The double mutant homozygous was more susceptible than *semmelweis* and *PGRP-LC*$^{\Delta E12}$, however *semmelweis* was more susceptible than *PGRP-LC*$^{\Delta E12}$. Panel C. Survival curves of the heterozygotes and homozygotes. The double homozygote was more susceptible than any of the heterozygotes and single homozygotes, hence both PGRPs are important against infection. Panel D.

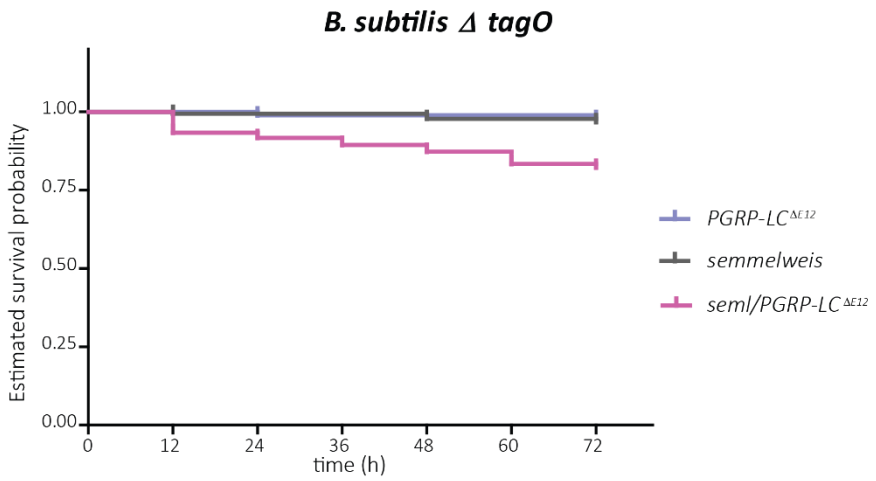
Log_{10}CFUS/Fly at 0h, 24h and 36h p.i.. In accordance to the survival probability data, *B. subtilis* bacteria were able to grow inside the flies regardless of the genotype, without detectable differences in the bacterial load between each genotype.

A.



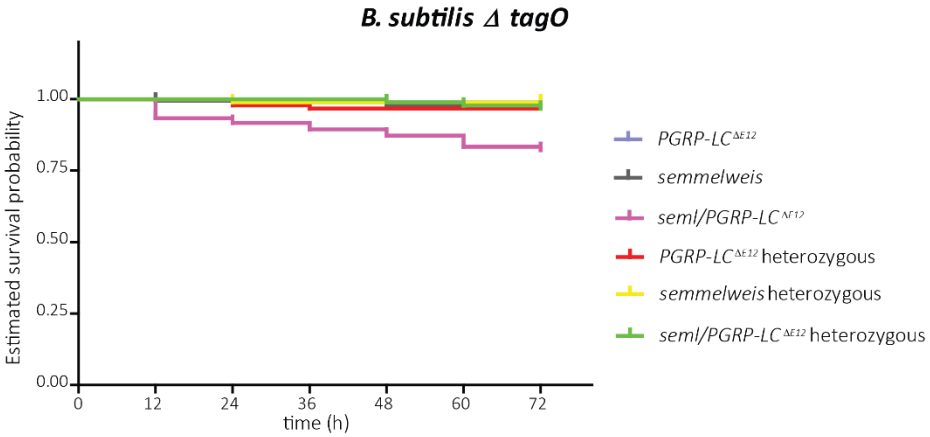
Heterozygous	*seml* vs. *PGRP-LC*$^{\Delta E12}$	ns
	seml vs. *seml/PGRP-LC*$^{\Delta E12}$	ns
	PGRP-LC$^{\Delta E12}$ vs. *seml/PGRP-LC*$^{\Delta E12}$	ns

B.



Homozygous	*seml* vs. *PGRP-LC*$^{\Delta E12}$	ns
	seml vs. *seml/PGRP-LC*$^{\Delta E12}$	***
	PGRP-LC$^{\Delta E12}$ vs. *seml/PGRP-LC*$^{\Delta E12}$	***

C.



Homozygous	*seml*	ns
vs.	*PGRP-LC* $^{\Delta E12}$	ns
Heterozygous	*seml/PGRP-LC* $^{\Delta E12}$	***

D.

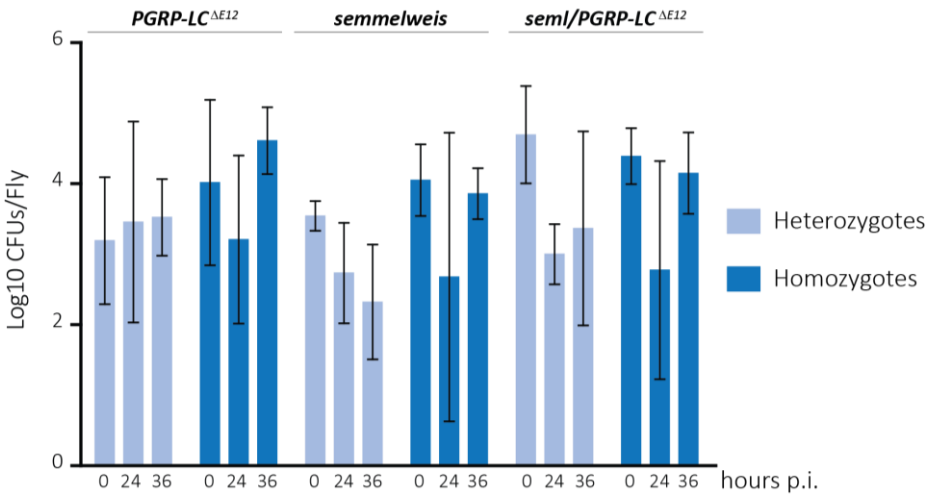
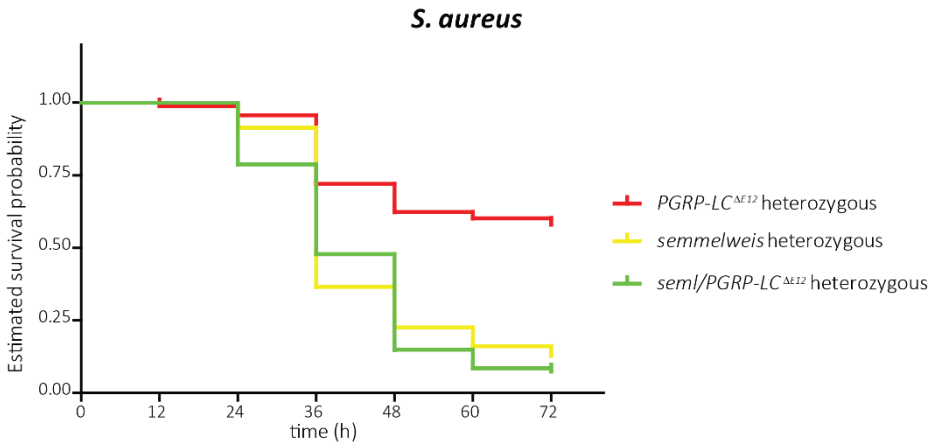


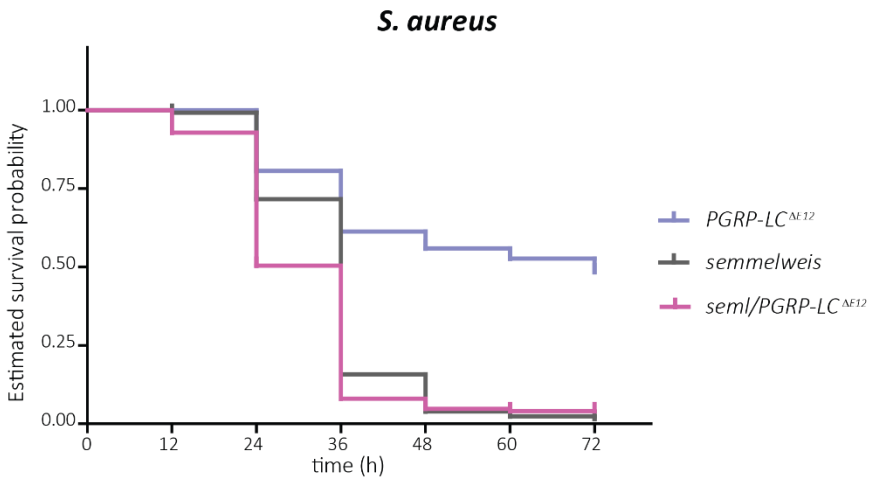
Figure 8. Survival curves for *B. subtilis* Δ *tagO* infections. Panel A. Survival curves of the heterozygotes. Panel B. Survival curves of the homozygotes. Panel C. Survival curves of the heterozygotes and homozygotes. Only the homozygous double mutant *semmelweis*/*PGRP-LC*$^{\Delta E12}$ was susceptible to infection. Panel D. Log_{10}CFUS/Fly at 0h, 24h and 36h p.i.. The heterozygous flies *semmelweis* and *semmelweis*/*PGRP-LC*$^{\Delta E12}$ showed a reduction of CFUs in contrast to the homozygotes whilst *PGRP-LC*$^{\Delta E12}$ homozygotes were able to control the CFUs levels, similar to the heterozygote.

A.



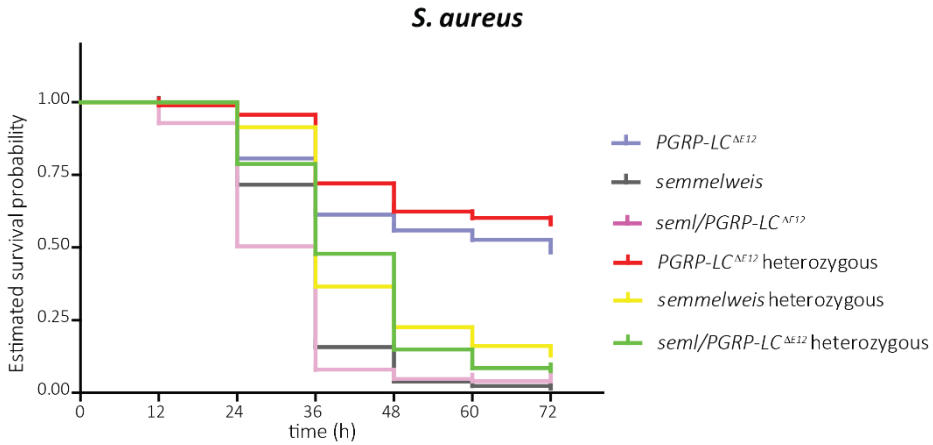
Heterozygous	*seml* vs. *PGRP-LC*$^{\Delta E12}$	***
	seml vs. *seml/PGRP-LC*$^{\Delta E12}$	ns
	PGRP-LC$^{\Delta E12}$ vs. *seml/PGRP-LC*$^{\Delta E12}$	***

B.



Homozygous	*seml* vs. *PGRP-LC*$^{\Delta E12}$	***
	seml vs. *seml/PGRP-LC*$^{\Delta E12}$	**
	PGRP-LC$^{\Delta E12}$ vs. *seml/PGRP-LC*$^{\Delta E12}$	***

C.



Homozygous	*seml*	***
vs.	*PGRP-LC*$^{\Delta E12}$	ns
Heterozygous	*seml/PGRP-LC*$^{\Delta E12}$	***

D.

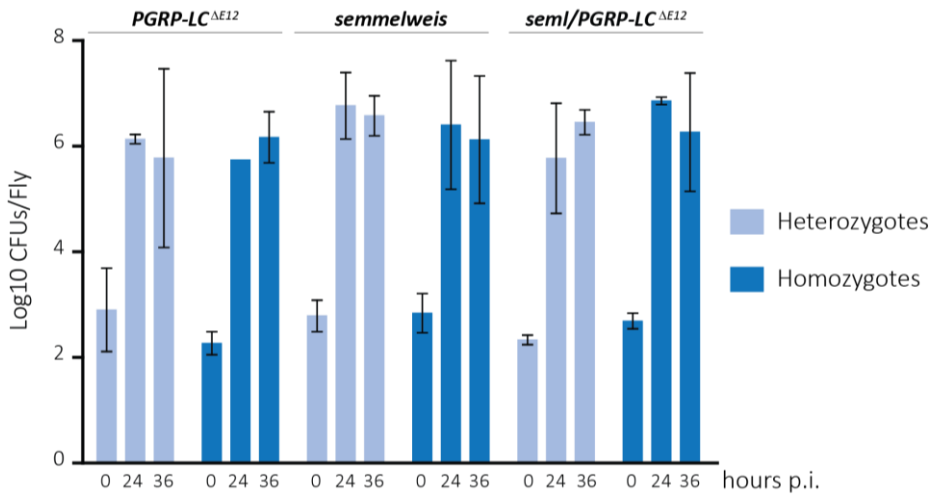
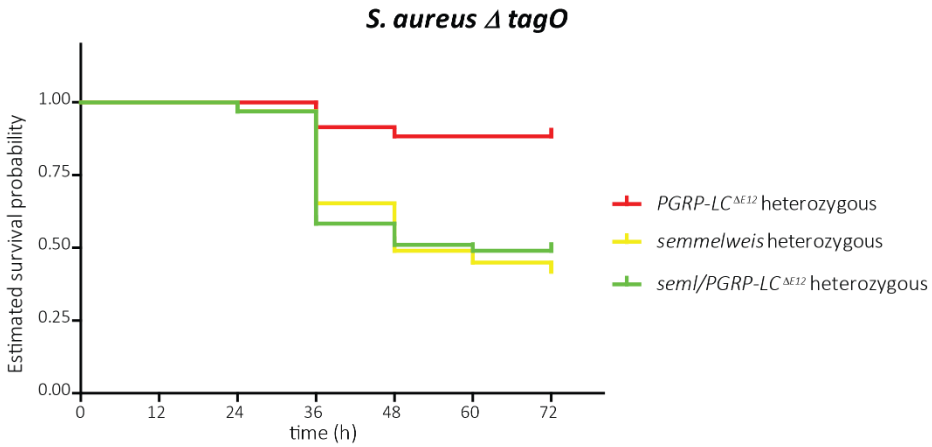


Figure 9. Survival curves for *S. aureus* infections. Panel A. Survival curves of the heterozygotes. Panel B. Survival curves of the homozygotes. Panel C. Survival curves of the heterozygotes and homozygotes. Panel D. Log_{10}CFUs/Fly at 0h, 24h and 36h p.i.. *PGRP-LC*$^{\Delta E12}$ heterozygous flies were more resistant to infection than both *semmelweis* and *semmelweis/PGRP-LC*$^{\Delta E12}$ heterozygous, which were indistinguishable. However, the double heterozygote succumbed earlier than *semmelweis* heterozygotes, hence it suggests the importance of PGRP-LC. Accordingly, the double homozygous mutant is more susceptible than both single mutants, although *PGRP-LC*$^{\Delta E12}$ is more resistant than *semmelweis*. *S. aureus* is able to

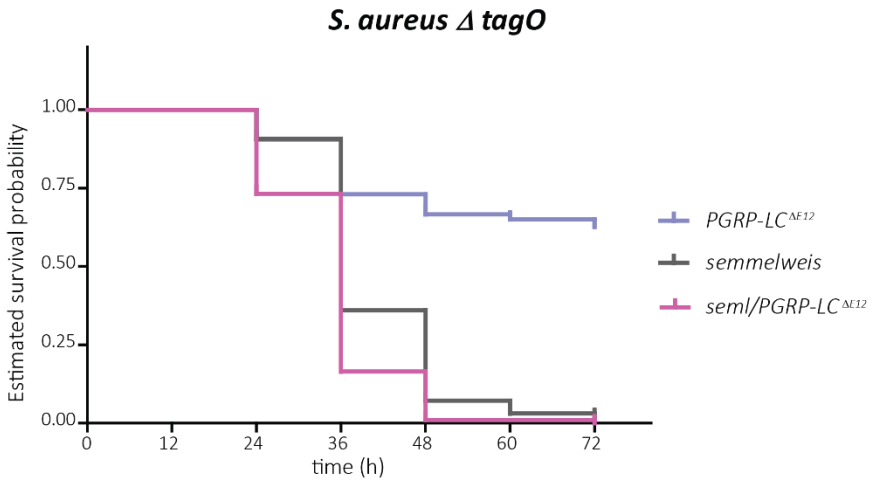
grow in the flies regardless of the genotype, without detectable differences in the bacterial load.

A.



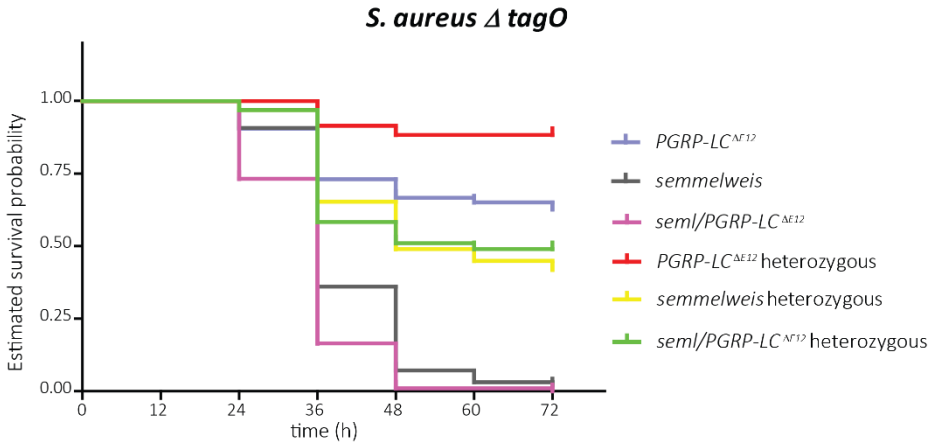
Heterozygous		
	seml vs. *PGRP-LC* $^{\Delta E12}$	***
	seml vs. *seml/PGRP-LC* $^{\Delta E12}$	ns
	PGRP-LC $^{\Delta E12}$ vs. *seml/PGRP-LC* $^{\Delta E12}$	***

B.



Homozygous		
	seml vs. *PGRP-LC* $^{\Delta E12}$	***
	seml vs. *seml/PGRP-LC* $^{\Delta E12}$	***
	PGRP-LC $^{\Delta E12}$ vs. *seml/PGRP-LC* $^{\Delta E12}$	***

C.



Homozygous	*seml*	***
vs.	*PGRP-LC* $^{\Delta E12}$	***
Heterozygous	*seml/PGRP-LC* $^{\Delta E12}$	***

D.

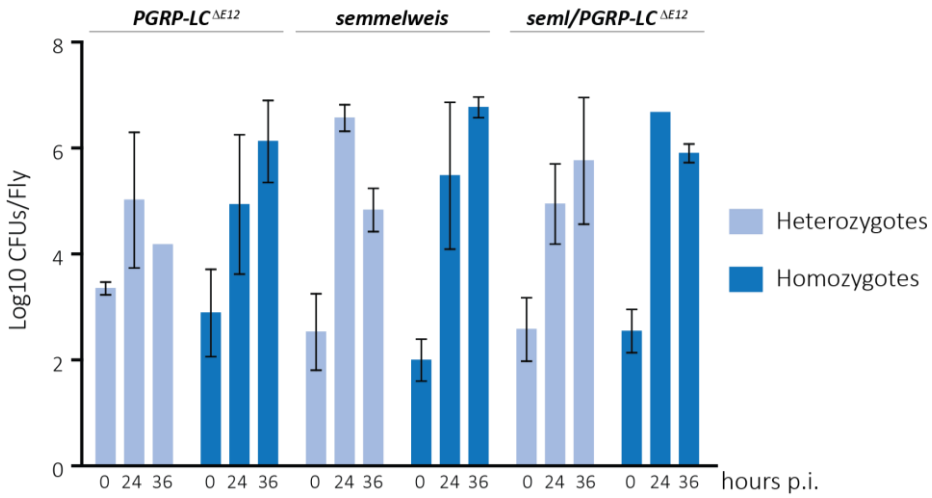


Figure 10. Survival curves for *S. aureus* Δ *tagO* infections. Panel A. Survival curves of the heterozygotes. *PGRP-LC*$^{\Delta E12}$ heterozygous was more resistant than *semmelweis* and *semmelweis*/*PGRP-LC*$^{\Delta E12}$ heterozygous that were equally susceptible. Panel B. Survival curves of the homozygotes. To reveal the importance of PGRP-LC both copies of the gene must be removed. The double homozygous mutant was more susceptible than the single mutants. Panel C. Survival curves of the heterozygotes and homozygotes. Panel D. Log_{10}CFUs/Fly at 0h, 24h and 36h p.i.. *S. aureus* Δ *tagO* was able to grow inside the Hosts and although the bacterial load was higher in the

homozygotes than the respective heterozygotes, significant differences were only seen at 36h for *semmelweis* heterozygous vs. *semmelweis* homozygous.

Figure 11 presents the survival curves in the double mutant *semmelweis*/*PGRP-LC*$^{\Delta E12}$ background for the *B. subtilis* and *S. aureus* wild-type and Δ *tagO* strains. The WTA mutants were less virulent than the parental strains and it corroborates the previous findings on the stereo-hindrance effect of the WTA in concealing the *S. aureus* PGN from PGRP-SA [72].

When injected with PBS, the flies did not succumb and remained healthy (Fig. 12), similar to the injection of the mutant *B. subtilis* Δ *tagO*, (apart from infection the homozygous *semmelweis*/*PGRP-LC*$^{\Delta E12}$ mutant).

Finally, Table 1 presents a summary of the results regarding the statistical analysis between the survivals of the infected flies.

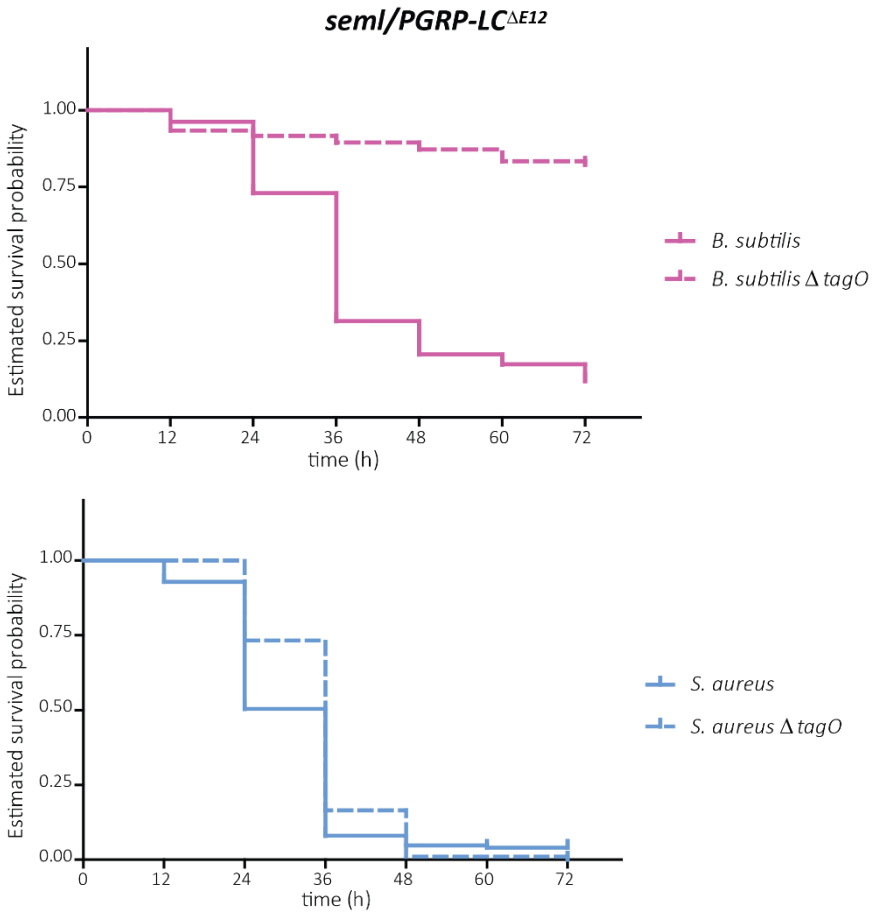


Figure 11. Survival curves of *semmelweis/PGRP-LC*$^{\Delta E12}$ flies. The graphic at the top shows the plot of the survival curves for the *B. subtilis* strains and bellow for the *S. aureus* strains. The WTA mutants were less virulent than the parental strains, *P*<0.0001 for *B. subtilis* vs *B. subtilis* Δ *tagO* and *P*=0.0086 for *S. aureus* vs *S. aureus* Δ *tagO*.

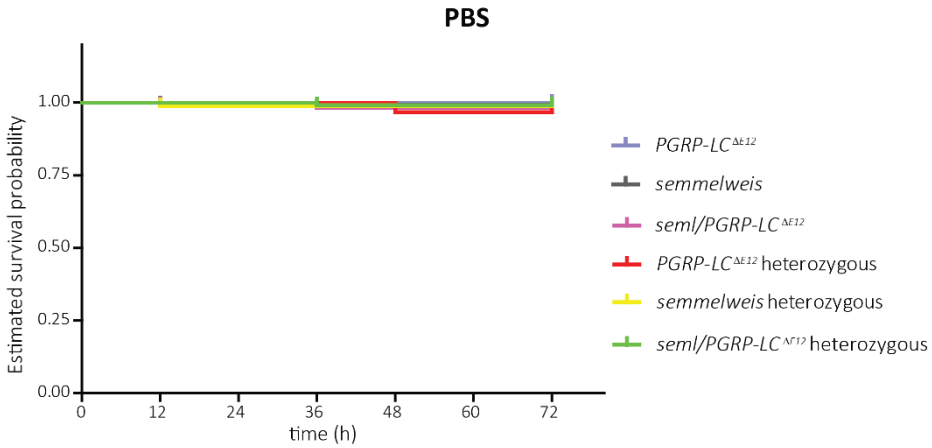


Figure 12. Survival curves upon PBS injection. The PBS injected/infection came from the same solution batch used to prepare the bacterial cells for infections. Thus, the survivals show that not only were the flies healthy but also that the PBS solution used was sterile and the volume injected did not affect the survival of the flies.

Table 1. Summary of the statistical analysis of the survival data.

	Statistics of the survival curves	*B. subtilis*	*B. subtilis* Δ *tagO*	*S. aureus*	*S. aureus* Δ *tagO*
Homozygous	*seml* vs. *PGRP-LC*$^{\Delta E12}$	***	ns	***	***
	seml vs. *seml/PGRP-LC*$^{\Delta E12}$	**	***	**	***
	PGRP-LC$^{\Delta E12}$ vs. *seml/PGRP-LC*$^{\Delta E12}$	***	***	***	***
Heterozygous	*seml* vs. *PGRP-LC*$^{\Delta E12}$	ns	ns	***	***
	seml vs. *seml/PGRP-LC*$^{\Delta E12}$	*	ns	ns	ns
Homozygous vs. Heterozygous	*PGRP-LC*$^{\Delta E12}$ vs. *seml/PGRP-LC*$^{\Delta E12}$	**	ns	***	***
	seml	**	ns	***	***
	PGRP-LC$^{\Delta E12}$	ns	ns	ns	***
	seml/PGRP-LC$^{\Delta E12}$	*	***	***	***

Discussion

The data here presented shows that both PGRP-SA and PGRP-LC are promiscuous for Lys- and DAP- type PGN and that both PGRPs detect the bacteria and contribute to survival of the host. Thus, this implies that it is not the peptide moiety of the PGN that determines PGRPs recognition. Rather, it is the accessibility through the WTA layer in the CW. This data is conflicting with the current model of bacterial PGN detection in the Fly.

Leulier and colleagues [65] were the firsts to study the *in vivo* effect of the injection of PGN on the expression of the AMPs in *Drosophila*. However, due to the procedure used for PGN purification, one can conclude that the activation of the IMD pathway by DAP-type PGN has been well characterized but the same might not be the case for the Lys-type PGN. The procedure to obtain pure PGN from Gram-negative and Gram-positive bacteria is dependent on the different CW compositions [77]. The first step of PGN purification is the boiling of the cells in an SDS solution. This step is common to both Gram-negative and Gram-positive bacteria. In Gram-negative bacteria, the SDS removes the outer membrane (OM), hence what is harvested is the PGN with (little) proteins attached. Thus, after treatment with Trypsin, DNase and RNase it is obtained pure PGN polymer. For purification of the Gram-positive PGN, three more steps need to be added to this procedure: 1) mechanical break of the cells so that the trypsin and the nucleases can pass through the PGN layer; 2) treatment with different solvents to remove non-covalently material attached to the PGN and 3) incubation with Hydrofluoric acid that specifically cuts the phosphodiester bonds between the WTA and the muramic acid. According to the materials and methods section of Leulier *et al.* 2003, it is not referred the incubation of the Gram-positive CW neither with acid or alkaline conditions. Therefore, it seems that it was injected Gram-negative PGN and Gram-positive CW. In addition, the amount of PGN injected from Gram–

negatives and the Gram-positive CW is then expected be very different since the WTA contribute substantially for the CW weight. Thus, when the authors speculated why the *B. subtilis* PGN is a less potent inducer of the IMD than the Gram-negative PGN by arguing the presence of amiDAP, it could be due also or instead to both the presence of WTA and the amount of *B. subtilis* PGN injected compared to the Gram-negative PGN injected. Nevertheless, the authors commented on the presence of the OM and the surprising recognition of PGN by PGRP-LC. They pointed out that the OM is a shield to PGN detection which it is implied in the data I present. Furthermore, assuming that the OM protects PGRP-LC from accessing the PGN, they speculated that PGRP-LC may recognise PGN fragments released by the turnover and growth of the bacteria. Indeed, it was later shown that PGRP-LCx recognises monomeric forms of PGN [70]. Interestingly, they observed that both the Gram-negative and Gram-positive PGN after muramidase treatment failed to activate an immune response in the Fly. Hence, they hypothesised that the size of the polymer chain and the 3D dimensional organization of the macromolecule is crucial for bacterial sensing.

Stenbak *et al.* 2004 [66] also studied the effect of PGN and PGN derivatives on the induction of AMPs expression. The authors concluded that the Fly discriminates between Gram-negative and Gram-positive bacteria by differentiating between DAP and Lys residues. Regarding the *B. subtilis* DAP-type PGN, they corroborate Leulier *et al.* 2003 as they also observed that it induces the IMD in a lower extent than the Gram-negatives. Since they used a similar protocol for PGN purification as Leulier and colleagues, it seems that the study was on Gram-positive CW and not pure PGN. Nevertheless, the authors propose that this less activation has to do with the amiDAP residue and the absence of the anhydrous-Mur*N*Ac form at the end of the glycan chains. In addition, they also propose that since the terminal anhydrous residue locks the Mur*N*Ac in a fixed

configuration, beta configuration, this is important for PGRP recognition. They argue that this might be the reason why only polymeric Gram-negative PGN and the anhydrous form but not the fragments generated by muramidase treatment have the capacity to strongly activate the IMD pathway. Thus, the absence of the anhydrous form in the muropeptides of the Gram-positive PGN, would result in the fact that only polymeric PGN but not muropeptides from Lys-type PGN could be recognised by PGRP. Again, they corroborate Leulier *et al.* 2003 [65] regarding the muramidase treatment after which Lys-type PGN does not induce the Toll target *Drosomycin*, similar to what Filipe *et al.* 2005 observed [31]. They concluded that perhaps a fixed Mur*N*Ac configuration is important for efficient recognition of both PGRP-LC and PGRP-SA and thus influence the recognition of PGN.

Studies with pure PGN however always come with limitations that may not be possible to completely remove. Regarding the Gram-negative PGN, the full length intact polymer can be injected, whereas for the Gram-positive PGN, due to the mechanical break of the cells, the polymer has inherently suffered some breakage. This may or may not influence to a certain level the *in vivo* detection by PGRPs or induction of AMPs expression. Moreover, as the PGN polymer is insoluble and the needles used to inject the fruit flies have a very small width, the amount of PGN that is injected will most likely have considerable variations that only with a wide number of biological repeats can be accurately assessed the systemic infection by pure PGN. Therefore, the use of live bacteria with exposed PGN at the surface presents itself as a way to overcome these constraints. In addition, another limitation of biochemical studies regarding host receptors, are the *in vitro* conditions used, which are usually far from the physiological conditions.

In this study, I used a buffer closer to the haemolymph composition than any other used so far and Gram-positive bacteria that do not possess

WTA but share an almost identical PGN composition as the parental strains (Fig. 13). Moreover, I confirmed that the CWs of both WTA mutants are totally devoid of polymers and furthermore they present a similar sugar composition (Fig. 14).

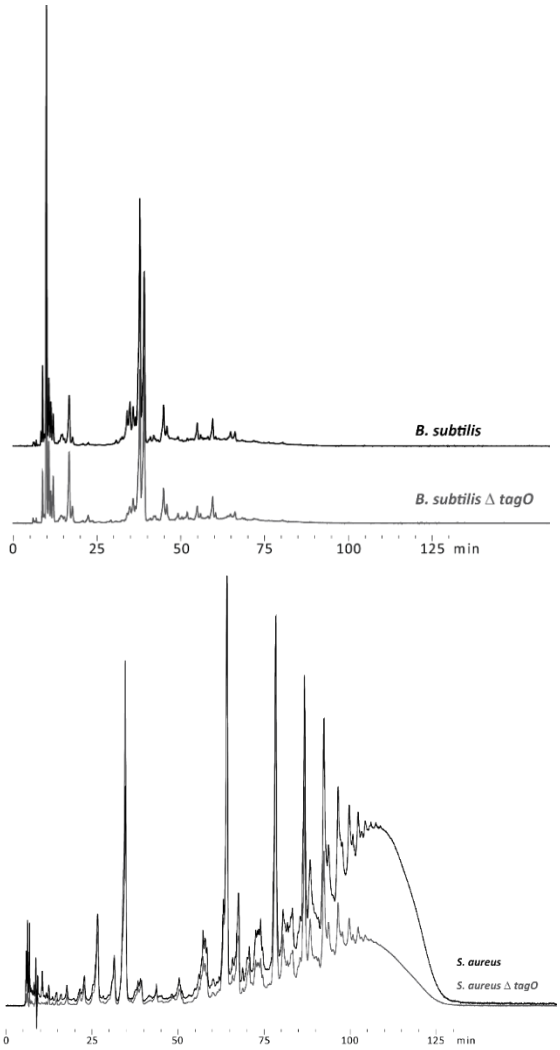


Figure 13. Muropeptide profiles of pure PGNs. Pure PGN was digested with the muramidase Mutanolysin and the resultant muropeptides were separated by HPLC. It can be observed that the Δ *tagO* mutants present a muropeptide composition similar to the parental strains. It is also observed the much reduced cross-linking of the *S. aureus* Δ *tagO* [74], which is not observed for the *B. subtilis* Δ *tagO* compared to the parental strain.

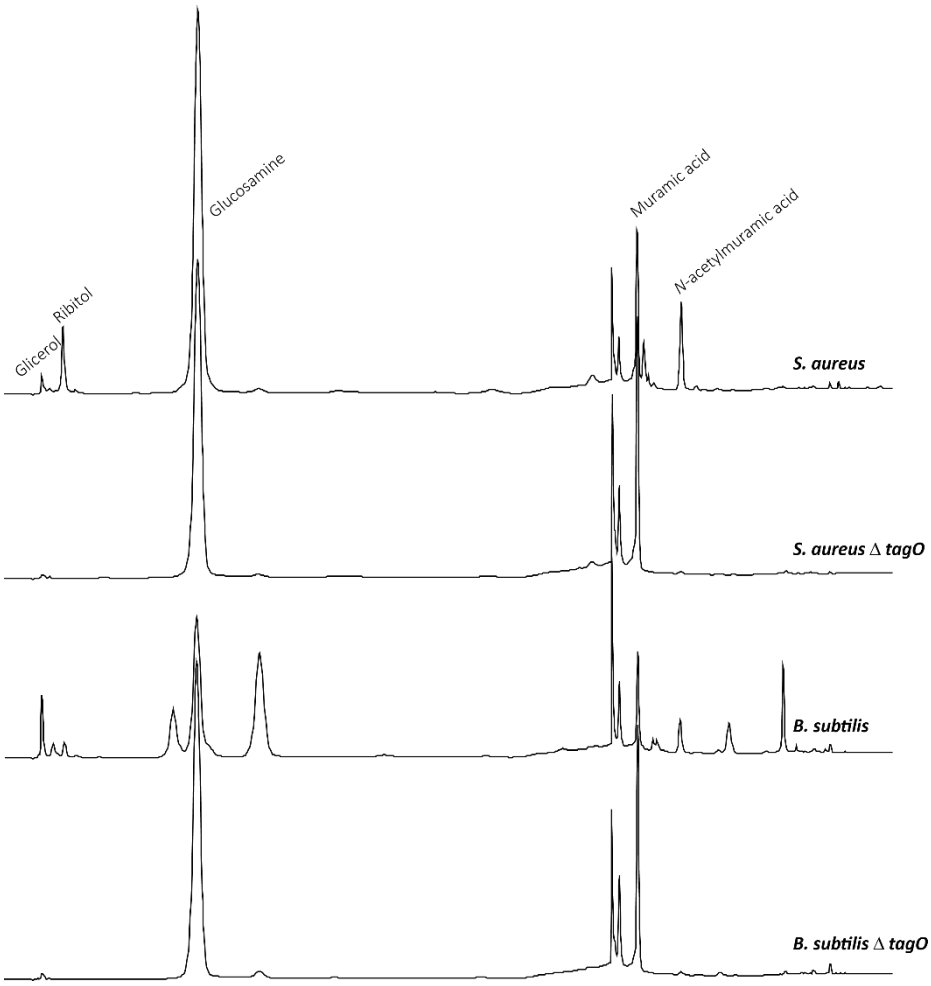


Figure 14. Sugar composition of pure CWs pre-incubated with HCl. Purified CW was incubated with HCl and the sugar species were separated by HPAEC-PAD. It can be concluded that both Δ *tagO* mutants present the same sugar composition, albeit different muropeptide specie, and are completely devoid of WTA or other type of CW polymers. The hydrolysis of the parental CWs was incomplete as it is still detected Mur*N*Ac residues, most likely due to the presence of the WTA that may affect the efficiency of the hydrolysis.

PGRP-SA and PGRP-LC bind to Lys- and DAP- type PGN.

As expected, when the WTA of *S. aureus* are absent it was detected the binding by mCherry_PGRP-SA and when the WTA of *B. subtilis* are absent it was detected the binding by mCherry_PGRP-LC (Fig. 4). However, contradicting what was expected according to the current model, it was

also detected binding of mCherry_PGRP-SA to *B. subtilis* Δ *tagO* and binding of mCherry_PGRP-LC to *S. aureus* Δ *tagO* (Fig. 4). These results suggest that PGRP-SA is able to recognise amiDAP-type PGN, PGRP-LCx is promiscuous for the third residue of the PGN and WTA can shield different PGN types from the recognition of different PGRPs, and not specifically from PGRP-SA.

Since PGRP-LCx is a transmembrane protein, although it has been reported that it can be found in the haemolymph the protein bearing the PGRP ectodomain [76]. Hence, the recombinant protein construct could possess a different level of affinity than the native protein, thus raising the possibility that in *in vivo* conditions, the transmembrane form could not be able to bind to the cells. To address this question, haemocytes expressing *GFP-PGRP-LCx* [76] were infected with the different bacterial types (Fig. 5). Similar results were obtained, *i.e.* the native protein bound to the surface of the WTA mutant bacteria of both *S. aureus* and *B. subtilis*. Moreover, GFP-PGRP-LCx could bind to the parental bacteria as well.

On one hand, the binding of the rPGRPs could be not so specific to the PGN since components of the CW could be promoting some non-specificity due to protein-protein interactions mediated by charge. On the other hand, if the PGRPs can indeed bind to both types of PGN when WTA are absent in live bacteria, then the same should be observed when using pure PGN of the parental strains. To address these questions, co-precipitation assays with pure quantified PGN were performed. Binding of the rPGRPs to the PGN of the parental strains showed that they are indeed able to bind to the pure substrate (Fig. 6A and 6B). Moreover, it is shown that under more physiological conditions such as the Hemo Buffer, PGRP-SA can bind to amiDAP. Thus, the reason why Chang *et al.* 2004 [2] and Leone *et al.* 2008 [30] failed to detect binding of PGRP-SA to amiDAP-type PGN was the use a non-physiological buffer. Regarding the binding of PGRP-LC, Mellroth *et al.* 2005 [69] had also detected that this PGRP was able to bind *in vitro* to

different types of PGN, in accordance with these results. However, the biological importance of these results were considered to be none. The work from Chang *et al.* 2006 [70] helped to define the current model whereby there is a structural discrimination between DAP- vs. Lys- type PGN by PGRP-LC and PGRP-SA, respectively. On the analysis of their data regarding the crystal structure of the complex TCT-PGRP-LCx-PGRP-LCa, the authors ascertain the stability of the PGRP-LC binding to the DAP residue in monomeric PGN. However, they refer that in the case of a polymeric PGN, a Lys residue would not destabilise the binding interaction as if it was present in a monomeric form similar to TCT. This interpretation corroborates my results and Mellroth`s regarding the non-specificity of PGRP-LCx to Lys-, Ornithine- or DAP- type PGN. In addition, Mellroth *et al.* 2005 also showed that PGRP-SA, like PGRP-LCx, possesses promiscuity towards the third residue of the stem peptide, although not addressing an amiDAP PGN. The binding of PGRP-LCx to the PGNs tested seemed to be similar to all, whereas the binding of PGRP-SA showed stronger and weaker affinities. This led the authors to conclude that PGRP-SA presents preferences for certain PGN substrates. However, this preferences might not be correlated to the third residue of the peptide chain, but rather to different PGN structures and the nature of the glycan chains. When Swaminathan *et al.* 2006 [71] concluded that PGRPs discriminate PGN through the stem peptide and the peptide cross-bridge, the authors refer that the binding of PGRPs to the PGN derivatives, although highly selective, was of low affinity. This low affinity could help to explain why they failed to observe binding to different PGN forms.

The rPGRPs showed similar bindings to the PGN of the parental and mutant strains (Fig. 6C). Therefore, the binding of the PGRPs to the mutant cells (Fig. 4 and 5) was indeed because of a more exposed PGN at the cell surface, corroborating the use of such mutant strains to study PGN recognition by host receptors. In addition, the binding of PGRP-LC to *B.*

subtilis PGN showed to be higher than the binding to the *S. aureus* PGN (Fig. 6C), in accordance with the binding to the live cells of the *tagO* mutants (Fig. 4B). Interestingly, the binding of PGRP-SA to the *B. subtilis* PGN is also higher than to the *S. aureus* PGN (Fig. 6C). This result may appear contradictory to the binding to the cells of the *S. aureus Δ tagO* mutant, where PGRP-SA shows better binding than to the *B. subtilis Δ tagO* cells. However, due to the characteristics of the physical-chemical properties of the *S. aureus Δ tagO* PGN, the comparison of the binding to this particular PGN with the *B. subtilis Δ tagO* PGN may come with limitations. Further studies would be needed to address this question.

PGRP-SA and PGRP-LC are important against infection by bacteria with either Lys- or DAP- type PGN.

To address the biological importance of the binding assays *in vivo*, I infected *semmelweis*, *PGRP-LC$^{\Delta E12}$* and *semmelweis/PGRP-LC$^{\Delta E12}$* mutant flies, both homozygotes and heterozygotes (Fig. 7-11 and Table 1). Upon *B. subtilis* and *S. aureus* infections, the heterozygotes *semmelweis* and *PGRP-LC$^{\Delta E12}$* were equally susceptible (Fig. 7A and 9A). However, for both bacterial infections, the homozygotes showed different susceptibilities, being *semmelweis* more susceptible than *PGRP-LC$^{\Delta E12}$* (Fig. 7B and 9B). In addition, the heterozygote vs. homozygote *semmelweis* show that the lack of two copies of the *PGRP-SA^{wt}* gene is more detrimental for the host than still having one wild-type copy (Fig. 7C and 9C). The same is not observed between one and two copies of the *PGRP-LC* gene. This suggests that although the rPGRP-LC binds better to both PGNs than rPGRP-SA (Fig. 6C), *in vivo* PGRP-SA may be more important for host survival against both bacterial types than PGRP-LC. Whereas *S. aureus* is a major opportunistic pathogen bearing an arsenal of virulence factors, *B. subtilis* is an innocuous soil bacteria. Then, it is not surprising to find that the Fly is much more susceptible to *S. aureus*. Consequently, the late humoral responses like the

AMPs, may not be crucial as a first step to fight infection. Hence, it is likely that the Fly is strongly relying on immediate immune responses against this infection. If indeed the immediate responses are paramount against an *S. aureus* infection and the *semmelweis* mutants are highly susceptible, more than PGRP-LC, this indicates that PGRP-SA has a role independent of the TOLL pathway whereby it participates in early immune responses. Accordingly, although *B. subtilis* is non-virulent, the number of bacteria injected is high. Thus, perhaps in the case of a high bacterial load, independently of the pathogenicity of the bacteria, the flies need to rely more on immediate responses, than on late responses. This rationale justifies the fact that, similar to *S. aureus*, upon *B. subtilis* infection the *semmelweis* flies were more susceptible than *PGRP-LC$^{\Delta E12}$* flies (Fig. 7 and 9).

Both WTA mutants of *B. subtilis* and *S. aureus* showed reduced virulence compared to the parental strains (Fig. 11). The *B. subtilis* Δ *tagO* mutant has a drastic loss of virulence since only the homozygote double mutant is susceptible to infection (Fig. 8 and 11). Nevertheless, after 24h p.i. the CFUs inside the Fly increased in the homozygote strains but decreased or stabilised in the heterozygotes (Fig. 8D). This could be due to the fact that when one of the receptors is missing, as the bacteria are not being so efficiently destroyed, it gives time for the bacteria to activate/access mechanisms of survival. It may be through suppressor mutations, and/or the finding of an ecological niche inside the host that allows them to hide from the defences, and/or induction from the environmental stimuli of the PhoP/PhoR system that induces the expression of teichuronic acids [78]–[80]. These hypothesis are not mutually exclusive. Upon infection with *S. aureus* Δ *tagO*, contrary to the parental infection, the homozygotes *PGRP-LC$^{\Delta E12}$* flies were more susceptible than the heterozygotes (Fig. 10C). Thus, only when the WTA are absent, can the role of PGRP-LC be detected. This indicates that indeed

the WTA are shielding the *S. aureus* PGN from PGRP-LC. Furthermore, the results indicate that a more exposed PGN is not turning the bacteria more susceptible to other host components such as lysozymes. If a more exposed PGN would render the bacteria more susceptible to lysozymes, then it should be observed an equal susceptibility between the heterozygotes and homozygotes for both *semmelweis* and *PGRP-LC$^{\Delta E12}$* backgrounds.

WTA impair recognition of Lys- and DAP- type PGN by both PGRP-SA and PGRP-LC, compromising the host survival.

In conclusion, the double homozygous mutant *semmelweis*/*PGRP-LC$^{\Delta E12}$* showed to be more susceptible to infection by any of the bacterial types than the correspondent heterozygote and the single hetero and homozygous mutants (Fig. 7-11 and Table 1). Therefore, both PGRP-SA and PGRP-LC are important for host survival whether by a Lys- or a (ami)DAP- type PGN bacteria. Furthermore, the WTA have a dramatic effect on the virulence of the bacteria. In the case of a bacteria devoid of pathogenicity factors like *B. subtilis*, WTA revealed to be essential for survival in the host. The absence of WTA in *B. subtilis* presents a dramatic impact on the growth and on the shape of the bacteria, which was a main issue during the construction of the mutant strain (Tomasz Czarny, personal communication). The extent to which these factors can influence the virulence of the bacteria upon a septic infection cannot be fully addressed with the work undertaken. Nevertheless, the data here presented shows that the WTA can impair the recognition of different types of PGN by both PGRPs and not specifically from PGRP-SA to Lys-type PGN bacteria [72], [73]. In the same way that the WTA impair accessibility of the PGRPs to the PGN, perhaps the OM exerts the same effect in the Gram-negative bacteria. This hypothesis is in accordance with Leulier and

colleagues [65] when they speculated that the OM could shield PGN from PGRP-LC. Hence, the studies from Michel *et al.* 2001 [1], Ramet *et al.* 2002 [3], Gottar *et al.* 2002 [4] and Choe *et al.* 2002 [5], were possibly the first reports on the effect of the CW composition in impairing PGN recognition. When Michel *et al.* 2001 [1] observed the differential susceptibility between Gram-positive and Gram-negative bacteria in *semmelweis* flies, they were perhaps the firsts to observe the impairment of CW factors on PGRP-SA accessibility to the PGN. They observed that *semmelweis* where more susceptible to a *Bacilli* specie that presents WTA and non-amiDAP-type PGN as they were to a Lys-type PGN bacteria. Thus, in the beginning of the study of PGRPs, there was no indication that the DAP and Lys residues where determinant for bacterial recognition. Accordingly, Chang and colleagues detected binding of rPGRP-SA to purified DAP-type PGN [2]. Choe *et al.* 2002 [5] clearly showed that PGRP-LCx is paramount for the expression of the AMPs induced by IMD pathway, but they did not show evidence for a preference of this receptor to a particular PGN type. In fact they showed that TOLL-dependent AMPs also failed to be expressed to normal levels in the PGRP-LCx mutants. In addition, these mutants, in contrast to the wild-type flies, 6h p.i. showed no detectable levels of *Diptericin* upon infection with the Gram-positive bacteria *M. luteus* and *B. subtilis* and the Gram-negative *Enterobacter cloacae*. These reports corroborate the data here presented and even suggest that there may be a cross-talk between IMD and TOLL that can be mediated by PGRP-LC. Corroborating a cross-talk between IMD and TOLL, Gottar *et al.* 2002 [4] also observed that PGRP-LC mutants did not express *Drosomycin* to the wild-type levels upon an *M. luteus* infection and, in addition, they also showed that albeit the PGRP-LC mutants showed a more dramatic phenotype, the *semmelweis* mutant presented, to some extent, decreased levels of IMD-dependent AMPs upon *E. coli* infection.

Conclusions

The results I present in this chapter are conflicting with the current model of bacterial recognition by PGRPs in *Drosophila melanogaster*. Regarding the recognition of PGN, I show that PGRP-SA and PGRP-LC are promiscuous for the peptide moiety of the PGN since they do not discriminate the Lys from the DAP residue and that PGRP-SA is able to bind to amidated DAP residues in polymeric PGN. This recognition by PGRPs is paramount against infection. PGRP-SA and PGRP-LC not only play roles against infection by both bacterial types but also seem to have redundant roles and to be able to compensate the absence of one another to some extent. When flies are deficient in both receptors, they are highly susceptible to infection, even when the bacteria have exposed PGN at the cell surface like the WTA mutant strains. Accordingly, it appears that CW composition and not the PGN composition determines the efficient detection of bacteria by PGRPs.

Since immune strategies are commonly shared by major bacterial pathogens, it is also intuitive to realise that the evasion mechanism provided by the WTA is transversal to Gram-positive bacteria and to different PGRPs. I propose a model whereby the OM of the Gram-negative bacteria and the WTA of Gram-positive bacteria shield PGN from the recognition by host receptors. Therefore, this model points towards a major role of the WTA, and its functional homologous OM, as conserved bacterial mechanisms of immune evasion. Ultimately, this suggests that classical virulence factors such as toxins might play a secondary role in comparison to the strategies of immune evasion, since whether it is a major pathogen or an innocuous soil bacteria, all of them present a protective barrier at their surface that covers their PGN layer. This corroborates the conclusions of Chapter II, since the data strongly suggests that impairment of PGN recognition is vital for bacterial survival in the

Host and that the virulence factors can promote the success of an infection, for as long as the bacteria can avoid PGN detection.

According to Janeway [81], the evolution of Pattern-recognition receptors (PRR) reaches a tie with the Pathogen Associated Molecular Patterns (PAMP). The PRR detect the conserved and exclusive components of the pathogens whose probabilities of change are greatly reduced. Hence, as the pathogen cannot change these PAMP, it has responded with an array of virulence factors and evasion strategies. It is then quite interesting why would Lys- and DAP- type PGNs be discriminated by PGRP-SA and PGRP-LC: 1) the third residue is the most variable position of the stem peptide and 2) PGRP-SA and PGRP-LC are main receptors of evolutionary conserved immune cascades that if so true, they would have limited capacities in detecting bacteria due to a discrimination of a single molecule. While being promiscuous for the PGN composition, particularly to the variable part of the macromolecule, it implies that the PGRPs are mainly recognizing the most conserved region, *i.e.* the glycans. Therefore, it is likely that these PGRPs might show different susceptibilities to secondary modifications of the macromolecule. In addition, chemically the DAP residue is a derivative of the Lys residue by the addition of a carboxyl group (Fig. 5, Chapter I), and the amiDAP is a modification of the DAP residue. Thus, the evolution of an Immune System bearing two principal PRR, PGRP-SA and PGRP-LC, that would discriminate Lys from DAP, and that one itself, PGRP-SA, would discriminate amiDAP from DAP, appears to be highly unlikely.

Materials and Methods

Bacterial strains and culture.

The plasmid IP15793 (Drosophila Genomics Resource Center) containing the PGRP-LCx isoform, was used to amplify the PGRP domain (region 966 bp to1500 bp of the CDS) with the pair of primers forward and reverse, respectively: "GGGAAGGGAATTCAACCAAACGGACTTGGATG" ; "TGCGGCCGCAAGCTTTTAGTGATGGTGATGGTGATGGATTTCGTGTGACCA GTGCGG" (sequences from 5` to 3`). Subsequently, the fragment was cloned into the EcoRI and EagI restriction sites of the pET21a backbones of the mCherry_PGRP-SA and rPGRP-SA vectors [72]. A 6x His-tag was added to the last coding triplet, before the stop codon, similar to the PGRP-SA constructs.

E. coli strains DH5α and BL21(DE3) (both for construction and expression of the recombinant proteins), *B. subtilis* EB6 and EB6 Δ *tagO* (EB1451) [75] strains were grown either in Luria–Bertani broth (LB; Difco, France)[10] or in Luria–Bertani agar (LA; Difco). *S. aureus* strains NCTC 8325-4 (reference strain from R. Novick) and NCTC Δ *tagO* [72] were grown either in Tryptic Soy broth (TSB; Difco) or in Tryptic Soy agar (TSA; Difco). All cultures were grown at 30ºC with aeration, except when infected flies were crushed and plated for CFUs, the LA and TSA plates were incubated at 25ºC.

[10] All reagents were purchased from Sigma-Aldrich (Germany), except when stated otherwise.

Fly Strains.

All flies, stocks and crosses, were grown on standard cornmeal-agar medium at 25ºC.

For construction of the double mutant *semmelweis*/*PGRP-LC*$^{\Delta E12}$, the I and III chromosome of *semmelweis* flies [1] and *PGRP-LC*$^{\Delta E12}$ flies [4] were balanced through cross with Bl#27336 flies (FBst0027336 from Bloomington Drosophila Stock Center, FlyBase, Genotype TI{TI}Pis^{1}/FM7a; P{hs-Pis.MYC}3/TM2). For each F1 of these two independent crosses, flies with both balancers were selected and the cross between female virgins *seml*/*FM7* and *TM2*/+ with males *FM7*/y and *TM2*/*PGRP-LC*$^{\Delta E12}$ was set. Of this progeny, it was selected female virgin flies that phenotypically presented both balancers and male flies that were not FM7 (hence were *seml* genotype) but presented TM2 phenotype. Several single crosses between such females and males were set. After the laid of the eggs and confirming the viability to larvae, the parents were killed and checked for the *PGRP-LC*$^{\Delta E12}$ gene. Briefly, gDNA was extracted and the *PGRP-LC*$^{\Delta E12}$ was amplified by PCR using the primers "CACACGCTGCCATATCAGAC" and "TATCGGTTTTCCTGGGTGAG" [82]. The PCR fragment of the *PGRP-LC* gene would be 9344 bp but it was not amplified since it was not allowed enough extension time. The *PGRP-LC*$^{\Delta E12}$ gene showed a 212 pb fragment. Only the single crosses of which both female and male showed amplification were kept. The *PGRP-LC*$^{\Delta E12}$ PCR products along with the PCR products of the *semmelweis/PGRP-SA* gene (primers in 5` to 3` sequence "TTAGATCTTAGCACATCAACATC" and "GACTACTGCAATTACTTGTAGTTG") were sent to sequencing to confirm the genotypes. The progeny of females and males that did not present balancers, *i.e.* homozygous for both *semmelweis* and *PGRP-LC*$^{\Delta E12}$ genes were collected. Finally, single crosses between these females and males were set to obtain a homogeneous population of homozygous flies for *seml* and *PGRP-LC*$^{\Delta E12}$. Three different lines were obtained, of which both the

parents and progeny genotype was confirmed by PCR and sequencing. For construction of the single mutants, the respective mutant flies balanced with the Bl#27336 flies were used and it was followed a similar approach as for the double mutant. Thus, the *semmelweis*, the PGRP-LC$^{\Delta E12}$ and the double mutant are in a similar genetic background.

For microscopy of the GFP::PGRP-LCx binding to live bacterial cells, *w* UAS-GFP::PGRP-LCx [76] female virgins were crossed with Hml$^{\Delta}$Gal4.

Purification of recombinant PGRP-SA and PGRP-LC proteins.

The recombinant proteins PGRP-SA and PGRP-LC with and without the mCherry fluorescent tag were purified as described in Chapter II.

Purification of Peptidoglycan.

The CWs and PGNs of *S. aureus* and *B. subtilis* parental and mutant strains were purified and analysed as previously described [31] and the procedure is here briefly described.

Cell wall purification.

Cultures were grown at 30ºC, 180 rpm in TSB or LB, accordingly, and when they reached an OD_{600nm} ~1.0 they were placed in an ice/ethanol bath. This quick chilling of the cultures avoids further activity of lytic enzymes that may modify the CW and PGN and serves in further removing some surface proteins. The cells were harvested and boiled in 4% (w/v) SDS for 30 min. The SDS was washed out using warm ddH_2O by centrifugation. Since the Gram-positive cell wall is resistant to breakage by SDS, the cell walls were mechanically broken (to maintain the peptidoglycan mesh intact from modifications by lytic enzymes). Broken cell walls were resuspended in 50 mM Tris-HCl pH 7.0 with $MgSO_4$ 20 mM final concentration, DNase to 10 µg/mL and RNase to 50 µg/mL and

incubated at 37ºC for 2 h with shaking. Trypsin at 100 µg/mL (Worthington Biochemical Corp., USA) and $CaCl_2$ at 10 mM final concentrations were added and incubated at 37ºC overnight (O/N). Trypsin degrades the enzymes added before and the cellular proteins. $CaCl_2$ prevents auto-degradation of trypsin, thus guarantees that the enzyme remains active cleaving all proteins/enzymes present in the suspensions. SDS to 1% (w/v) final concentration was added and the mixes were boiled for 15 min. After harvest of the suspensions, pellets were treated with 8 M LiCl, 100 mM EDTA pH 7.0 and pure Acetone-ultrasound water bath. These treatments remove any non-covalently linked compounds that may be bound to the cell walls. After washing with ddH_2O, the final resuspension is made in MilliQ H_2O and transferred into pre-weighted tubes and lyophilised O/N (without heat) in a Speed Vac apparatus. The pure cell wall dry-weight is calculated and they are adjusted to a final concentration of 20 mg/mL with MilliQ H_2O. They can be stored for long periods of time at -20ºC.

PGN purification.

PGN was purified by incubation the CWs with 46% Hydrofluoric Acid (HF) in a ratio of 2 mL of HF per 10 mg of CW, at 4ºC for 48 h, stirring. Under these conditions, HF cleaves the phosphodiester bonds between the wall teichoic acids and the Mur*N*Ac residues. As a non-controlled secondary reaction, it is likely that the acetate is lost from *O*-acetylated Mur*N*Ac of the *S. aureus* PGN, because the ester bond of *O*-linked acetate is significantly weaker than the amide bond of N-linked acetate, even in mild alkaline or acidic conditions [83]. After HF treatment, the pure PGN was washed with 100 mM Tris-HCl pH 7.0 until complete removal of the acid and the pH values of the supernatants were neutral. Finally, the purified PGNs were resuspended in MilliQ H_2O and transferred into pre-weighted tubes and lyophilised O/N (without heat) in a Speed Vac apparatus. The

dry-weight was determined and the PGNs were suspended in MilliQ H_2O to a final concentration of 10 mg/mL. They can be stored for long periods of time at -20ºC.

Separation and analysis of the muropeptides.

50 µL of PGN (10 mg/mL) was incubated in 50 µL of 25 mM $NaHPO_4$ pH 5.5 and 2 µL of Mutanolysin at 10 mg/mL, O/N at 37ºC, 1200 rpm. Mutanolysin is a muramidase more robust than most Lysozymes to glycan modifications. 100 µL of MilliQ H_2O was added to the samples followed by 5 min boiling to denature the mutanolysin. The suspensions were centrifuged to remove the denatured mutanolysin and any polymeric PGN that was still insoluble. The supernatants were collected into a fresh tube to which it was added 200 µL of 0.5 M Borate Buffer pH 9.0 with 25 µL of fresh 50 mg/mL $NaBH_4$ and incubated at room temperature (RT) for 2 h. The reduction of the muropeptides ensures that the PGN species will be latter on separated regarding the size without charge interferences. The reaction is stopped by lowering the pH to 2.0 with 85% ortho-phosphoric acid. Finally the samples were centrifuged and the supernatants were collected into a fresh tube and stored at -20ºC until further use. The muropeptides were separated in an HPLC system using a Reverse Phase C-18 Hypersil column (ODS-Hypersyl, 5 µm, 4.6×250 mm Thermo Scientific) at a flow rate of 0.5 mL/min for 160 min with a Methanol gradient from 5% to 30% (v/v) in 100 mM NaH_2PO_4 pH 2.0. The eluted muropeptides were detected by UV absorption at 206 nm.

Quantification of Peptidoglycan using HPAEC-PAD.

Sugar components of the purified PGNs were analysed using HPAEC-PAD (High Performance Anion Exchange Chromatography coupled with Pulsed Amperometric Detection). After quantification of the

Glucosamine and Muramic acid in each PGN sample, the final PGN suspensions were adjusted to the same amount of Muramic acid for further studies.

Hydrolysis of PGN into its basic constituents.

Three repeats for each PGN sample and three different batches of quantification were performed because as PGN is insoluble in aqueous solution, these repeats minimise potential errors in the quantification. The pure PGN suspension was left O/N stirring at RT for maximum homogenization. 20 µL of the suspensions were hydrolysed in 3 M HCl at 95ºC for 2 h (150 µL reaction volume). The hydrolysed suspension was lyophilized until it was completely dried and resuspended in 500 µL of MilliQ H_2O and lyophilized O/N. Finally, the acid-free hydrolysed material was resuspended in 150 µL MilliQ H_2O.

Separation of the monosaccharides.

10 µL of the samples were injected in a Thermo Scientific™ *Dionex*™ *ICS-5000* system, in 18 mM NaOH constant and a gradient of 1 M $NaCH_3COO$ and MilliQ H_2O. The MilliQ H_2O as all the eluents were in MilliQ H_2O of resistivity ≥ 18 M**Ω**, filtered with 0.2 µm ϕ pore filter and degassed for 15 min in an ultrasonic bath. Between the eluents pump and the injection valve it was used a BorateTrap™ column to remove borate contamination from eluents. An AminoTrap™ (Thermo Scientific™ Dionex™ AminoTrap™) was used as a pre-treatment column to remove the amino acids from the samples, thus only the sugars passed to the CarboPac PA10 column (Thermo Scientific™ CarboPac™ PA10) where they were separated. Each injection was done in triplicates.

Control samples.

Controls for the HCl hydrolysis.

To access if the hydrolysis had been complete, it was injected non-HCl treated Glucosamine, Muramic acid, *N*-acetylglucosamine and *N*-acetylmuramic acid. If the hydrolysis was complete, only Glucosamine and Muramic acid should be detected because the *N*-acetyl groups were removed by the acid treatment. Also these same samples HCl treated were injected, to determine if the hydrolysis had not destroyed the sugar molecules. After HCl treatment, only Glucosamine and Muramic acid should be detected and the areas of the peaks should be similar to the areas of the non-hydrolysed Glucosamine and Muramic acid standards because, theoretically, it was injected the same concentration for all of them.

Controls to confirm the purity of the PGN.

Injection of 1 mM of Ribitol, Glycerol and Mannosamine (HCL and non-HCL treated) to determine whether the PGN was indeed pure or still had WTAs attached.

Controls for quantification of Glucosamine and Muramic acid.

Injection of fresh solutions of Glucosamine and Muramic acid, at different concentrations - 1 mM followed by 1/2 dilutions until 31.25 μM. The area of these peaks in the chromatogram were used to plot the calibration curve that if $r^2 \sim 1$, the equation was used for the quantification of Glucosamine and Muramic acid in each PGN sample. It is then assumed that all the molecules of *N*-acetylglucosamine and *N*-acetylmuramic acid were converted into Glucosamine and Muramic acid, respectively.

Quantification of Glucosamine and Muramic acid.

The area of each peak corresponding to either Glucosamine or Muramic acid was quantified using the Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System software and thus the correspondent number of moles was calculated. PGN suspensions to be used in the co-precipitation assays were made in MilliQ H_2O and they were diluted to the same number of moles of Muramic acid of the PGN that was more diluted.

Co-precipitation assays.

The co-precipitation or pull down assays intended to determine the binding affinity of the PGRPs to both pure PGN and to live cells, *i.e.* whole bacteria. The PGN pull down assays are quantitative assays performed with the non-fluorescent tagged rPGRPs and analysed by SDS-PAGE [84]. Due to the non-solubility of the PGN in aqueous solutions, the amount of protein that binds is harvested with it. The bacterial binding assays are qualitative and were performed with the mCherry_PGRPs for microscopy visualisation.

PGN co-precipitation assays.

PGN suspensions at $2.89x10^{-1}$ nmol of Muramic acid/µL of PGN were thoroughly mixed and 20 µL were taken and spun down for 3 min at RT, 16.1 x1000 *g*. The supernatants were carefully removed, in particular the NCTC Δ *tagO* who's PGN does not pellet well like the others and hence it is easily aspirated. To the pellets it was added 0.3 mg/mL final concentration of either PGRP-SA or PGRP-LC in a 200 µL reaction volume, filling up the volume with the reaction buffer (20 mM Tris-HCl pH 8.0; 300 mM NaCl or PBS 1X pH 6.0 or Hemo Buffer – 7.3 mM $Na_2SO_4.10H_2O$; 34 mM $NaH_2PO_4.H_2O$; 5 mM $Na_2HPO_4.7H_2O$; 25 mM KCl; 2 mM $CaCl_2$; 14.4 mM

$MgCl_2.6H_2O$; pH 6.0-6.2 adjusted with NaOH). The mixes were incubated at 25ºC for 30 min, without shaking and then centrifuged for 5 min at RT 0.8 x 1000 *g*. The pellets were washed with 200 µL of reaction buffer and centrifuged for 5 min at RT, 3.2 x 1000 *g*. The pellets were finally resuspended in 20 µL of 2X SDS Loading Buffer and boiled for 5 min. The samples were centrifuged for 3 min at RT, 16.1 x 1000 *g*. The supernatants were recovered (20 µL) into a fresh tube. This collection step allows the loading on the gel to be quick, clean and guarantee that only the supernatant and not bits of PGN were loaded, which is important for comparing and quantifying the binding affinities. The full supernatant volume was loaded on a 12% SDS-PAGE gel. The bands were visualized by Coomassie Blue Staining and both imaging and quantification were performed using a Gel Doc™ EZ Gel (Bio-Rad, USA). For quantification purposes of the bindings, each experiment was repeated in two different days, with two different batches of buffers (made fresh). The error bars represent the mean with standard deviation. The data was plotted and analysed using GraphPad Prism 5 (GraphPad Software, Inc.).

Live bacteria co-precipitation assays.

The binding assays of mCherry_PGRP-SA and mCherry_PGRP-LC with live cells were conducted as before, expect that it was used Hemo Buffer instead of PBS 1X pH 6.0. The agarose slides were performed as before because it does not dissolve in Hemo buffer. Images were acquired with a GE Healthcare DeltaVision Elite integrated imaging system, in the conventional mode at 25ºC using an Olympus 150x 1.45 NA TIRF Objective (Olympus, USA).

Survival curves and CFUs.

Bacterial cultures were grown overnight and the OD_{600nm} was adjusted with the appropriate medium culture as follows: *B. subtilis* 5.0, *B. subtilis* Δ *tagO* 7.0, *S. aureus* 0.5 and *S. aureus* Δ *tagO* 0.7. 500µL of these cultures was centrifuged at RT for 10min, 3380 x *g*. The cells were washed once with 1mL of PBS and resuspended in 500µL of PBS. The suspensions of the *S. aureus* strains were further diluted 1/100 in PBS. 32.2 nL of bacterial suspension was injected in thorax of 3-5 day old flies using a nanoinjector (Nanoject II; Drummond Scientific, Broomall, PA). The infected flies were kept at 25ºC and monitored for 72h every 12h. Estimated survival curves were analysed using the Log-rank (Mantel-Cox) test to determine statistical significance between the curves. For determination of the CFUs, infected flies were collected at different time-points, homogenized and plated in the appropriate media and incubated at 25ºC for 24h-48h. The number of flies infected for survival curves following the order *semmelweis*, *PGRP-LC$^{\Delta E12}$*, *semmelweis*/*PGRP-LC$^{\Delta E12}$*, *semmelweis* heterozygote, *PGRP-LC$^{\Delta E12}$* heterozygote, *semmelweis*/*PGRP-LC$^{\Delta E12}$* heterozygote were: *B. subtilis* – 181, 92, 182, 98, 63, 93; *B. subtilis Δ tagO* – 181, 91, 180, 91, 92, 90; *S. aureus* – 127, 93, 125, 93, 93, 94; *S. aureus Δ tagO* – 97, 126, 97, 95, 94, 96. CFUs were determined for at least 2 independent infections and the statistical analysis was performed by Unpaired Student´s-t test. The error bars represent the mean with standard deviation. All data was plotted and analysed using GraphPad Prism 5 (GraphPad Software, Inc.).

Time-lapse microscopy.

Macrophage preparation.

Three third instar larvae were washed in 1 mL of ddH_2O followed by 50% (v/v) bleaching and vortex. The larvae were quickly washed three

times with autoclaved MilliQ H_2O and left swimming whilst the slides were prepared. Into previously washed metal slides (100% (v/v) Ethanol) with a 1.0 mm coverslip it was put Schneider medium supplemented with 5% (v/v) Fetal Calf Serum. The larvae were bled into the medium and the macrophages were let settle for 1h at RT in a humid chamber.

Preparation of the bacteria.

200 µL of an O/N culture were harvested at RT and washed with Schneider medium. The cells were resuspended in 200 µL of Schneider with 0.5 µL of a DAPI solution (at 1 mg/mL in MilliQ H_2O) and incubated for 5 min at RT, without shaking. The cells were washed with 200 µL of Schneider and resuspended in 100 µL of Schneider. Finally, they were dilute 1/10 in Schneider in a 200 µL final volume.

Imaging of the macrophages with the bacteria.

To the macrophages slide, 200 µL of the bacterial suspension was added and an YSI 5775 Standard Membrane (YSI Incorporated, Japan) was glued on top. Acquisition was done at 25ºC in a humid chamber for 12 h every 15 min in a GE Healthcare DeltaVision Elite integrated imaging system, using an Olympus MPLAPON-Oil immersion objective (100X 1.40A).

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CHAPTER IV

PGRP-SA is involved in bacterial clearance

Contributions to this Chapter:

Filipa Vaz[1,2] performed all the experimental procedures, designed the experiments and analysed the data.

Richard Parton and Ilan Davis[1] provided the *GFP-Moesin* flies, helped in the development of the time-lapses microscopy regarding both biological and microscopy techniques. Participated in the analysis of the microscopy data.

Sergio Filipe[2] and Petros Ligoxygakis[1] designed the experiments and analysed the data.

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Publications in this Chapter:

F. Vaz and S.R. Filipe, "Preparation and Analysis of Crude Autolytic Enzyme Extracts from *Staphylococcus aureus*," *Bio-protocol,* vol. 5, Iss 24, pp. 1–12, 2015.

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Dr. Maria João Gravato-Nobre, Department of Biochemistry University of Oxford, for letting me do experiments with PGRP-SA whilst optimizing/performing experiments for her paper (M.J. Gravato-Nobre, F. Vaz, S.R. Filipe, R. Chalmers and J. Hodgkin, "The invertebrate lysozyme effector ILYS-3 is systemically activated in response to danger signals and confers antimicrobial protection in *C. elegans," Plos Pathog*, 15;12(8):e1005826), 2016). It was with these side experiments that I could gather the data that put me in the right track to (finally) prove the lytic activity of PGRP-SA and design my own assays to conclude this study.

Dr. Alberto Baena, Sir William Dunn School of Pathology University of Oxford, for advice and the help in analysing the behaviour and death of the macrophages.

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Summary

The data presented in Chapters II and III suggest that PGRP-SA plays a TOLL-independent role in early immune responses. Results from Chapter II allow to infer that recognition of PGN by PGRP-SA can aid in the clearance in an AMP-independent manner. Survival data from both Chapters II and III, show that the flies succumb early in infection, particularly before or coincident with the reach of the AMPs synthesis. Thus, an efficient response appears to be dependent in immediate constitutive responses of clearance. Furthermore, flies lacking a functional PGRP-SA showed reduced survival compared to flies lacking PGRP-LC, which suggests that PGRP-SA has a particular role associated with these immediate responses. Thus, I aimed to understand whether PGRP-SA is involved in phagocytosis. Here, I show that PGRP-SA participates in phagocytosis and clearance of both Lys- and DAP- type PGN bacteria, together with PGRP-LC. I show that PGRP-SA has optimal binding and lytic activity at low pH, suggesting that it is active inside the phagolysosomes. Moreover, I show that the WTA impair the phagocytosis-clearance process by shielding the PGN from these PGRPs.

In addition, I demonstrate that the macrophages present two ways of clearance upon phagocytosis. One is the already established canonical route of phagocytosis-exocytosis. The other is the induction of apoptosis upon phagocytosis. This later strategy appears to be employed when the macrophages find hard to control and destroy the bacteria, whether due to pathogenicity factors whether due to a high bacterial load which unbalances the rate of phagocytosis and clearance. Finally, the process of apoptosis to promote clearance is impaired when the macrophages are compromised both in PGRP-SA and PGRP-LC. Thus, this apoptotic process is triggered by factors associated with the clearance of the bacteria.

Introduction

Phagocytosis and *Drosophila* immunity

Etymologically, phagocytosis is a Greek word that comes from "phagein" – to eat – , "kytos" – a big animal – and "-osis" that means process. Thus, phagocytosis is the process by which a cell engulfs large particles, usually over 0.5 μm in diameter. This primordial and fundamental process present from unicellular to multicellular organisms was first described by Élie Metchnikoff in 1882. This discovery rendered him and Paul Elrich the Nobel Prize in 1908. Phagocytosis is paramount for homeostasis, because it is the first step of clearance. In development and tissue remodelling, phagocytosis is the final step of the apoptotic process [1], [2] and in immune responses, clearance occurs upon phagocytosis of the microorganism. Metazoans present cells that specifically perform phagocytosis, the professional phagocytes, such as the macrophages, which patrol the host to promote clearance of dying cells and microorganisms. Therefore one can assume that macrophages are primordial components of the Innate Immune System as they or their homologues are found in all Metazoans. The *Drosophila melanogaster* blood cells, haemocytes, are responsible for phagocytosis.

Phagocytosis is initiated upon the interaction of opsonins[3] at the surface of the particle with receptors at the surface of the macrophage. Upon phagocytosis, the internalized particle will be destroyed through a sequential process of engulfment, destruction and excretion (Fig. 1). The macrophages are composed by lamellipodia and filopodia. The lamellipodia are extensions of the actin cytoskeleton to the outer of the cell

[3] Any component that binds to a cell and turns it more susceptible to be phagocytosed.

surface. They propel the macrophage or anchor them onto a surface. The filopodia are cytoplasmic projections that extend beyond the lamellipodia. They participate in the sensing of chemicals and other stimuli from the surrounding environment, in macrophage movement and migration and in the engulfment of pathogens. Thus, all phagocytic signalling pathways activate cytoskeleton-remodelling molecules and the dynamics of the cytoskeleton is exploited in order to achieve particle internalisation. For instance, the *Drosophila* TM9SF4 is important for bacterial phagocytosis due to its function in the morphology of cell adhesion and actin cytoskeleton [3]. Different phagocytic systems show different cytoskeleton requirements and are composed of a series of ligands, receptors and signalling pathways.

The history of phagocytosis is intimately associated with Immunology and the understanding of host resistance to infection. Conversely, pathogens developed different mechanisms to evade or subvert the phagocytosis or the clearance. Many microorganisms have conceived strategies of evasion whether by remaining extracellular or invading other type of host cells. Others have found ways to not only survive but even to replicate within macrophages. While it may be advantageous for an intracellular pathogen to preserve the function of the host cell for its replication, most intracellular pathogens can actively induce programmed cell death, such as apoptosis.

Phagocytosis participates in host defences through the uptake and clearance of infectious agents and contributes to inflammation. It is a type of cellular response that in Insect Immunity it belongs to the constitutive and early immune responses. Phagocytosis is conducted by the *Drosophila* blood cells, haemocytes, which have been successfully used as a model of 'professional' mammalian phagocytosis [4].

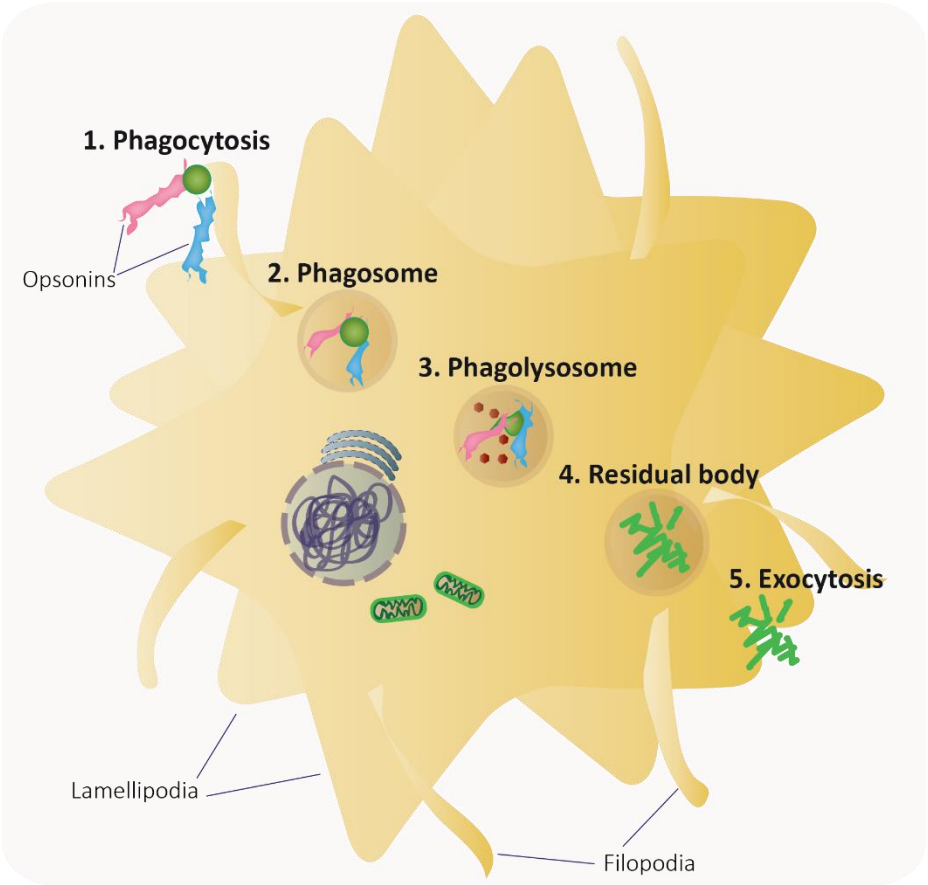

Figure 1. Steps of the phagocytosis-exocytosis process. 1. Phagocytosis, *i.e.* internalization of the particle through engulfment; 2. Formation of the phagosome by the fusion of the cell membrane around the particle; 3. Fusion of the phagosome with a lysosome that contains hydrolytic enzymes resulting in the phagolysosome containing the particle to be digested and the enzymes at a low pH. It is within this vesicle that the particle is destroyed, *i.e.* the clearance occurs; 4. Upon clearance, the residual body contains the debris and undigested material; 5. Exocytosis with release of the residual body contents to the outside of the cell.

There are three types of functional haemocytes that have different roles throughout the developmental stages and respond to environmental stimuli: plasmatocytes, crystal cells and lamellocytes. Plasmatocytes represent ca. 90% of the total circulating haemocytes in all developmental stages. They are involved in phagocytosis of apoptotic bodies and of pathogens. They are the *Drosophila* professional macrophages and in this Chapter, I will address them as macrophages. Crystal cells participate in

the melanization process and engulfment of parasites whose size is too large to follow a canonical phagocytosis route. These two types of haemocytes circulate in the haemolymph and/or can be found sessile. Lamellocytes participate in the encapsulation of foreign bodies that are too large to be phagocytosed and are exclusively differentiated in response to specific immune challenges, such as wasp parasitism.

Several genes involved in phagocytosis, both receptors and components involved in actin-cytoskeleton and cell-cell contact have been identified in *Drosophila*. Regarding the phagocytic receptors, some of the ligands are yet to be identified. Up to date, ca. 12 phagocytic receptors have been identified: Croquemort [5], [6], Draper [7], and Nimrod C4/Six-microns-under (NimC4/SIMU) [8], Eater [9], Nimrod C1 [10], Down syndrome cell adhesion molecule (Dscam) [11], Peste [12], *Drosophila* scavenger receptor class C, type I (dSR-CI), Integrin βυ [13], [14] , Integrin αPS3/βυ [15], PGRP-LC [16] and PGRP-SC1 [17].

Phagocytosis and Programmed Cell Death

It is thought that macrophages were primordially involved in removal of apoptotic bodies and their role in pathogen uptake is an adaptive function [5]. In accordance to this line of thought, it has recently been shown that macrophages require priming through phagocytosis of apoptotic corpses to function in response to foreign bodies [18]. Indeed, programmed cell death (PCD) is vital in multicellular organisms for development and homeostasis and thus the clearance of such dying cells is essential (Table 1). The concept of PCD was defined in 1964 by Lockshin and Williams, as a process that occurs in predictable places at predictable times during embryogenesis, implying that cells are programmed to die during the development of the organism [19]. Later, Kerr *et al.* 1972 described the morphological changes of cell death during development

and proposed the term apoptosis. Three morphologically distinct types of physiological cell death were identified in tissues of mouse and rat embryos [20]: type I (apoptotic cell death), type II (autophagic cell death), and type III (necroptotic cell death).

Table 1. Types of Programmed Cell Death.

	Apoptosis	Necrosis	Autophagy
Concept	Active programmed process of autonomous cellular dismantling that avoids eliciting inflammation.	Passive process lacking underlying signalling events and resulting from environmental perturbations with uncontrolled release of inflammatory cellular contents. Necroptosis: programmed necrosis. Pyroptosis: inflammatory cytokines.	Degradation of cellular components within the dying cell in autophagic vacuoles.
Morphological changes	- Cellular shrinkage with condensation of the cytoplasm. - Sharply delineated chromatin lying against the nuclear membrane. - Nuclear fragmentation (karyorrhexis). - Membrane-confined apoptotic bodies containing a variety of cytoplasmic organelles and nuclear fragments. - Mitochondria appear to be normal or shrunken rather than dilated or swollen.	- Swelling of the organelles and the cytoplasm (oncosis). - Collapse of the plasma membrane and lysis of the cells	Presence of autophagic structures with a double membrane: autophagosomes. - Vacuolization. - Degradation of cytoplasmic contents. - Slight chromatin condensation.
Outcome	Apoptotic bodies engulfed by phagocytes.	Recognition by phagocytes is less clearly understood than recognition of apoptotic cells.	If cellular stress continues, cell death may continue by autophagy or develop apoptotic or necrotic features.

Apoptosis is an active mode of cell death in the sense that it is a death process that is initiated by the cell in a well-orchestrated and controlled manner. Paradoxically, it is a passive and peaceful mode of death because it evokes minimal inflammation and avoids disturbance to the neighbouring cells and onto the surrounding medium. It is the type of PCD that cells undergo during development, tissue remodelling and wound healing. As such, the apoptotic cell death (type I cell death) is characterized by a sequence of specific morphological changes. There is condensation of the organelles and cytoplasm, thus the cell volume decreases and the cell density increases [21]. The cell membrane starts to form buds, the budding process, and it is accompanied with shrinkage of the entire nucleus and DNA fragmentation or karyorrhexis, which is a hallmark of apoptosis. It is commonly observed sharply delineated chromatin masses lying against the nuclear membrane and persistence of the nucleolar structure until

very late stages [22]. Also, there is loss of specialized surface structures such as microvilli and cell–cell contacts. Subsequently, there is the formation of membrane-confined apoptotic bodies containing a variety of compacted but well-preserved cytoplasmic organelles and nuclear fragments [23]. Phagocytic ingestion is the ultimate fate of cells undergoing apoptosis. Ingestion of apoptotic cells occurs very rapidly to prevent the exposure of surrounding cells and tissues to any potentially harmful contents [20]. In the absence of phagocytosis, apoptotic cells proceed to a stage called secondary necrosis. Secondary necroptotic cells resemble necrotic cells, but differ in having gone through an apoptotic stage, thus their nuclei are fragmented or condensed.

Necrosis is often considered a passive process since it lacks the signalling events of controlled cell death and it is a rapid and sudden type of death. In opposite to apoptosis, the cells burst and release in an uncontrolled manner all of the content to the surrounding medium. Thus, it induces strong inflammation and stress to the neighbouring cells. It typically occurs under extreme physicochemical conditions. However, there is growing evidence that there are types of programmed necrosis, designated necroptotic cell death. It appears it may be as well controlled and programmed as apoptotic cell death and that it results from extensive cross talk between several biochemical and molecular events at different cellular levels [24]. A particular type of necroptosis is pyroptosis, which is a death by necrosis that is highly inflammatory and generally occurs after infection of intracellular pathogens. Necrosis (type III cell death) is characterized by swelling of the organelles and the cytoplasm, followed by collapse of the plasma membrane and lysis of the cells [20]. The cytoplasm becomes increasingly translucent and, finally, the plasma membrane is disrupted, and cell contents start to leak out, hence causing inflammation. As a consequence of the prominent swelling of the cytoplasm, this type of cell death is also designated as oncosis [25].

Autophagy (type II cell death) is characterized by the presence of double membrane autophagic vacuoles [20], [26]. It is most prominently a survival mechanism activated in cells undergoing different forms of cellular stress. When the cellular stress continues, cell death may continue by autophagy alone, or may develop apoptotic or necroptotic features [27].

Is PGRP-SA involved in phagocytosis?

Taking into account the data presented in the previous Chapters, *Staphylococcus aureus* kills the flies very rapidly. Its pathogenicity is probably attributed to its arsenal of virulence factors. Nevertheless, upon an *S. aureus* infection one must consider that perhaps what is paramount for Host survival are processes involved in the immediate immune responses. The data presented in Chapter II, shows that the reason for the reduced virulence of the *atl* mutant is due to a better recognition of PGN by PGRP-SA which may be promoting clearance in an AMP-independent manner. Furthermore, as observed in Chapter III, the mutant flies *semmelweis* and *semmelweis*/*PGRP-LC*$^{\Delta E12}$ succumb earlier than the heterozygotes flies upon infection (Fig. 7 of Chapter II) and in a manner much more dependent on PGRP-SA than PGRP-LC (Fig. 7 and [28], [29]). Therefore, if immediate immune responses are key against *S. aureus* infection and PGRP-SA is particularly important for host survival, then the results suggest that PGRP-SA is involved in immediate early responses. Accordingly, it has been reported by the Host groups, that the *semmelweis* flies are more susceptible to an *S. aureus* Δ *tagO* infection than *Dif-Key* flies, which are flies impaired in AMP expression [28]. So far, this topic has been overlooked in *Drosophila* immunity and the focus on the late AMPs expression and induction of the immune cascades has been extensively studied using several different bacterial types. In addition, PGN has been

shown to be required for phagocytosis of *S. aureus* [14] and two PGRPs have been identified as phagocytic receptors [16], [17].

In sum, the data points towards the importance of immediate responses that are dependent on PGRP-SA. I aimed to understand the impact of cellular immunity and particularly whether PGRP-SA plays a role in such responses. Therefore, I studied whether this PGRP is involved in phagocytosis, since PGRP-LC has already been identified as a phagocytic receptor [16]. In addition, PGRP-LC was identified as being exclusively involved in phagocytosis of *Escherichia coli* due to its specificity to DAP-type PGN. As in the previous Chapter, I demonstrated that such specificity does not exist (for both receptors), I also aimed to understand if in the absence of WTA, *i.e.* with a more exposed PGN at the cell surface, both PGRP-SA and PGRP-LC could play roles in phagocytosis, similar to the recognition and activation of the immune cascades.

I performed *ex vivo* time-lapse microscopy studies using *D. melanogaster* macrophages. Moesin belongs to the conserved family of ERM (ezrin-radixin-moesin) proteins. They function as molecular linkers between the actin cytoskeleton and the transmembrane receptors, but also localise at the nucleus [30]. Therefore, tracking Moesin localisation allows to follow the dynamics of the phagocytic processes at the cell membrane. Flies expressing a *GFP-Moesin* fusion have been used to study changes in cell shape during developmental stages [31]. Hence, I used *GFP-Moesin* larvae as a reference for a wild-type background.

To study the effect of both PGRP-SA and PGRP-LC, I used larvae from the crosses of female *semmelweis* or *semmelweis*/*PGRP-LC*$^{\Delta E12}$ virgin flies with *GFP-Moesin* male flies. The larvae collected for these experiments regarding the role of the PGRPs were male larvae. Thus, the macrophages extracted were homozygous for *PGRP-SA*^{seml}, since the gene is present in chromosome I and heterozygous for *PGRP-LC* (chromosome III).

To address whether both PGRPs could also be promiscuous in phagocytic processes for the Lys- vs. DAP- type PGN, I used the same mutant bacterial strains of *S. aureus* and *Bacillus subtilis*. Lastly, if indeed cellular responses are vital against infection from the parental strains, then perhaps the WTA may also be involved as immune evasion mechanisms against phagocytosis and bacterial clearance.

Briefly, third instar larvae were bled and the haemolymph was collected. The macrophages were allowed to settle and attach to the microscope slide (Video 1A). Afterwards, they were infected with the bacteria and imaged for 18h every 3min acquisition (Video 1B). The macrophages were visualised under the FITC channel for imaging of the GFP-Moesin which allowed to follow the phagocytic processes and the outcome upon infection (shown in green). The bacteria were previously stained with DAPI (shown in purple-pink) and when complementation experiments were conducted, it was added the TRITC channel for visualization of the mCherry_PGRP-SA protein (shown in blue). Unfortunately, mCherry_PGRP-LC showed to be toxic to the macrophages, thus it was not possible to perform complementation studies of this receptor in the *semmelweis/PGRP-LC*$^{\Delta E12}$ macrophages (data not shown).

A. **B.**

Macrophage.mov

Macrophage with bacteria.mov

Video 1. Macrophages attached to the microscope slide in Schneider media or infected with bacteria. Video A. shows fixed macrophages attached to the slide through their lamellipodia. Filopodia are typically observed upon movement of the macrophages or during the engulfment of particles when they extend as “arms” to grab the material to be internalised, as it can be seen in Video B. when they are in the presence of bacteria.

Results

Wild-type bacteria induce a fast mode of death in macrophages.

Upon infection with *S. aureus*, the macrophages quickly show signs of high levels of cellular stress. They appear to enter a route of apoptotic death (Video 2 and Fig. 2). As described in Table 1, this type of cellular death is characterized by shrinking of the cell volume and karyorrhexis. On the other hand, *B. subtilis* are innocuous bacteria devoid of such virulence factors. Although it does not induce such a fast death of the macrophages like *S. aureus*, it was still observed the beginning of an apoptotic death, suggested by the shrinking of the cells (Video 4 and Fig. 2). In contrast, in the absence of bacteria, the macrophages did not enter a route of cellular death (Video 6). When macrophages were infected with heat-killed *S. aureus* cells, there were no signs of induction of cellular death of the macrophages. The macrophages appeared healthy and able to control the infection through a canonical route of clearance phagocytosis-exocytosis after 18h post infection (p.i) (Video 7). When death of the macrophages happened, it appeared to be due to other factors than the presence of bacteria.

WTA mutant bacteria are less virulent to the macrophages.

The absence of WTA rendered the bacteria more susceptible for phagocytic-clearance processes. In the case of *S. aureus* Δ *tagO*, the macrophages survived for a longer period and initiated later a cellular death (Video 3 and Fig. 2). With the parental bacterial strain, the macrophages death started shortly after infection, ca. 1h15 p.i., whereas with the *S. aureus* Δ *tagO* mutant it was detected beginning of death ca. ~4h30 p.i.. As for *B. subtilis* Δ *tagO*, the macrophages did not die nor

presented any indication of cellular stress, in contrast to an infection with the parental strain (Video 5 and Fig. 2).

GFPMoe_Saureus.mov

Video 2. *GFP-Moesin* macrophages infected with *S. aureus* bacteria.

GFPMoe_Saureus_tagO.mov

Video 3. *GFP-Moesin* macrophages infected with *S. aureus* Δ *tagO* bacteria.

GFPMoe_Bsubtilis.mov

Video 4. *GFP-Moesin* macrophages infected with *B. subtilis* bacteria.

GFPMoe_Bsubtilis_tagO.mov

Video 5. *GFP-Moesin* macrophages infected with *B. subtilis* Δ *tagO* bacteria.

GFPMoe_Schneider.mov

Video 6. *GFP-Moesin* macrophages in Schneider media.

GFPMoe_HeatKilled_Saureus.mov

Video 7. *GFP-Moesin* macrophages infected with heat-killed *S. aureus* cells.

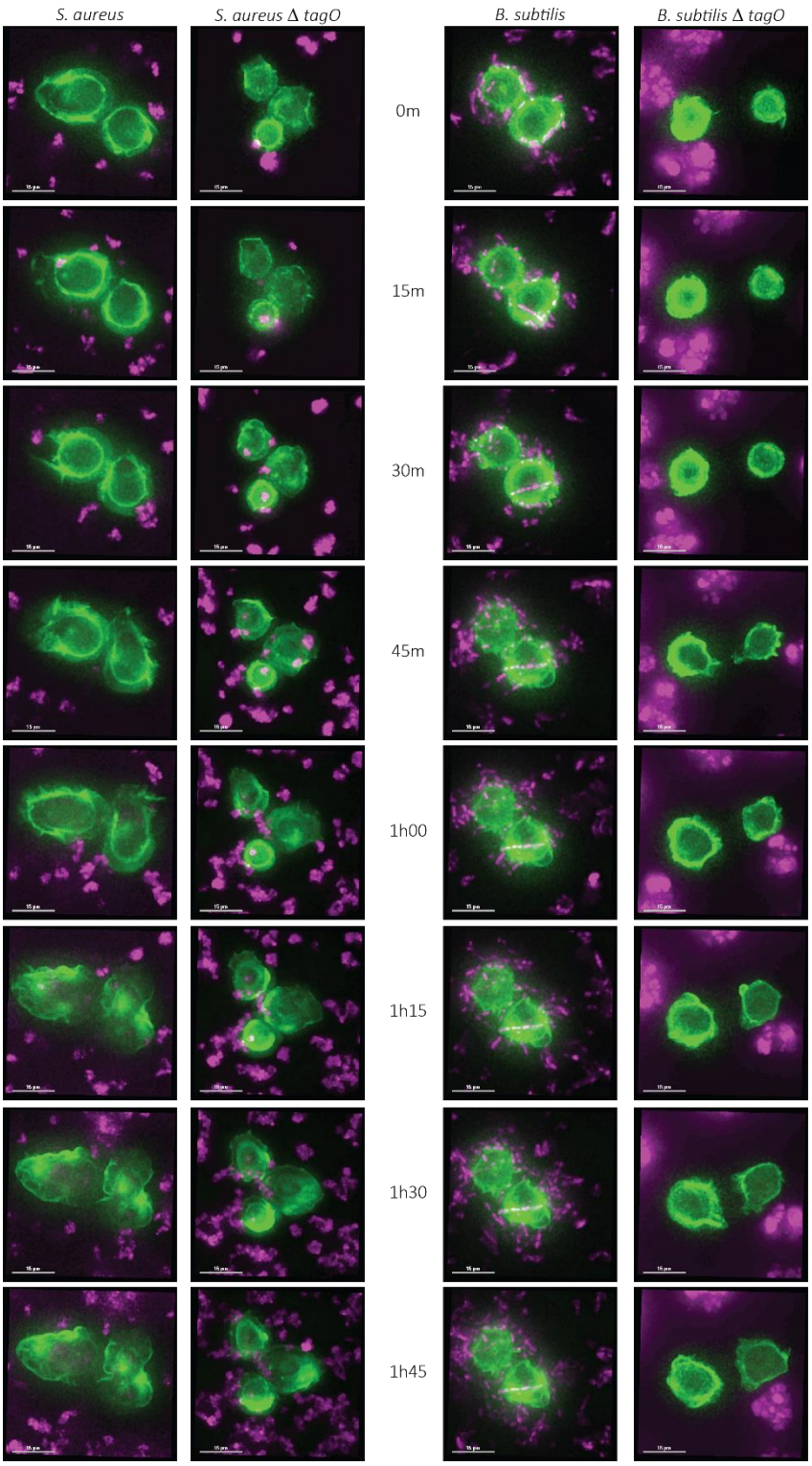
GFP-Moesin macrophages
S. aureus
S. aureus Δ tagO
B. subtilis
B. subtilis Δ tagO
0m
15m
30m
45m
1h00
1h15
1h30
1h45

Figure 2. Snapshots of *GFP-Moesin* macrophages infected with bacteria. It is shown snapshots of every 15min acquisition of the macrophages infected with bacteria from the videos 2-5, from time points 0-1h45 p.i.. It is clear that infection with *S. aureus* is very virulent to the macrophages when compared to any other bacteria. After 1h15 they cease the dynamics of the filopodia and actin cytoskeleton. On the contrary, the *B. subtilis* Δ *tagO* mutant is devoid of virulence since the macrophages remain healthy and start spreading to engage in the phagocytic processes.

PGRP-SA appears to be involved in bacterial clearance.

When *semmelweis-GFP-Moesin* macrophages were infected with each of the bacteria, I observed the same outcome of the macrophages, albeit the mutant macrophages appeared less healthy and succumbed to infection earlier than the *GFP-Moesin* macrophages (Videos 8-11 and Fig. 3). Upon infection with *S. aureus* and *S. aureus* Δ *tagO*, the macrophages seem less dynamic in their movements of the filopodia and actin cytoskeleton (Videos 8 and 9). This may be an indication that perhaps there is some impairment in the canonical route of phagocytosis of these bacteria and there is an early triggering of an apoptotic death. An infection with *B. subtilis* strongly triggered death of the macrophages, when compared to the wild-type background *GFP-Moesin*, and some macrophages do not survive the 18h of the time-lapse experiment (Video 10). Regarding infection with *B. subtilis* Δ *tagO*, although I could not observe cellular death, some macrophages presented signs of cellular stress: they ceased to be as dynamic as normal, over-expressed *GFP-Moesin* and the cells started to shrink (Video 11).

Common to all infections, is the presence of bacteria inside the macrophages that seem to retain their shape. Thus, it appears that the bacteria are phagocytosed but accumulate in the cytoplasm because they are not being cleared and destroyed. This observation suggests that PGRP-SA may have a role in bacterial clearance (see discussion).

The addition of mCherry_PGRP-SA to the media followed by infection, restored the phenotypes to levels similar to the *GFP-Moesin* macrophages (Videos 13-16 and Fig. 4). The macrophages survive longer

and initiated later an apoptotic cell death when compared to the control mutant macrophages in non-rescue conditions (absence of mCherry_PGRP-SA).

Regarding cellular localisation, whether in the absence of bacteria (Video 17), whether upon infection (Videos 13-16), mCherry_PGRP-SA was found mainly inside the macrophages. It was detected co-localised with the DAPI signal of the bacteria, extracellularly and intracellularly. Intracellularly, it was also found both at the cytoplasm and at the cell surface, most likely at the cell membrane.

GFPMoe_seml_Saureus.mov

Video 8. *Semmelweis-GFP-Moesin* macrophages infected with *S. aureus* bacteria.

GFPMoe_seml_Saureus_tagO.mov

Video 9. *Semmelweis-GFP-Moesin* macrophages infected with *S. aureus* Δ *tagO* bacteria.

GFPMoe_seml_Bsubtilis.mov

Video 10. *Semmelweis-GFP-Moesin* macrophages infected with *B. subtilis* bacteria.

GFPMoe_seml_Bsubtilis_tagO.mov

Video 11. *Semmelweis-GFP-Moesin* macrophages infected with *B. subtilis* Δ *tagO* bacteria.

GFPMoe_seml_Schneider.mov

Video 12. *Semmelweis-GFP-Moesin* macrophages in Schneider media.

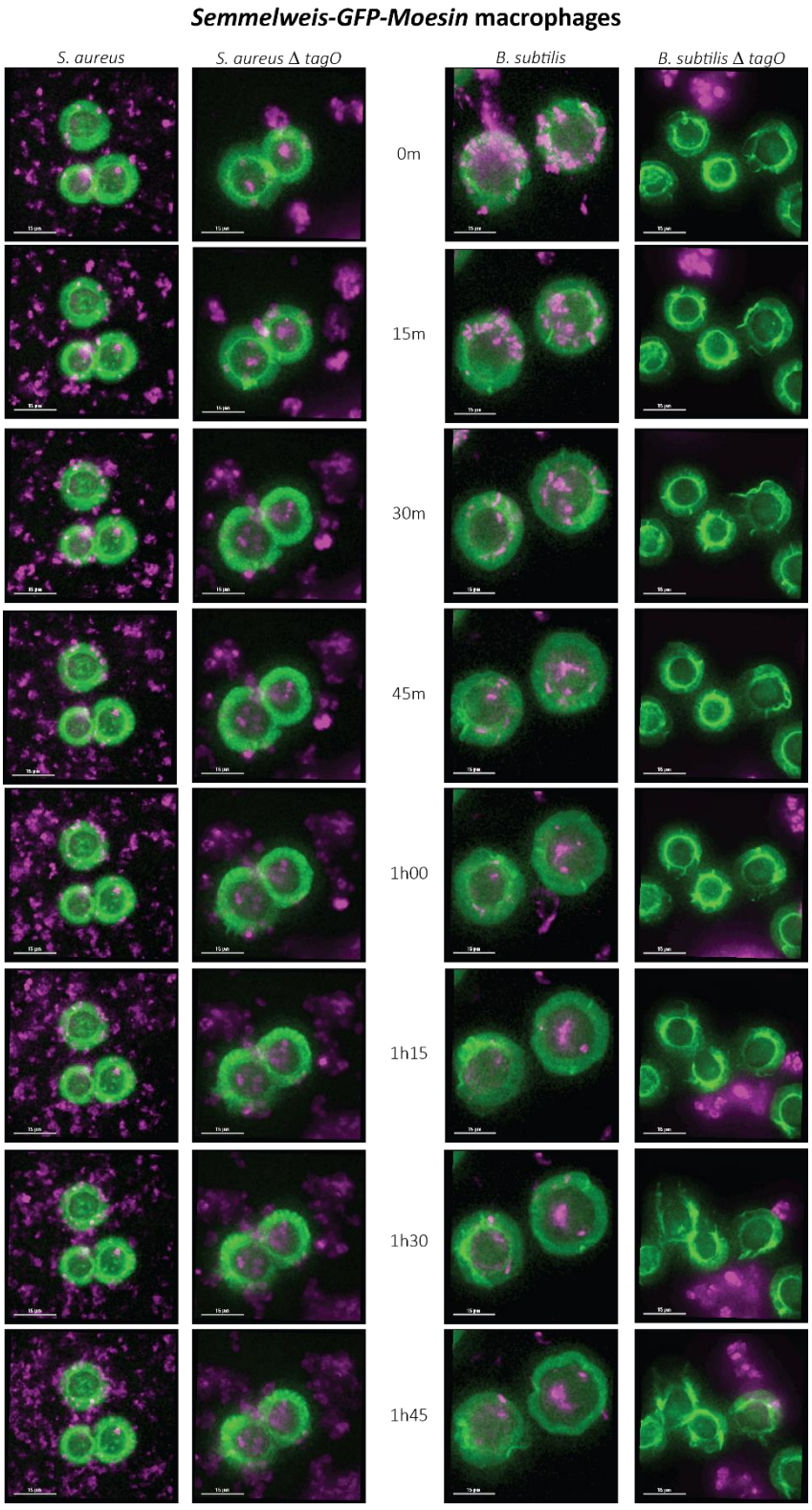
Semmelweis-GFP-Moesin macrophages
S. aureus
S. aureus Δ tagO
B. subtilis
B. subtilis Δ tagO
0m
15m
30m
45m
1h00
1h15
1h30
1h45

Figure 3. Snapshots of *Semmelweis-GFP-Moesin* macrophages infected with bacteria. It is shown snapshots of every 15min acquisition of the macrophages infected with bacteria from the videos 8-11, from time points 0-1h45 p.i.. It is visible the presence of bacteria that retain almost their natural shape, particularly for *S. aureus* Δ *tagO* and *B. subtilis*, accumulating inside the macrophages.

GFPMoe_seml_Saureus_mChPGRPSA.mov

Video 13. *Semmelweis-GFP-Moesin* macrophages infected with *S. aureus* bacteria rescued with mCherry_PGRP-SA.

GFPMoe_seml_Saureus_tagO_mChPGRPSA.mov

Video 14. *Semmelweis-GFP-Moesin* macrophages infected with *S. aureus* Δ *tagO* bacteria rescued with mCherry_PGRP-SA.

GFPMoe_seml_Bsubtilis_mChPGRPSA.mov

Video 15. *Semmelweis-GFP-Moesin* macrophages infected with *B. subtilis* bacteria rescued with mCherry_PGRP-SA.

GFPMoe_seml_Bsubtilis_tagO_mChPGRPSA.mov

Video 16. *Semmelweis-GFP-Moesin* macrophages infected with *B. subtilis* Δ *tagO* bacteria rescued with mCherry_PGRP-SA.

GFPMoe_seml_mChPGRPSA.mov

Video 17. *Semmelweis-GFP-Moesin* macrophages in Schneider media rescued with mCherry_PGRP-SA.

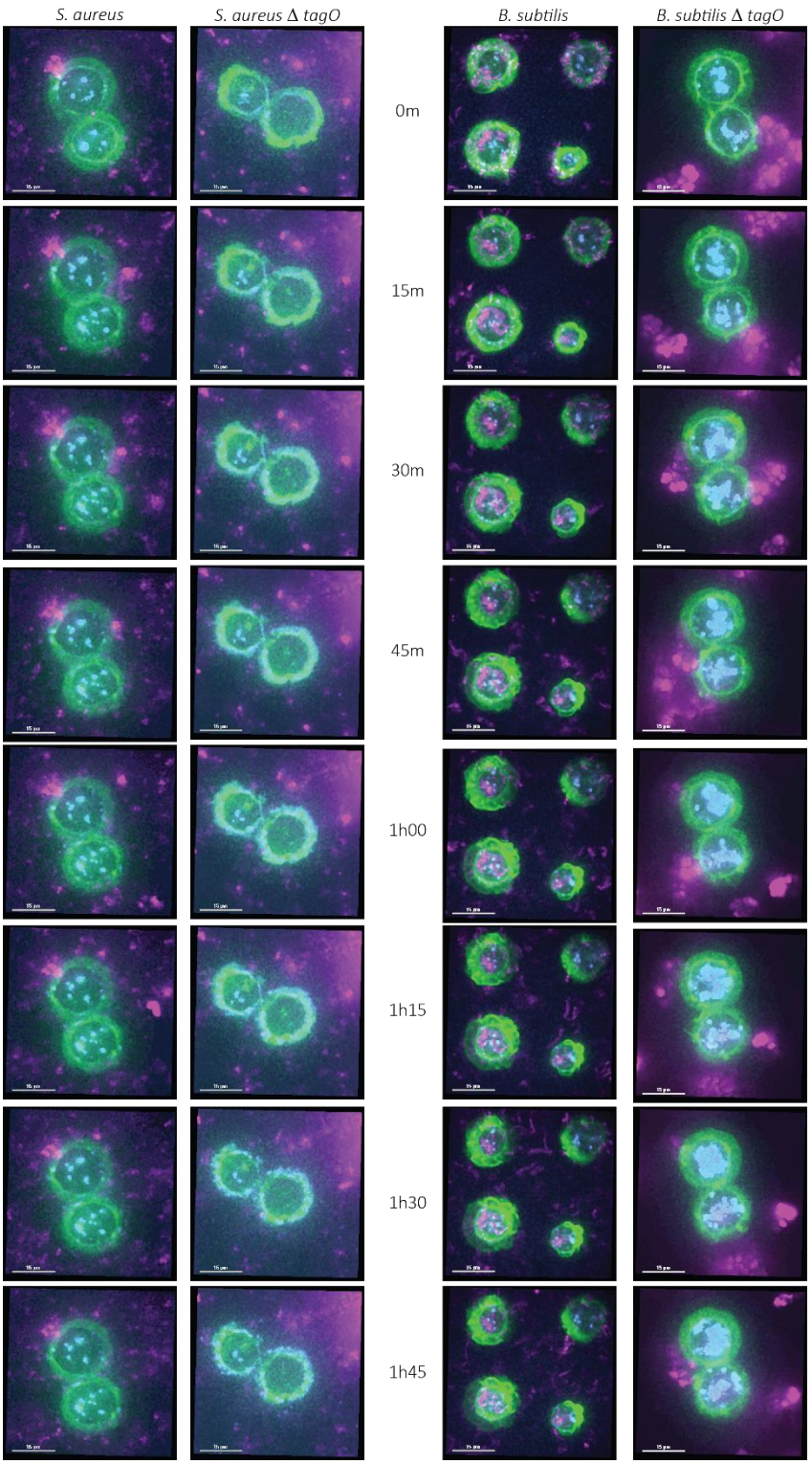
Semmelweis-GFP-Moesin macrophages with mCherry_PGRP-SA
S. aureus
S. aureus Δ *tagO*
B. subtilis
B. subtilis Δ *tagO*
0m
15m
30m
45m
1h00
1h15
1h30
1h45

Figure 4. Snapshots of *Semmelweis-GFP-Moesin* macrophages complemented with mCherry_PGRP-SA infected with bacteria. It is shown snapshots of every 15min acquisition of the macrophages infected with bacteria from the videos 13-16, from time points 0-1h45 p.i.. Interestingly, mCherry_PGRP-SA seems to mainly localise inside the cell, rather on the outside and it co-localises with the DAPI signal, hence it is bound to the bacteria.

Both PGRPs seem to contribute to bacterial clearance.

When *semmelweis/PGRP-LC$^{\Delta E12}$-GFP-Moesin* macrophages were infected with *S. aureus*, there was still death by apoptosis (Video 17 and Fig. 5). However, in contrast to what had been observed before, when these mutant macrophages were infected with *S. aureus* Δ *tagO* and *B. subtilis* bacteria, the macrophages died through necroptosis (Video 18-19 and Fig. 5). In addition, these double mutant macrophages were also more susceptible to the non-virulent *B. subtilis* Δ *tagO* mutant (Video 20 and Fig. 5).

Similar to the single mutant background *semmelweis-GFP-Moesin* macrophages, these double mutant macrophages appear to be less dynamic regarding the filopodia movements with both *S. aureus* strains (Video 18). Moreover, it is also seen that the phagocytosed bacteria accumulate in the cytoplasm retaining almost their shape. This suggests that they were not being efficiently destroyed and so both PGRP-SA and PGRP-LC are involved in the clearance processes and the absence of both is more severe than the absence of a functional *PGRP-SA*.

When these mutant macrophages were rescued with mCherry_PGRP-SA, the phenotypes upon infections were almost restored to the *GFP-Moesin* phenotypes (Videos 22-25 and Fig. 6) and I could no longer observe accumulation of bacteria inside the macrophages.

GFPMoe_semILC_Saureus.mov

Video 18. *Seml/PGRP-LC$^{\Delta E12}$-GFP-Moesin* macrophages infected with *S. aureus* bacteria.

GFPMoe_semILC_Saureus_tagO.mov

Video 19. *Seml/PGRP-LC$^{\Delta E12}$-GFP-Moesin* macrophages infected with *S. aureus* Δ *tagO* bacteria.

GFPMoe_semILC_Bsubtilis.mov

Video 20. *Seml/PGRP-LC$^{\Delta E12}$-GFP-Moesin* macrophages infected with *B. subtilis* bacteria.

GFPMoe_semILC_Bsubtilis_tagO.mov

Video 21. *Seml/PGRP-LC$^{\Delta E12}$-GFP-Moesin* macrophages infected with *B. subtilis* Δ *tagO* bacteria.

GFPMoe_semILC_Schneider.mov

Video 22. *Seml/PGRP-LC$^{\Delta E12}$-GFP-Moesin* macrophages in Schneider media.

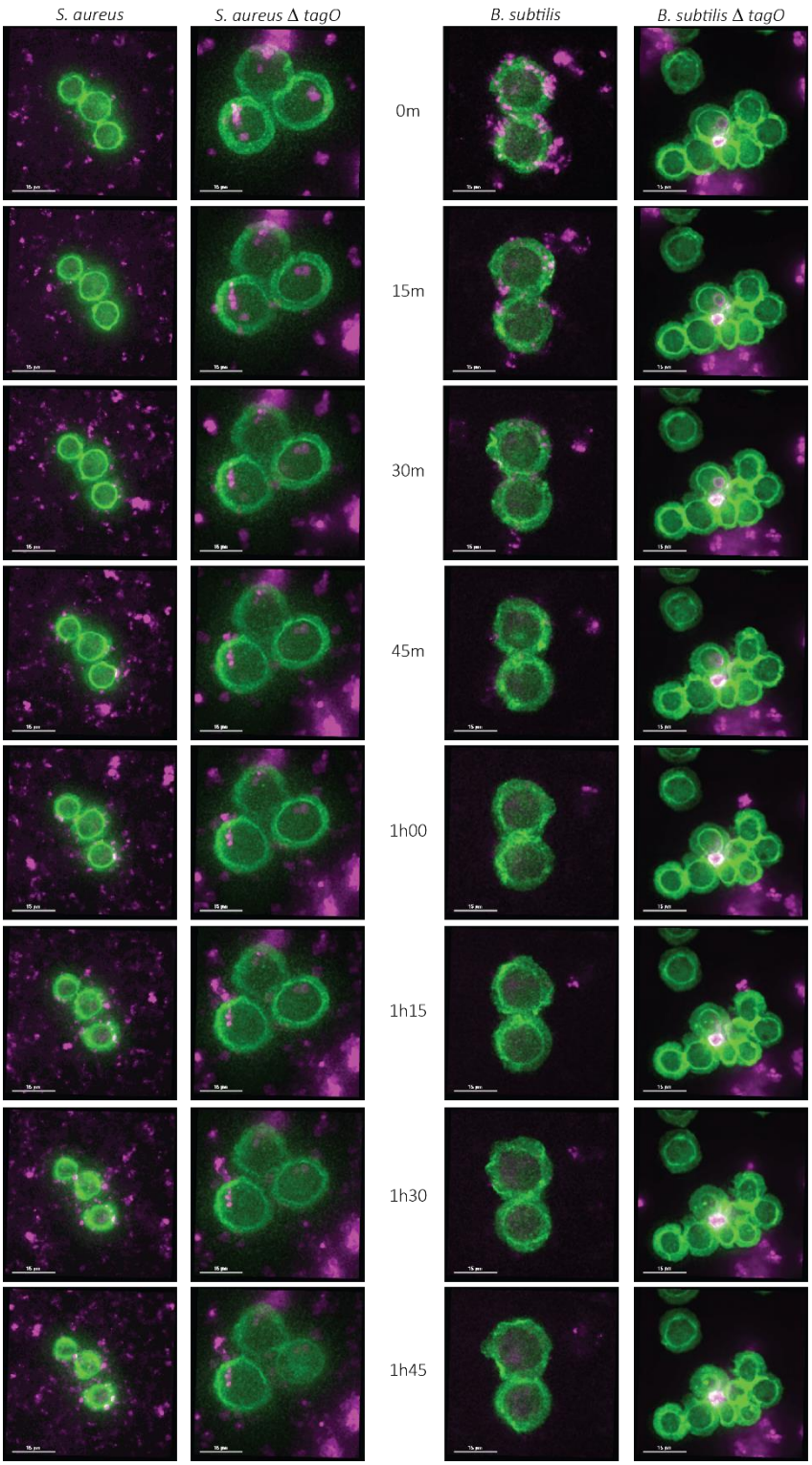
seml/PGRP-LC^NF12-GFP-Moesin macrophages
S. aureus
S. aureus Δ tagO
B. subtilis
B. subtilis Δ tagO
0m
15m
30m
45m
1h00
1h15
1h30
1h45

Figure 5. Snapshots of *seml/PGRP-LC$^{\Delta E12}$-GFP-Moesin* macrophages infected with bacteria. It is shown snapshots of every 15min acquisition of the macrophages infected with bacteria from the videos 18-21, from time points 0-1h45 p.i..Comparatively to the other macrophages, the macrophages infected with *S. aureus* appear very unhealthy and initiate an apoptotic death shortly after infection (the lamellipodia is retracted and the cells are shrinking).

GFPMoe_semlLC_Saureus_mChPGRPSA.mov

Video 23. *Seml/PGRP-LC$^{\Delta E12}$-GFP-Moesin* macrophages infected with *S. aureus* bacteria rescued with mCherry_PGRP-SA.

GFPMoe_semlLC_Saureus_tagO_mChPGRPSA.mov

Video 24. *Seml/PGRP-LC$^{\Delta E12}$-GFP-Moesin* macrophages infected with *S. aureus* Δ *tagO* bacteria rescued with mCherry_PGRP-SA.

GFPMoe_semlLC_Bsubtilis_mChPGRPSA.mov

Video 25. *Seml/PGRP-LC$^{\Delta E12}$-GFP-Moesin* macrophages infected with *B. subtilis* bacteria rescued with mCherry_PGRP-SA.

GFPMoe_semlLC_Bsubtilis_tagO_mChPGRPSA.mov

Video 26. *Seml/PGRP-LC$^{\Delta E12}$-GFP-Moesin* macrophages infected with *B. subtilis* Δ *tagO* bacteria rescued with mCherry_PGRP-SA.

GFPMoe_semlLC_Schneider.mov

Video 27. *Seml/PGRP-LC$^{\Delta E12}$-GFP-Moesin* macrophages in Schneider media rescued with mCherry_PGRP-SA.

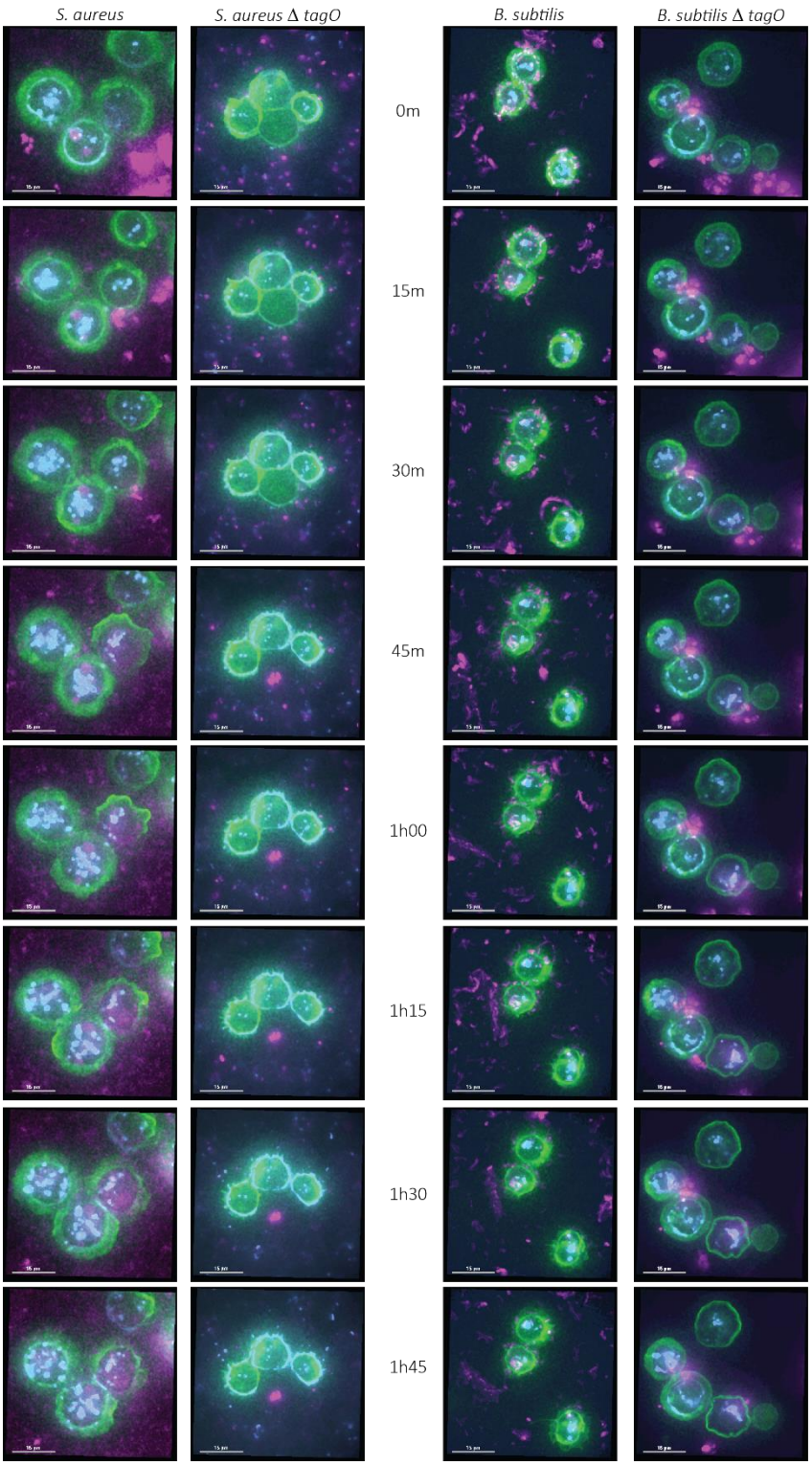
seml/PGRP-LC^ΔF12-GFP-Moesin macrophages with mCherry_PGRP-SA
S. aureus
S. aureus Δ tagO
B. subtilis
B. subtilis Δ tagO
0m
15m
30m
45m
1h00
1h15
1h30
1h45

Figure 6. Snapshots of *seml/PGRP-LC$^{\Delta E12}$-GFP-Moesin* macrophages complemented with mCherry_PGRP-SA infected with bacteria. It is shown snapshots of every 15min acquisition of the macrophages infected with bacteria from the videos 23-26, from time points 0-1h45 p.i.. In the *S. aureus* Δ *tagO* video, it is observed an example of death by necrosis from a macrophage that had not up taken mCherry_PGRP-SA.

PGRP-SA shows binding and lytic activity at low pH.

In the absence of a functional PGRP-SA, the macrophages accumulated bacteria in the cytoplasm and seemed to be impaired in clearance (Videos 7-10 and 17-20). This suggested that PGRP-SA is involved both in bacterial recognition and clearance processes. Upon phagocytosis, the phagosome containing the bacteria fuses with the lysosomes containing hydrolytic enzymes (Fig. 1). It is inside this phagolysosome that the bacteria will be destroyed, hence where it occurs the process of clearance. Typically, phagolysosomes present a very low pH that aids in the clearance process and enzyme activity. Hence, if indeed PGRP-SA is involved in the clearance process, it should be found with the bacteria enclosed in the phagolysosomes. Consequently, it should be able to bind to the bacteria and exert lytic activity at low pH.

Accordingly, when wild-type bacteria *S. aureus* and *B. subtilis* were incubated with mCherry_PGRP-SA at pH 3, the protein showed a very high binding ability to these cells (Fig. 7). In contrast, when the pH approached the physiological pH of the haemolymph (pH 6), this binding was no longer detected (Fig. 8 and Fig. 2 of Chapter II).

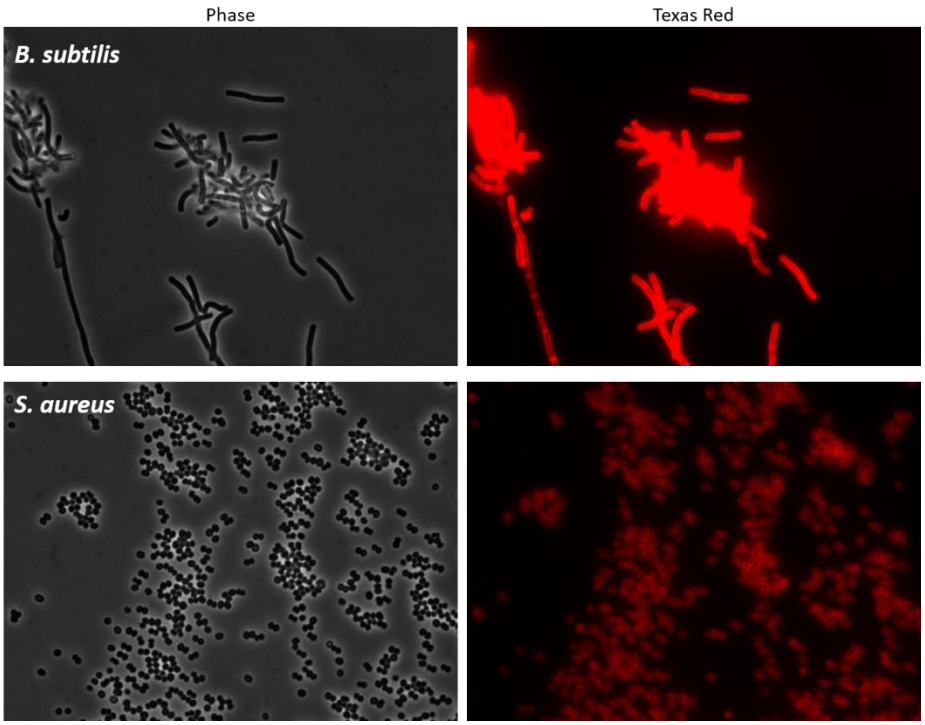

Figure 7. Co-precipitation of live bacterial cells with mCherry_PGRP-SA at pH 3. Cultures in early exponential phase of the parental strains of *B. subtilis* and *S. aureus* were incubated with mCherry_PGRP-SA in 25 mM Glycine Buffer pH 3. The cells were imaged with a Texas Red filter at a very low exposition time of 30 ms. When using the same microscope and setting conditions the analysis of the these bindings are normally performed using an exposition time of 500 ms [28], [29].

In order to analyse a potential lytic activity of PGRP-SA, I performed Zymogram assays. Briefly, these assays consist of an SDS-PAGE gel where it is incorporated a substrate. The proteins to be studied are ran on the gel using standard protocol conditions. After the run, instead of staining the proteins, the gel is incubated in a buffer that will allow the proteins to renaturate. Finally, instead of staining the proteins as in a standard SDS-PAGE, it is stained the gel, *i.e.* the substrate. If the protein has lytic activity the substrate is degraded, therefore in the region where the proteins lies in the gel there is no substrate present. Therefore, it is visualised a clear band at the place of the protein in the gel.

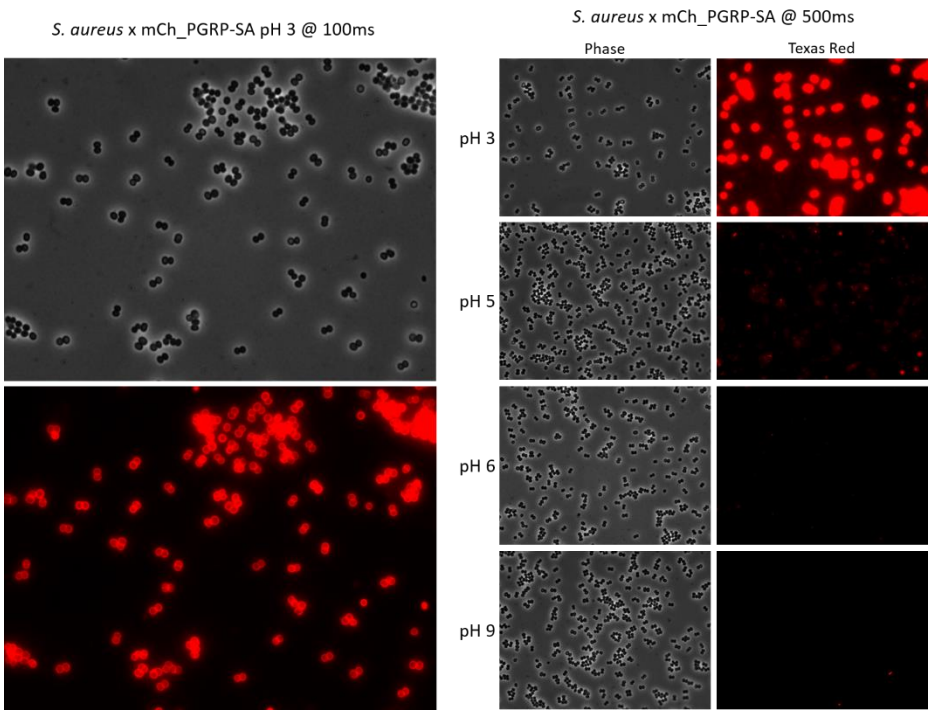

Figure 8. Co-precipitation of live *S. aureus* cells with mCherry_PGRP-SA at different pH. The left panel shows the binding of mCherry_PGRP-SA at pH 3 to *S. aureus* cells using optimal exposition time of 100 ms for visualisation of the binding. The right panel presents the binding of mCherry_PGRP-SA at different pH. The scale intensities were adjusted to the binding at pH 3. It was used a higher exposure time of the Texas Red as it would be possible to not detect binding at other pH due to using very low exposition times.

Autoclaved cells of *Micrococcus luteus*, *Microbacterium nematophilum* and *S. aureus* were used as a substrates. When both mCherry_PGRP-SA and rPGRP-SA were analysed through the zymogram assay, I detected lytic acitvity of the protein, particularly at pH 3 (Fig. 9 and Fig. 10). *M. nematophilum* is a natural pathogen of *C. elegans* [32]. It is a Gram-positive bacterium that appears to have a very particular and intricate PGN composition resistant to the muramidase Mutanolysin from *Streptomyces globisporus* (personal communication from Hodgkin and Filipe Lab). In sum, it can be concluded that PGRP-SA possesses lytic activity, being able to cleave different types of PGN substrates while not inhibited by major PGN modification like most of the Lysozymes and

Muramidases (such as *M. nematophilum*) and to the *O*-acetylation of the muramic acid residue (such as *S. aureus*).

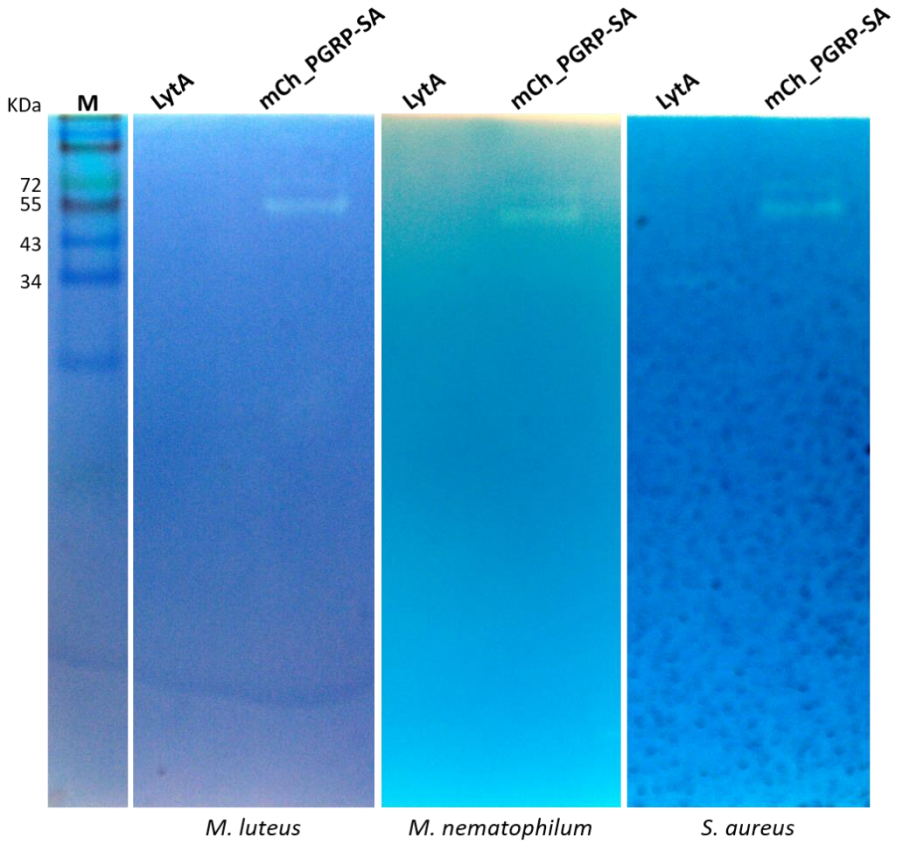

Figure 9. Zymography assay of the lytic activity of mCherry_PGRP-SA at pH 3. The gels were incubated O/N at 37ºC in a Renaturing Buffer Tris-Glycine at pH 3. LytA is the major autolysin of *Streptococcus pneumoniae* with amidase activity and it was used as a negative control of the assay. The different colours of blue in the gels are associated with the different properties of these cells to be stained by the Methylene Blue.

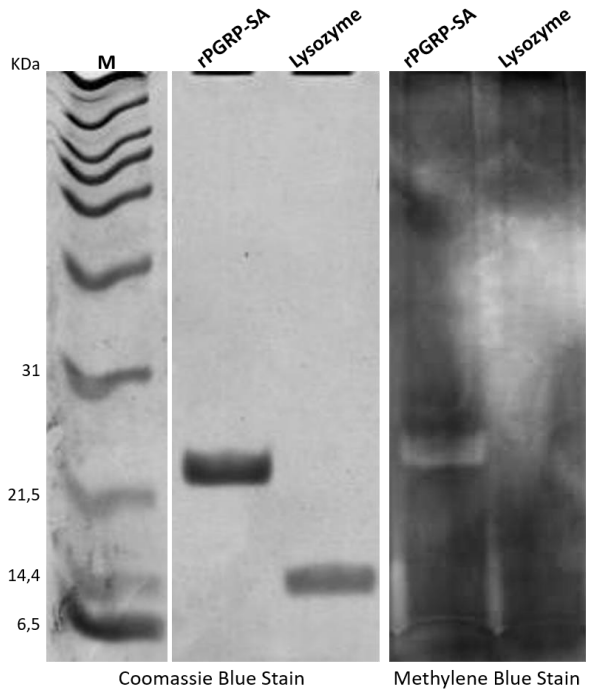

Figure 10. Zymography assay of the lytic activity of rPGRP-SA at pH 6. To ascertain that the observed activity was related with the PGRP-SA protein and not by a non-specific activity of the mCherry_PGRP-SA, an assay with the rPGRP-SA was performed using *S. aureus* cells. In this zymogram it was used a buffer at pH 6 and a higher concentration of rPGRP-SA than the one used for mCherry_PGRP-SA. Lysozyme was used as a negative control.

Discussion

The process of bacterial clearance is believed to be highly dependent on the cellular responses of the Innate Immune System. Professional phagocytes, *i.e.* macrophages, uptake the pathogen through phagocytosis. Phagocytosis is inherently linked to the process of destruction and clearance. Up to date, the canonical route of clearance is the phagocytosis-exocytosis unidirectional flow (Fig. 1). However, it has been recently reported that this elegant and simple mechanism of pathogen destruction does not occur most of the times. Instead, the macrophages activate an apoptotic death upon phagocytosis [33]. Non-pathogenic bacteria and bacteria that are easily killed by the macrophages have been reported to induce apoptosis after phagocytosis [33]. Evidence suggests that phagocytosis-induced apoptosis may be a natural response of the macrophages to different bacteria. In addition, an efficient clearance process is inherently dependent of an efficient recognition of the bacteria by the host receptors, as the phagocytosis process begins with the action of the opsonins that promote the uptake of the bacteria by the macrophages.

There are three main modes of PCD which morphologically present characteristic distinctive features: apoptosis, necrosis and autophagy [20]. Nevertheless, the discrimination between cell death processes should rely on the observation of the morphology of the dying cells, on the detection of biochemical parameters of cell death and ultimately on the interaction of the macrophages that will phagocyte the dying cells [34]. In the present study, only morphological data was acquired regarding the death of the macrophages. Therefore, the analysis of the mode of death of the macrophages may be subject to re-adjustments upon future experiments. Hence, the interpretation of the results that I present next should be taken

as working hypothesis which will guide me to plan future experiments and conclude the work I present in this chapter.

Bacterial-induced apoptosis.

Pathogenic bacteria present numerous ways to subvert the host immune responses. *S. aureus* is a major Gram-positive pathogen that presents a number of virulence factors which compromise cellular responses, such as the expression of haemolysins and toxins (Fig. 11). Although it has been identified several mechanisms by which *S. aureus* subverts the phagocytosis and clearance, there is still not clarity in the field regarding the interaction with the macrophages. Nevertheless, it has been shown that it is able to replicate within human macrophages [35], [36] and that once inside the macrophages it upregulates antiapoptotic factors [37].

Upon infection with *S. aureus*, the macrophages very early show signs of stress that culminated in cellular death (Video 2). It was observed ruffling of the membrane, retraction of the lamellipodia and ceasing of the filopodia movement. Moreover, it was evident the shrinkage of the cell size accompanied with the condensation of the DNA. Ultimately, the DNA presented signs of karyorrhexis. These observations strongly suggest that *S. aureus* infection induced apoptotic death of the macrophages. Therefore, the following data interpretation is based on the hypothesis that *S. aureus* triggered an apoptotic death in the macrophages.

In contrast, infection with heat-killed *S. aureus* cells did not trigger death and the dead bacteria were easily up taken and cleared (Video 7). These results suggest that the macrophages trigger apoptosis as a response towards the activity of the bacterial virulence factors, although to some extent the process of heat-killing might compromise at some level the integrity of the cell wall and render these cells more easily recognised and killed. This helps to explain the previous observed upregulation of antiapoptotic factors by *S. aureus* [37]. In addition, the need that the

bacteria have to impair macrophages apoptosis is an indirect evidence that induction of apoptosis upon phagocytosis is a natural immune response to promote the clearance. Moreover, this also helps to understand that the reported activation of caspases by the α–haemolysin [38], is not due to the haemolysin itself but rather it is a response of the cell towards the effect of the toxin. Before the cell is destroyed by the activity of the haemolysin, it induces apoptosis.

When the macrophages were infected with the *S. aureus* Δ *tagO* mutant, the cellular death by a presumed apoptosis, happened later on in the infection course (Video 3). There was still induction of death (apoptosis), because the mutant bacteria bears the virulence factors of the parental strain, considering that the absence of WTA is not significantly impairing the activity of virulence factors (Fig. 11). Hence, the delay in the induction of apoptosis is due to a faster clearance of these bacteria (see last topic of the Discussion). As they are destroyed faster, it also takes longer for the activity of the virulence factors to achieve the wild-type levels required for the triggering of the apoptosis.

To this type of apoptosis induced by bacterial factors, I will onwards refer to as “bacterial-induced apoptosis” (Fig. 12). This type of apoptotic death is a response of the macrophages against harm caused by the bacteria and it is different from the reported phagocytosis-induced apoptosis [33]: 1) the bacterial-induced apoptosis is dependent on the pathogenicity of the bacteria; 2) the apoptotic death is a slow process of death, in contrast to this apoptotic death which is unusually fast because it was triggered by a high sudden stress; 3) when the bacteria preserve their pathogenicity, the induction of apoptosis is delayed only when they are more easily destroyed, albeit the process of death is still faster than a death that is not caused by harmful virulence factors.

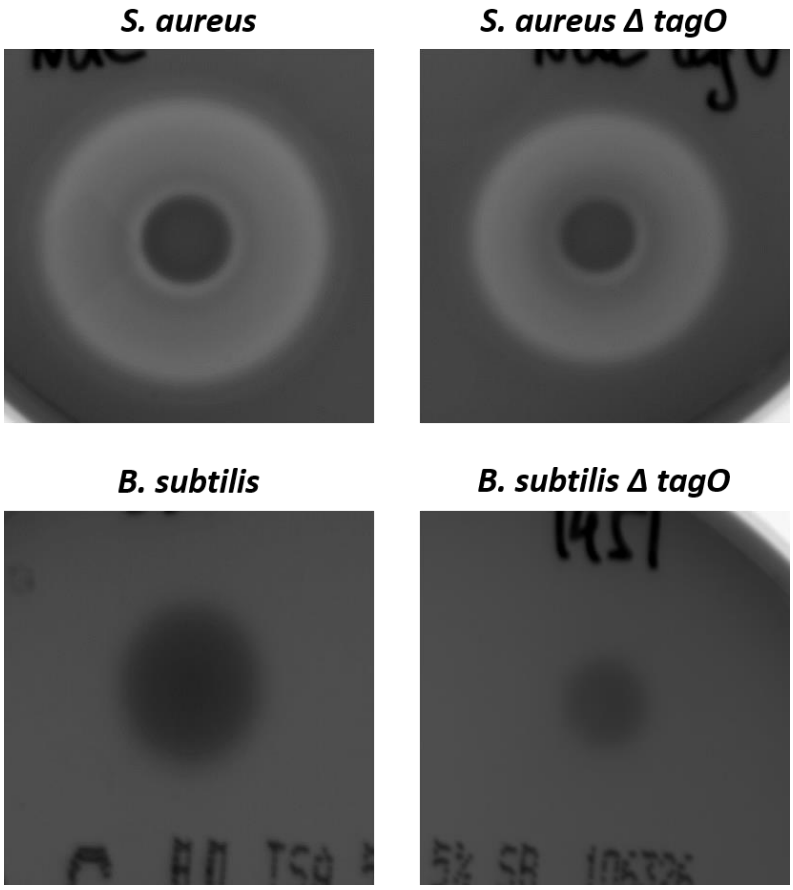

Figure 11. Haemolytic expression pattern in sheep blood. *S. aureus* presents haemolysins that are able to degrade red blood cells. The *S. aureus Δ tagO* mutant seems to retain the ability to express the same type of haemolysins to which the red blood cells of sheep are sensitive to. In contrast, *B. subtilis* is devoid of such lysins.

Phagocytosis-induced apoptosis.

Infection with the innocuous soil bacteria *B. subtilis* also showed signs that can be interpreted as an apoptotic mode of death in the macrophages, although in a less potent manner than *S. aureus*. After 18h of infection, the macrophages had not yet finished the process of (apoptotic) death (Video 4). In contrast, the *B. subtilis* Δ *tagO* mutant did not induce any visible stress to the macrophages and appears to be easily phagocytosed and killed, through the canonical way of phagocytosis-

exocytosis (Video 5). As *B. subtilis* is devoid of virulence and both wild-type and mutant strains present the same PGN muropeptide composition (Fig. 13, Chapter II), it can be concluded that a more exposed PGN is rendering the cells more vulnerable to clearance and thus the macrophages are able to easily contain the infection. This implies that due to a more exposed PGN, the macrophages are able to efficiently and rapidly destroy the mutant cells. Hence, there is no cellular stress conditions that would require/induce an apoptotic death in order to control the infection. Therefore, the presence of the WTA is a bacterial cell wall factor that prevents an efficient killing after phagocytosis (see last section of the Discussion).

In sum, upon infection with non-virulent bacteria, when the bacterial load is high and the rate of phagocytosis is higher than the rate by which the bacteria can be destroyed, the macrophages may trigger an apoptotic death – the phagocytosis-induced apoptosis (Fig. 12) [33]. This phagocytosis-induced apoptosis (Fig. 12) [33], may be a strategic mechanism of clearance by which the macrophages cope with the saturation of bacteria attached to their lamellipodia and inside the cell coupled with accumulation of bacteria in phagosomes/phagolysosomes.

Apoptosis may be a Host mechanism of bacterial clearance.

The fact that when bacteria are more easily cleared, the apoptosis upon phagocytosis becomes delayed or unnecessary, is an indication that apoptosis is a mechanism to promote bacterial clearance.

Apoptosis presents itself as a type of PCD that helps the macrophages to continue/finish the process of clearance as it culminates in phagocytosis. The macrophages promote the clearance by forming the apoptotic bodies containing the bacteria and cellular fragments that will be phagocytosed (Fig. 12). This may be an advantage to the Host, rather than a death by necroptosis. The death by necroptosis results in the burst

of the cell with release of its contents, it induces inflammation and it would release the still viable bacteria and incomplete digested bacterial products into the blood stream. In table 2 it is presented the three types of proposed pathways of phagocytosis-clearance.

Table 2. Phagocytosis-clearance pathways.

Bacterial-induced apoptosis	Phagocytosis-induced apoptosis	Phagocytosis-Exocytosis
Pathogenic bacteria	Non-pathogenic bacteria	Low numbers of non-pathogenic bacteria which are easy to be killed
It is an immediate protective response against the activity of virulence factors	It is a response towards an unbalance rate of phagocytosis and the number of bacteria to be cleared and/or how easy are certain bacteria killed	Bacteria that lost virulence (mutations)
Fast apoptotic death	Slow apoptotic death	Dead or dying bacteria
The levels of virulence factors, strongly determine the degree of acute infection, thus when the time upon infection that the macrophages trigger apoptosis	The bacterial load determines when the macrophages trigger apoptosis	May depend on the bacterial load
Little accumulation of bacteria inside the macrophage	Accumulation of bacteria inside the macrophage	Macrophages may enter the route of phagocytosis-induced apoptosis

A new look into the phagocytic ligand-receptors.

There has been growing evidence of a dual role of phagocytic receptors both in phagocytosis of apoptotic cells and of bacteria. The *Drosophila* phagocytic receptors present in haemocytes that are involved in phagocytosis of apoptotic bodies are: Croquemort [5], [6], Draper [7], and NimC4/SIMU [8]. As for bacterial phagocytosis, the haemocyte phagocytic receptors are: Eater [9], Nimrod C1 [10], Dscam [11] and Peste [12]. Other bacterial phagocytic receptors expressed in other tissues than haemocytes are the PGRP-LC [16] and PGRP-SC1 [17].

The dSR-CI is thought to be involved in phagocytosis of both apoptotic bodies and microorganisms due to its broad polyanionic binding ability [39]. Integrin βυ [13], [14] and Integrin αPS3/βυ [15] have also been identified as phagocytic receptors of both apoptotic bodies and bacteria. Interestingly, later on Draper was reported to also be involved in phagocytosis of *S. aureus* [40].

Integrin βυ was reported to bind to the *S. aureus* PGN and that this

binding was required for phagocytosis [23] and Draper was shown to bind to LTA of *S. aureus* [40]. According to a line of thought in which apoptosis may be a mechanism of bacterial clearance, it is then possible that the apoptotic bodies can present bacterial products at the surface to promote their engulfment. Hence, perhaps the authors have observed the phagocytosis of apoptotic bodies formed upon bacterial phagocytosis. . The binding of Draper to LTA is an indirect evidence of this, since it has been shown that these wall polymers are not exposed at the surface and access to them requires the degradation of PGN [41].

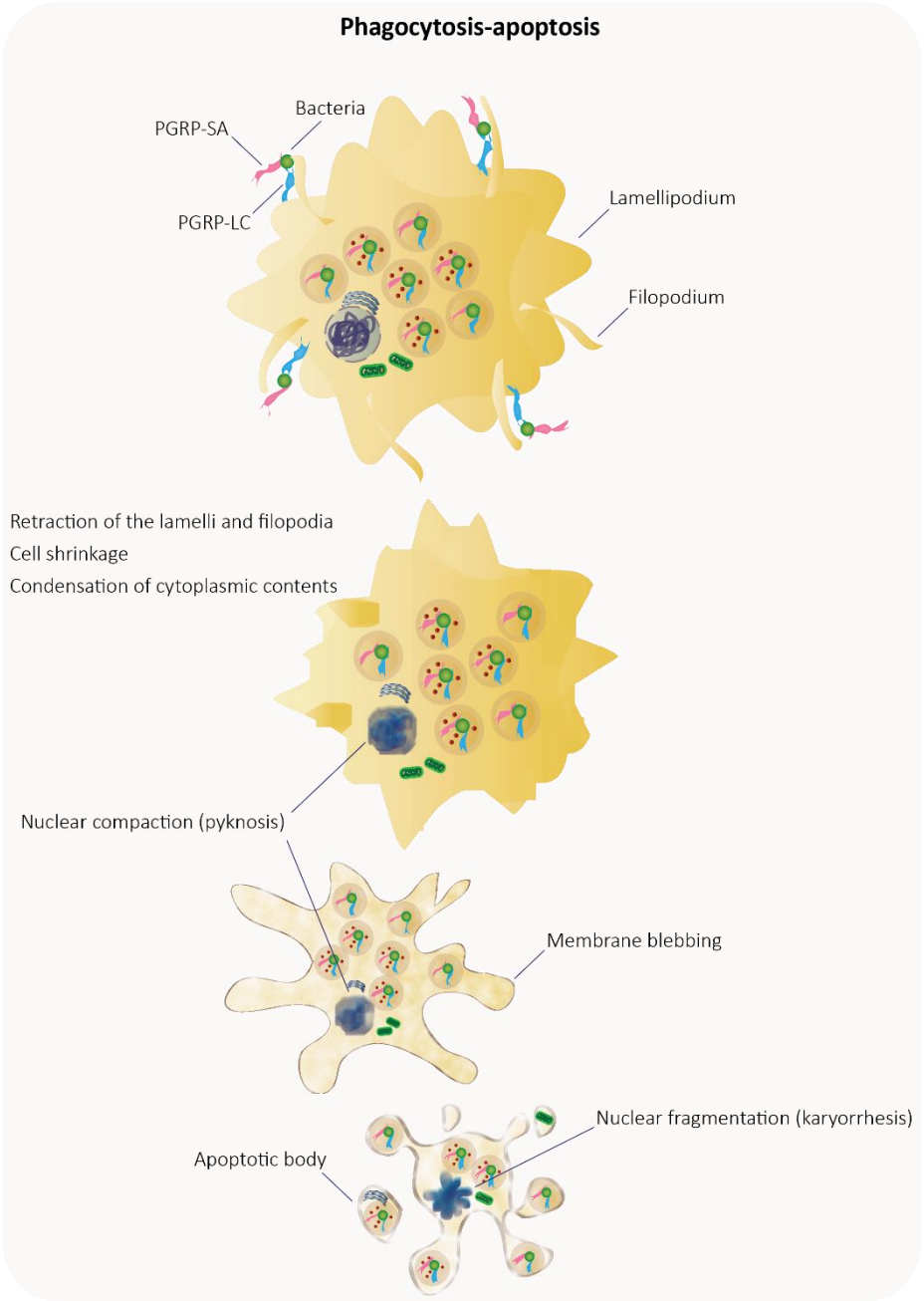

Figure 12. Phagocytosis-apoptosis process. The macrophages induce apoptosis whilst containing bacteria enclosed in phagosomes and phagolysosomes. These will be incorporated by the apoptotic bodies. The bacteria will be phagocytosed again and a new cycle of phagocytosis-clearance is initiated. This process is likely to be repeated for as long as it is necessary to completely destroy the bacteria.

PGRP-SA and PGRP-LC participate in clearance of both Lys- and DAP- type PGN bacteria.

When functional PGRP-SA is absent, it appears that the phagocytic ability is not compromised, i.e. the internalisation of the bacteria is not affected (Videos 7-10). In s*emmelweis-GFP-Moesin* macrophages, it was observed bacteria inside the macrophages that seem to retain their shape, and thus are likely to be alive, in contrast to what was observed in the wild-type *GFP-Moesin* macrophages. In the absence of bacteria, when the *semmelweis* macrophages were complemented with mCherry_PGRP-SA, although predicted to be extracellular and circulating in the haemolymph, the protein was found localised inside the cell (Video 12-16). When these macrophages were infected with bacteria, then the protein co-localised with the bacteria thus being found both outside, inside at the cytoplasm overlapping with the bacterial DAPI staining or even at the cell surface of the macrophage. Moreover, it was no longer detectable so many intact bacteria inside the macrophage. These results show that these mutant macrophages are compromised in the clearance and thus PGRP-SA plays a role in this process for both Lys- and DAP- type PGN bacteria.

Similar to the single mutant macrophages, the *semmelweis*/*PGRP-LC*$^{\Delta E12}$*-GFP-Moesin* macrophages also showed accumulation of phagocytosed bacteria in their cytoplasm which retained almost their shape, again suggesting that the bacteria are not been being efficiently destroyed (Videos 18-21). Since these macrophages showed to be much more compromised in the response upon infection than the *semmelweis* macrophages (see next section of the Discussion), PGRP-LC is also involved in the clearance whether by a Lys- or DAP- type PGN bacteria.

PGRP-SA and PGRP-LC are opsonins of both Lys- and DAP-type PGN bacteria.

So far it has been shown that apoptosis is triggered upon phagocytosis when the process of clearance is compromised. Moreover, it was observed that both PGRP-SA and PGRP-LC engage in the process of clearance. As such, the observation of an impaired clearance resulting in the uncontrolled death of the double mutant macrophages, corroborates the above findings (Videos 18-21 and Fig. 4).

Regarding the *S. aureus* infection, the macrophages in a very early stage of infection show signs of apoptotic death, suggesting that the infection is very acute (Video 16 and Fig. 5). Hence, from a very initial stage of interaction, the double mutant macrophages sense the virulence of *S. aureus* as the wild-type macrophages do, responding quickly against infection through a fast triggering of apoptosis.

When *semmelweis/PGRP-LC$^{\Delta E12}$–GFP-Moesin* macrophages were infected with *S. aureus* Δ *tagO* and *B. subtilis* bacteria, the macrophages died by necroptosis (Videos 19-20 and Fig. 5).

Perhaps, due to a high number of bacterial cells and/or difficulty in the clearance by the presence of WTA, *B. subtilis* infection triggers phagocytosis-induced apoptosis under most circumstances. Upon absence of both PGRPs, the clearance of the bacteria is more compromised than in the absence of PGRP-SA. Therefore, the growth of *B. subtilis* is not so well contained and the macrophages cannot manage an apoptotic death and succumb by necroptosis.

Similarly for *S. aureus* Δ *tagO*, even with a more exposed PGN, because these PGRPs are needed for clearance and not just recognition, the macrophages are highly impaired in controlling the bacterial growth.

As for the infection with *B. subtilis* Δ *tagO*, the macrophages die through apoptosis. Perhaps because this mutant strain is highly compromised in growth and cell division, on one hand it takes longer to

induce signalling dangers and on the other hand it is this feature that allows the macrophage to detect lack of clearance and still have time to begin an apoptotic death.

When these double mutant macrophages are rescued with mCherry_PGRP-SA, the phenotypes upon infections are almost restored to the *GFP-Moesin* phenotypes (Videos 21-24 and Fig. 6). Then, that the presence of PGRP-SA can compensate to some extent the absence of one copy of PGRP-LC.

As an overall, the data so far allow to infer that PGRP-SA and PGRP-LC participate in the processes of phagocytosis-clearance and that the presence of WTA impair this clearance. Thus, both receptors can act as opsonins, participate in the clearance and may help to retain the bacteria and their non-totally digested products enclosed in a subcellular space until the apoptotic body is formed and phagocytosed. Through this mechanism, PGRP-SA and PGRP-LC limit the infection by helping to prevent the spreading of the bacteria that would occur if the cell did not induce its apoptotic pathway or rather would succumb by a necroptotic death. Finally, as it was not observed impaired uptake of the bacteria by any of the mutant macrophages tested, but it was observed earlier/later phagocytosis-induction apoptosis associated with impairment of the clearance, then this natural mechanism of apoptotic death is triggered not by the uptake/engulfment, but rather upon the formation of the phagolysosome and possibly the balance between the rate of uptake and the rate of clearance.

PGRP-SA is a lytic enzyme that aids in bacterial clearance upon phagocytosis.

PGRP-SA is a secreted protein that circulates in the haemolymph of the Fly. As such, it came as a surprise to find that the protein localised mostly in the macrophages, either at the cytoplasm or at the cell surface,

rather than almost exclusively in the extracellular medium (Videos 12-16 and 21-24). Since the phenotype upon infection of the mutant macrophages was restored with the addition of mCherry_PGRP-SA and the fluorescent signal of the mCherry co-localised with the DAPI signal of the bacteria, what was observed was the localisation of the fusion protein and not of a cleaved form separating the mCherry from the PGRP-SA protein. Hence, how come a secreted protein seems to be mostly found inside the cell? Since it was not observable differences in the uptake of the bacteria, but it seemed that they accumulated in the cytoplasm whilst not being cleared, then when inside the cell, PGRP-SA is participating in clearance. Moreover, as it was observed that the binding of mCherry_PGRP-SA to the bacteria seemed to be higher when the bacteria were inside the cell which suggests that PGRP-SA binds better when inside rather than outside the cell. Consequently, if indeed PGRP-SA participates in bacterial clearance, it should be present with the bacteria inside the phagolysosomes. Thus, the protein ought to bind and possess hydrolytic activity at a low pH.

Accordingly, when live cells of either parental strains from *S. aureus* and *B. subtilis* were incubated with mCherry_PGRP-SA at pH 3, the protein showed optimal ability to bind to these cell walls (Fig. 6), in contrast to what had been observed when the assays were performed in the haemolymph physiological pH (Fig. 2, Chapter II). As the pH rises, the binding decreases, thus there is an inverse proportionality between the pH and the binding ability of PGRP-SA (Fig. 7). In addition, at pH 3 mCherry_PGRP-SA showed to be able to cleave the PGN from different bacterial types (Fig. 8). The reason for an optimal binding of the protein of the bacterial cell surface, might be related to a partial breakdown of the WTA polymers upon acidic conditions [42], coupled with the denaturation of the cell wall proteins. The optimal activity of the protein at low pH for both binding and lytic activity favours the hypothesis of it being involved in bacterial clearance and present inside the phagolysosomes. Perhaps, the

reason for PGRP-SA having such strong activity at low pH is so that the destruction happens in a confined compartment in order that the fragments of destroyed bacteria are not spread and circulate in the haemolymph as it could provoke toxicity, inflammation and/or an anaphylactic shock.

WTA impair phagocytosis and clearance because degradation of the PGN is vital for bacterial destruction.

Both *S. aureus* Δ *tagO* and *B. subtilis* Δ *tagO* mutants showed decreased virulence towards the macrophages than the correspondent parental strains. Since the only difference between these mutants and the wild-type strains is a more exposed PGN at their cell wall due to the lack of WTA, it implies that WTA are impairing phagocytosis and clearance.

The results also suggest that for efficient killing, the degradation of PGN is paramount for bacterial destruction inside the phagolysosomes. As a consequence, one may assume that WTA impair the effect of the hydrolytic PGN enzymes present inside the phagosome, perhaps in a similar fashion as they prevent PGRP recognition. Thus, WTA are impairing the binding of PGRP-SA and PGRP-LC as receptors and their activity in the clearance upon phagocytosis.

Conclusions

In this Chapter I present novel data regarding phagocytosis and bacterial clearance by the macrophages of *Drosophila melanogaster*.

I show evidence that possibly the macrophages present two different canonical ways of phagocytosis-clearance processes: 1) phagocytosis-exocytosis and 2) phagocytosis-apoptosis. The occurrence of one process or the other seems to be highly dependent on the type of bacteria – virulent/non-virulent, easily killed or not – and also on the bacterial load. Apoptosis is triggered by the cell to promote clearance as a response towards: 1) high levels of pathogenicity that harm the macrophages and 2) high numbers of bacteria to be phagocytosed which is in turn dependent on how efficient the process of PGN degradation is inside the phagolysosomes. The formed apoptotic bodies will then be phagocytosed and this process of phagocytosis-induced apoptosis may happen several times until the bacteria are completely cleared. It is possible that the apoptotic macrophages use bacterial components as ligands to promote/improve their phagocytosis. The non-phagocytosed bacteria are again recognised by PGRP-SA and PGRP-LC, which will play their roles as receptors that trigger the late-induced AMPs by the TOLL and IMD pathways. The secreted systemic AMPs target the bacteria that were not contained by the cellular responses.

I present data that suggests that both PGRP-SA and PGRP-LC engage in cellular responses and both participate in the clearance of the bacteria. Moreover, also at the level of clearance, the WTA impair the activity of these PGRPs.

Lastly, I showed that PGRP-SA possesses lytic activity. Since it showed promiscuity towards the binding and cleavage of different types of PGN, it is probable that the protein cleaves at the glycan chains. This proposed activity is different from the L,D-carboxypeptidase activity

proposed by Chang *et al.* 2004 [43] and from the serine proteases activity proposed by Reiser *at al.* 2004 [44]. Furthermore, since the glycans are the most conserved moiety of the PGN, it is intuitive to think that a protein involved in pathogen degradation (as well as recognition) would target the most conserved region of the molecule. To compensate for the existence of lysozymes in the host, it is possible that PGRP-SA may possess a glucosaminidase activity.

I propose a model whereby PGRP-SA and PGRP-LC detect bacteria through recognition of their PGN and act as opsonins promoting phagocytosis. Upon phagocytosis, these PGRPs are also involved in bacterial clearance. PGRP-SA is likely to localise at the phagolysosome where it exerts a strong lytic activity towards the PGN at very low pH.

In order to ascertain the interpretations that I presented, further work needs to be done which will continue to make the most of the microscopy techniques. The use of microscopy, in particularly time-lapse microscopy, is still the first method of choice to study cell death, since it allows to follow the morphological changes and the kinetics of the processes. Nevertheless, it is important to correlate these observable morphological changes with the kinetic of the different biochemical parameters that distinguish apoptosis from necroptosis. A simple and common method consists in the detection of propidium iodide and phosphatidylserine. The propidium iodide is a fluorescent membrane-impermeable dye (it stains the nuclei). The phosphatidylserine is actively localized on the inner leaflet of the plasma membrane in healthy cells. Thus, they allow to determine loss of cell membrane integrity. In most cases, phosphatidylserine positivity precedes the propidium iodide positivity in apoptotic cells, whilst the two events coincide in necrotic cells [34]. Subsequently, to further characterise the apoptotic process, it is possible to perform caspase activity assays. Regarding the co-localisation of PGRP-SA in the phagolysosome, experiments of tracking of

lysosomes/phagolysosomes, should show the presence of mCherry_PGRP-SA and the bacteria inside these acidic compartments. Finally, to properly characterise a lytic activity of PGRP-SA, not only should other controls be included, such as the mCherry protein alone, but it should also be identified the cleavage site at the PGN molecule.

Materials and Methods

Bacterial Strains and culture, Flies and Protein purification.

The bacterial parental and mutant strains of *S. aureus* and *B. subtilis* and the fly strains *semmelweis* and *semmelweis/PGRP-LC*$^{\Delta E12}$ used were the same and were grown as described in Chapter III. For the zymogram assays it was used *M. luteus* DMS20030 [45], *M. nematophilum* CBX102 (provided by M. J. Gravato-Nobre, Hodgkin Lab), *S. aureus* NCTC 8325-4 and NCTC 8325-4 Δ *oat* Δ *tagO* [28]. *GFP-Moesin* flies [31] were used as a wild-type background for the *ex vivo* assays of the macrophages.

The proteins rPGRP-SA and mCherry_PGRP-SA were purified as described previously. LytA was also purified from BL21 cells, using a DEAE cellulose resin and Lysozyme was purchased from Sigma-Aldrich (Germany).

Haemolytic assays were performed as described in Chapter II.

Time-lapses microscopy experiments.

Macrophage preparation.

When the role of PGRP-SA was accessed, only male larvae from a cross of *semmelweis* or *semmelweis/PGRP-LC*$^{\Delta E12}$ female virgins with *GFP-Moesin* were collected for the experiments. Three third instar larvae were washed in 1 mL of ddH_2O followed by 50% (v/v) bleaching and vortex. The larvae were quickly washed three times with autoclaved MilliQ H_2O and left swimming whilst the slides were prepared. Into previously washed metal slides (100% (v/v) Ethanol) with a 1.0 mm coverslip it was put Schneider medium supplemented with 5% (v/v) Fetal Calf Serum. The larvae were bled into the medium and the macrophages were let settle for 1h at RT in a humid chamber.

Preparation of the bacteria.

200 µL of an O/N culture were harvested at RT and washed with of Schneider. The cells were resuspended in 200 µL of Schneider with 0.5 µL of a DAPI solution (at 1 mg/mL in MilliQ H_2O) and incubated for 5 min at RT, without shaking. The cells were washed with 200 µL of Schneider and resuspended in 100 µL of Schneider. Finally, they were dilute 1/10 in Schneider in a 200 µL final volume.

Imaging of the macrophages with the bacteria.

To the macrophages slide, 200 µL of the bacterial suspension was added and a YSI 5775 Standard Membrane (YSI Incorporated, Japan) was glued on top. For rescue of the PGRP-SA phenotype, it was added 180 µL of mCherry_PGRP-SA at 0.3 µg/µL in Schneider (fresh solution) and 20 µL of non-diluted cells. Acquisition was done at 25ºC in a humid chamber for 18h every 3min in a GE Healthcare DeltaVision Elite integrated imaging system, using an Olympus MPLAPON-Oil immersion objective (100X 1.40A). The Photometrics Evolve-512 EMCCD camera for high-sensitivity imaging was used to allow lower intensities of excitation light. For denoising, it was used Denoise Prism with default parameters except: no. of iterations 3, adaptability 10 and patch radius 3. Analysis of the data was performed using *softWoRx*® (AppliedPrecision®, USA), which permitted the conversion of the time-lapse images into a ".mov" file at 5 frames/sec.

Live bacteria co-precipitation assays.

The binding assays of mCherry_PGRP-SA with live cells were conducted as before, expect that it was used a 25 mM Glycine pH 3. Images were acquired with a Zeiss Axio ObserverZ1 microscope equipped with a Photometrics CoolSNAP HQ2 camera (Roper Scientific using Metamorph software, Meta Imaging series 7.5) and analysed using the ImageJ software.

Zymography assay.

The zymogram presented in Figure 9 was performed as described in Vaz and Filipe, 2015 [46], except that the renaturing buffer was replaced by 250 mM Tris; 20 mM Glycine pH 3 and 5 μg of mCherry_PGRP-SA and LytA were loaded on the gels. The protocol of Figure 10 was performed with some differences, since it was the original protocol for Zymography, from which it was optimized into what is now the protocol described by Vaz and Filipe, 2015 [46].

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CHAPTER V
Bacteria present mechanisms to evade cellular and humoral responses mediated through peptidoglycan recognition by PGRP-SA and PGRP-LC

In this thesis I present novel data regarding the interaction of the Host and the Bacteria, particularly in the Fruit Fly. I believe that these results will help to further understand the intricate and complex interactions that happen when the Host meets the pathogen.

I started by unravelling how PGRP-SA can detect a bacteria through recognition of its peptidoglycan (PGN). The recognition relies on the access to PGN that is surface exposed at the septum during cell division. The binding of PGRP-SA is dependent on the probability of meeting a bacteria that is in a certain stage of cell division that temporarily presents newly synthesised PGN at the septum which has not yet been incorporated into the polymer mesh. Thus, the coordination of the synthesis of new PGN, degradation of old chains and the joining of the new material with the old, is crucial for the bacteria not only to survive but also to escape from PGRP-SA recognition. It is likely that other PGN receptors may target the same region. I identified that Sle1 has a vital role for cell splitting and re-shaping of the septum by giving physical space through cleavage of old PGN that permit the incorporation of the new material. I propose a model in which efficient evasion of PGN detection during cell division is determined by the activity of the autolysins in coordination with the incorporation of new PGN. In addition, I showed that less virulent bacteria, induce lower levels of antimicrobial peptides (AMPs). Therefore, I inferred that it was because of a lower bacterial load in circulation which in turn is due to a better cellular early response. As these responses proved to be highly dependent on PGRP-SA recognition, I hypothesised that PGRP-SA could be involved in

cellular responses of clearance. However, what was evident from the data gathered at that moment, was that recognition of PGN is key for a potent and efficient immune response. Since the virulence by haemolysins, which target the haemocytes, was not affected in the non-virulent *atl* mutant, recognition of PGN, up to certain levels, is sufficient for survival of the Host. Subsequently, for as long as the PGN is not accessible by PGRP-SA (and probably other PGRPs), the bacteria can efficiently employ its virulence factors. Thus, protection of PGN from Host receptors is paramount for bacteria to establish an infection. Accordingly, the subsequent findings corroborated these interpretations and conclusions.

I next addressed how the Host discriminates (or not) different PGN types. I showed that PGRP-SA and PGRP-LC can recognise both Lys- and DAP- type PGN and act as bacterial receptors of both phagocytosis and late induced AMPs responses. Upon recognition of PGN by PGRP-SA and PGRP-LC, there is the triggering of phagocytosis. It is likely that bacteria that survive the phagocytosis-clearance processes, are again recognised by both PGRPs which trigger the TOLL and IMD cascades. This results in the expression of the AMPs, whose primary function is to kill the remaining bacteria and protect the Host from reincidence of infection.

Regarding the phagocytic processes, these proteins appear to not only act as receptors but also to be involved in the clearance of the bacteria. The results suggest that upon phagocytosis, the bacteria-PGRPs complex are internalised into the phagosome and seems to continue to the stage of the phagolysosome. At this structure, with a low and optimal pH for PGRP-SA binding and lytic activity, the protein helps in the cleavage of the PGN. The biological reason for this lytic activity to be best at low pH may have to do with the fact that it helps to eliminate the bacteria in a controlled manner. When inside a vesicle, the products released from PGRP-SA cleavage are contained in an enclosed environment. If PGRP-SA would possess a high activity at the haemolymph`s pH, it could be detrimental for

the Host as it would perhaps accumulate bacterial debris and provoke toxicity and acute inflammation. As PGRP-SA is promiscuous for different PGN types and since the glycans are the most conserved region of the macromolecule, PGRP-SA probably cleaves the glycan backbone.

Moreover, the data points towards two phagocytosis-clearance pathways employed by macrophages: 1) phagocytosis-exocytosis and 2) phagocytosis-apoptosis. The phagocytosis-exocytosis is the canonical route and it occurs only when the bacteria is not pathogenic and when it is a bacteria that can be easily destroyed in the phagolysosomes. Under certain cases, it is likely that the phagocytosis-exocytosis might end up in the phagocytosis-apoptosis route. For instance, under stress conditions that the macrophages may feel compromise in the clearance processes, or if it is the case that even a very susceptible bacteria may be present in very high numbers. The phagocytosis-apoptosis seems to be triggered under two different situations. One is in response to the pathogenicity of the bacteria. It is a protective mechanism to avoid a death caused by the bacterial factors which would result in an uncontrolled death and therefore, the inability to control the infection. Through apoptosis, the macrophages aim to contain the already internalized bacteria and drive them into a new process of phagocytosis. The other situation occurs when non-pathogenic bacteria are present in high number. In this scenario, the macrophages are saturated with bacteria at their surface ready to be phagocytosed whilst accumulating the phagosomes/phagolysosomes to clear the bacteria. As the rate of clearance does not level up with the amount of bacteria to be internalised, the macrophages trigger apoptosis.

As a result of apoptosis, the bacteria are contained inside the apoptotic bodies. These bodies may present at their surface bacterial components originated from the initial bacterial degradation. These would serve as ligands for the phagocytic receptors, thus promoting the phagocytosis and hence the clearance process by other macrophages. It is

likely that this process of phagocytosis-apoptosis repeats itself, until complete clearance of the bacteria.

In addition, efficient early and late immune responses are dependent on the access of PGRP-SA and PGRP-LC to the PGN. Bacteria that have a more exposed PGN are easier to be detected thus, in terms of the time course of infection, as the responses can be triggered more quickly, the resolution of the infection is quicker for the Host. Also, a more exposed PGN renders the bacteria more susceptible both to the destruction by the Host lytic enzymes, via the phagocytosis-clearance route and to the activity of the late induced AMPs.

I propose a model whereby from the side of the bacteria, the WTA and the OM are conserved immune evasion mechanisms that are transversal to bacteria. They compose a protective barrier that shields the PGN from the outer environment. It is the access of the PGRPs through the cell wall that dictates the recognition of the PGN. From the side of the Host, I propose a model whereby it possesses two major receptors, PGRP-SA and PGRP-LC. They are promiscuous to different types of PGN composition and they participate in both cellular-early and humoral-late responses.

I believe that the work presented in this thesis helps to re-shape the current model of bacterial detection in the Fly and perhaps, at some level, it may be possible to extrapolate for other Hosts. I hope that it opens up new questions that will bring answers regarding how we perceive bacteria, how do they hide and subvert our protective responses and what can we do to achieve a balance when this interaction is detrimental to us, Humans, as Hosts.

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