NimB2 Enhances *Staphylococcus aureus* Recognition by Macrophages in *Drosophila*

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By

Prince Kumar Sah

Indian Institute of Science Education and Research Pune

Dr. Homi Bhabha Road,

Pashan, Pune 411008, INDIA.

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Under the guidance of

Supervisor: Prof. Bruno Lemaitre,

Professor, Global Health Institute
School of Life Science, École Polytechnique Fédérale de Lausanne

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Certificate

This is to certify that this dissertation “NimB2 Enhances *Staphylococcus aureus* Recognition by Macrophages in *Drosophila*” towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents work carried out by Prince Kumar Sah at École Polytechnique Fédérale de Lausanne, EPFL under the supervision of Prof. Bruno Lemaitre, School of Life Science, during the academic year 2024-2025.

Lausanne March 13rd 2025

Prof. Bruno Lemaitre
Global Health Institute, SV, EPFL

Prof. Girish Ratnaparkhi
Department of Biology, IISER Pune

This thesis is dedicated to my elder brother for his unwavering support and encouragement throughout my journey.

Declaration

I hereby declare that the matter embodied in the report entitled “NimB2 Enhances *Staphylococcus aureus* Recognition by Macrophages in *Drosophila*” are the result of the work carried out by me at the school of Life Science, École Polytechnique Fédérale de Lausanne (EPFL), under the supervision of Prof. Bruno Lemaitre and the same has not been submitted elsewhere for any other degree. Wherever others contribute, every effort is made to indicate this clearly, with due reference to the literature and acknowledgement of collaborative research and discussions.

Prince Kumar Sah

Prince Kumar Sah

Roll No: 20201009

Date: 16-03-2025

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Abstract

The innate immune system relies on rapid recognition and clearance of pathogens to maintain homeostasis. Phagocytosis, a crucial defence mechanism, enables the elimination of both microbial invaders and apoptotic cells. In *Drosophila melanogaster*, haemocytes act as professional phagocytes, coordinating immune responses against infections. While several regulators of phagocytosis have been identified, the role of NimB2 in this process remains unexplored. Here, we investigate the function of NimB2 in bacterial clearance, specifically its role in recognizing and eliminating *Staphylococcus aureus*. Using *NimB2* null mutant and infection assays, we show that the loss of *NimB2* significantly impairs *S. aureus* clearance, leading to increased bacterial load and heightened susceptibility to infection. Flow cytometry and microscopy analyses confirm a phagocytic defect in *NimB2* mutants, highlighting its role in haemocyte-mediated bacterial uptake. Furthermore, our findings suggest that NimB2 functions as an opsonin, facilitating the recognition of *S. aureus* by haemocytes and promoting efficient bacterial clearance. This study identifies NimB2 as a secreted Nimrod protein essential for *S. aureus* phagocytosis and provides new insights into its role in cellular immunity.

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Finally, I would like to thank each and every member of the Lemaitre lab for their kindness and support, which made my time here an enriching and enjoyable experience.

Contributions

Contributor name	Contributor role
Prof. Bruno Lemaitre, Prince Kumar Sah	Conceptualization Ideas
Prof. Bruno Lemaitre, Prince Kumar Sah	Methodology
-	Software
Prince Kumar Sah	Validation
Prince Kumar Sah	Formal analysis
Prince Kumar Sah	Investigation
EPFL, PT-BIOP, PTCF and PTPSP	Resources
-	Data Curation
Prince Kumar Sah	Writing - original draft preparation
Prof. Bruno Lemaitre	Writing - review and editing
Prof. Bruno Lemaitre	Visualization
Prof. Bruno Lemaitre	Supervision
Prof. Bruno Lemaitre	Project administration
Prof. Bruno Lemaitre	Funding acquisition

Chapter 1. Introduction

In all multicellular organisms, innate immunity acts as the initial defence mechanism, providing a rapid and essential response against microbial infections by recognizing and eliminating potential threats before they establish an infection(Alberts *et al.*, 2002). Unlike adaptive immunity, which is found in vertebrates and involves antigen-specific responses, innate immunity is evolutionarily ancient and relies on germline-encoded receptors to detect conserved microbial signatures. This system is highly conserved across species, from insects to mammals, underscoring its fundamental role in host survival. A key aspect of this defence is phagocytosis, a well-preserved cellular process where immune cells engulf and break down foreign particles—such as bacteria, fungi, and apoptotic cells—by forming a membrane-derived vesicle called a phagosome.(Mechnikov *et al.*, 1988). Figure 1.1 presents a schematic illustration of this process. Phagocytosis is an essential mechanism for host defence and immune homeostasis, as it helps in the clearance of pathogens and antigen presentation to adaptive immune components(Rosales and Uribe-Querol *et al.*, 2017). *Staphylococcus aureus* (*S. aureus*) is one of the most clinically significant pathogens encountered by the immune system, causing a broad spectrum of infections ranging from mild skin conditions to severe diseases like pneumonia, sepsis, and endocarditis.(Tong *et al.*, 2015). Understanding the molecular mechanisms governing the recognition and engulfment of *S. aureus* is crucial for elucidating host-pathogen interactions and identifying novel therapeutic targets.

Drosophila melanogaster, commonly known as the fruit fly, has become a valuable model organism for studying innate immune responses, as its conserved immune signalling pathways closely resemble those found in mammals. (Lemaitre *et al.*, 2007, Westlake *et al.*, 2024). The immune response in *Drosophila* functions as a multi-layered defence mechanism, safeguarding the organism against microbial invasion. A physical barrier, consisting of the chitinous cuticle, tracheal system, and intestinal epithelium, serves as the first line of defence. When these barriers are breached, a local immune response is activated, leading to antimicrobial peptides (AMPs) production and reactive oxygen species (ROS) to combat pathogens (Banerjee *et al.*, 2019; Rizki and Rizki *et al.*, 1984; Tang *et al.*, 2023). If the infection

spreads into the haemolymph, *Drosophila* triggers a systemic response that integrates both cellular and humoral elements. Cellular immunity, involving key processes like phagocytosis and encapsulation, acts to directly eliminate pathogens, whereas humoral immunity involves the production and secretion of AMPs and other immune effectors into the haemolymph, where they target pathogens and prevent further proliferation(Davis and Engström *et al.*, 2012). Figure 1.2 presents a schematic depiction of these immune pathways. This multi-layered system allows *Drosophila* to respond efficiently to diverse microbial threats, making it a robust model for studying immune function and pathogenesis. The conserved nature of these immune pathways makes *Drosophila* an excellent system to explore how innate immunity has evolved to protect against a wide variety of pathogens, providing valuable insights into immunity in higher organisms.

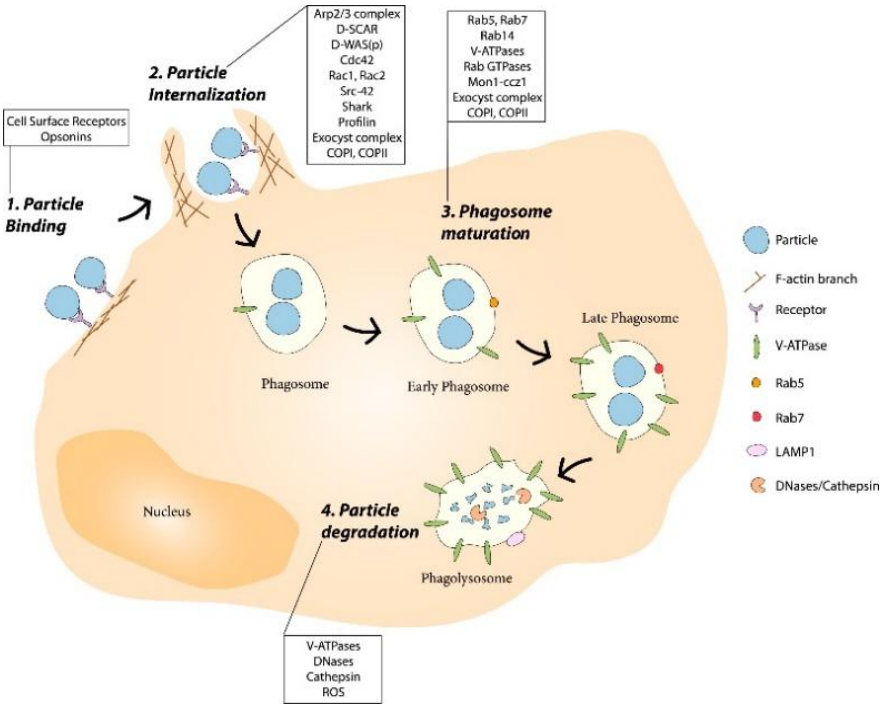

Figure 1.1: The Process of Phagocytosis in Macrophages. *The Process of Phagocytosis in Macrophage. Phagocytosis begins with the recognition and binding of the target particle to the macrophage surface. Upon binding, the macrophage undergoes actin actin cytoskeletal rear. Image derived from (Melcarne et al., 2019).*

Drosophila's humoral immune response plays a vital role in defending against diverse pathogens and is primarily regulated by the fat body, an essential immune organ functionally comparable to the mammalian liver. When a pathogen is detected,

the fat body initiates key immune pathways, including the Toll and Imd pathways, leading to the production of antimicrobial peptides (AMPs) that aid in fighting infections. (Imler and Bulet et al., 2005). Toll pathway is primarily triggered by Gram-positive bacteria and fungi, which initiates the subsequent production of genes encode AMPs like *Drosomycin* and *Bomanins*, which target and neutralize the invading microbes(Lemaitre et al., 1996 ; Lemaitre et al., 2007; Westlake et al., 2024). Alongside AMP production, this pathway also regulates other immune responses, including melanisation, clotting, and haematopoiesis, ensuring a coordinated defence response(Rutschmann et al., 2005; Qiu et al., 1998; Schmid et al., 2014). Unlike the Toll pathway, the Imd pathway mainly responsible for the immune-response against Gram-negative bacteria and works in tandem with the Toll pathway to ensure that the fat body produces the necessary AMPs to control infections(Uttenweiler-Joseph et al., 1998). The molecular components and signalling cascades involved in these pathways are illustrated in Fig 1.3. Importantly, the fat body also regulates the production of transferrin, which plays a critical role in limiting pathogen growth by sequestering iron, an essential nutrient for microbial survival, thereby reducing the pathogen's ability to grow(Iatsenko et al., 2020). Together, these processes create an intricate and highly effective systemic immune response that enables *Drosophila* to respond to various pathogens efficiently.

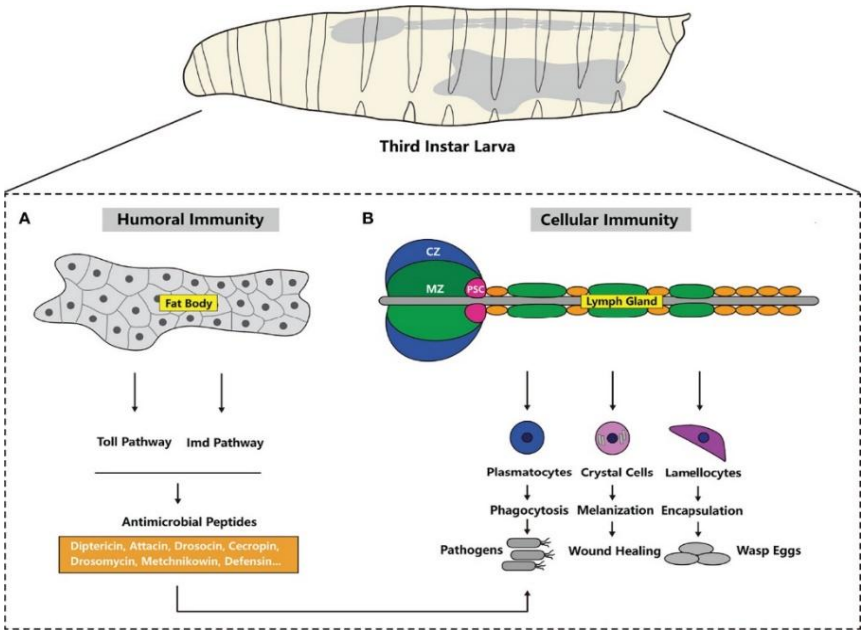

Figure 1.2: General description of Drosophila Immunity. *Drosophila immunity is composed of both cellular and humoral responses. The humoral response is activated upon bacterial*

infection, leading to the induction of the Toll and IMD pathways, which regulate the antimicrobial peptides (AMPs) production that combat bacterial pathogens. The cellular response involves various haemocyte types: plasmatocytes, which are responsible for phagocytosis; crystal cells, which contribute to melanisation and wound healing; and lamellocytes, which encapsulate foreign objects like parasitoid wasp eggs. Image derived from (Yu et al., 2022).

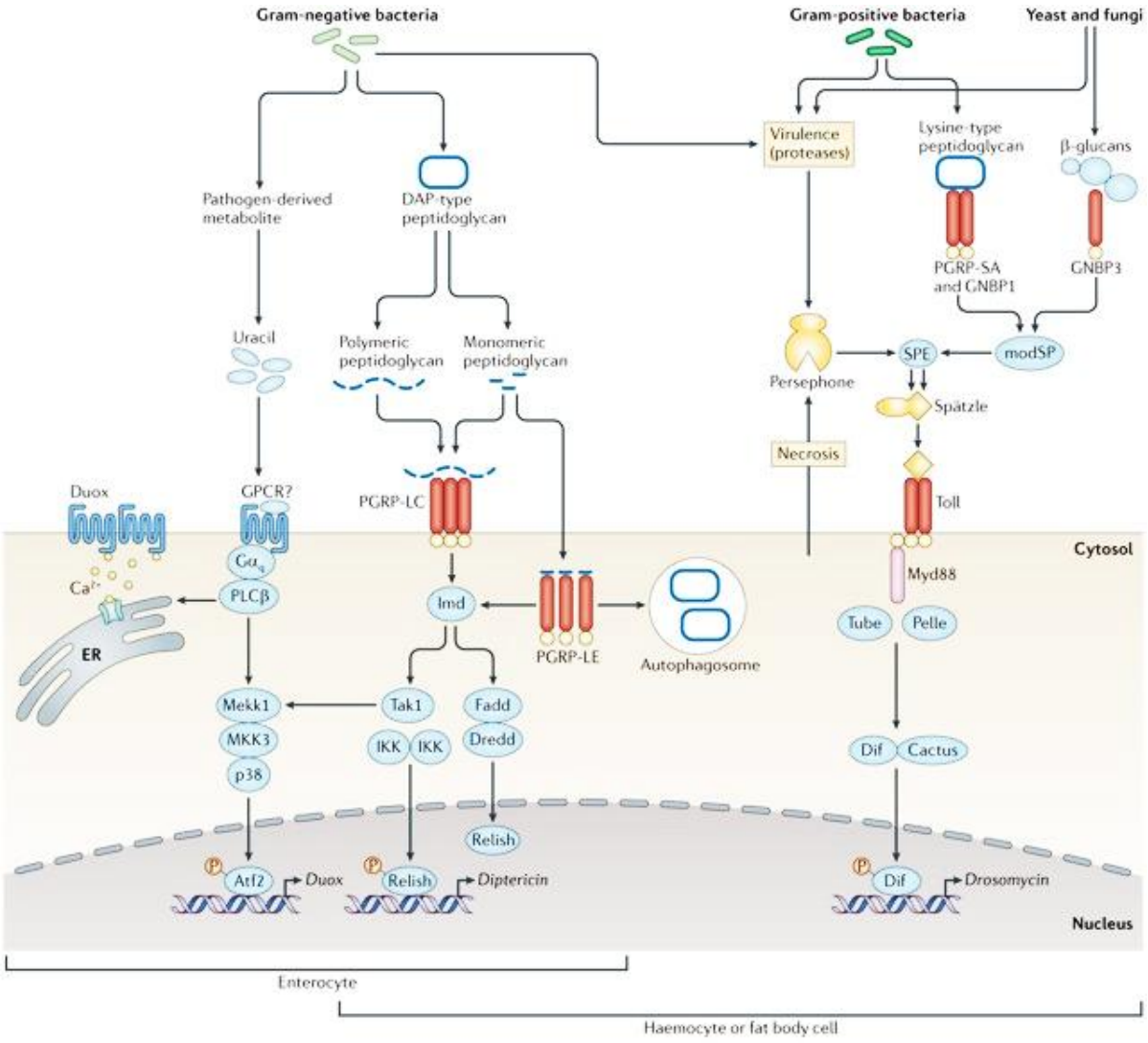

Figure 1.3: Molecular Mechanism of Toll and IMD Pathways in Drosophila Immunity. *The Toll and IMD pathways are essential for the humoral immune response in Drosophila. The Toll pathway is mainly triggered by fungal and Gram-positive bacterial infections, activating the NF-κB transcription factor Dif through a signalling cascade involving Spätzle, Toll, MyD88, Tube, Pelle, and the degradation of Cactus. In contrast, the IMD pathway is activated by Gram-negative bacterial infections, leading to the activation of the NF-κB transcription factor Relish via the sequential activation of PGRP-LC, IMD, FADD, Dredd, and the TAK1-TAB2 complex. Both pathways ultimately result in the transcriptional activation of antimicrobial peptides (AMPs), which help to neutralize and eliminate the invading pathogens. Image adapted from (Buchon et al., 2014).*

Drosophila possesses special cells called haemocytes that are highly versatile and play a role in various processes, including development, immunity, metabolism, and wound healing (Honti *et al.*, 2014 ; Hultmark and Andó *et al.*, 2022). These haemocytes originate during embryonic development, where the first wave of primitive haematopoiesis generates a few hundred blood cells that populate the embryo. This is followed by a second phase of definitive haematopoiesis in the larval lymph gland, a specialized hematopoietic organ that functions as a reservoir for blood cells. During larval development, these haemocytes proliferate extensively, leading to the formation of approximately 5,000 circulating and sessile haemocytes by the third instar(Banerjee *et al.*, 2019). These haemocytes are distributed across three major compartments: the lymph gland, which serves as a reservoir for undifferentiated progenitors; the circulating haemolymph, where immune surveillance and rapid response occur; and sessile patches, which are localized aggregates of haemocytes found between the cuticle and muscle layers, often acting as immune hubs(Crozatier and Meister *et al.*, 2007).

The primary haemocyte types include plasmatocytes, which comprise around 95% of the haemocyte population and function similarly to macrophages by engulfing pathogens and apoptotic cells, and crystal cells, which contain crystallized prophenoloxidases essential for melanisation and wound healing(Lanot *et al.*, 2001). Additionally, in larvae, lamellocytes can differentiate in response to immune challenges such as parasitic infection, particularly during wasp infestation, where they encapsulate foreign invaders through a specialized immune reaction(Anderl *et al.*, 2016). The balance and regulation of these haemocyte populations are crucial for maintaining immune homeostasis, and disruptions in their function can compromise the ability of *Drosophila* to mount effective immune responses.

Plasmatocytes, as mentioned, they are analogous to mammalian macrophages, play a vital role in pathogen recognition, phagocytosis, and antimicrobial peptide production. These immune cells utilize a diverse array of phagocytic receptors to detect and internalize microbes(Fu and Harrison et al., 2021). Some well-known phagocytic receptors, including Eater and Draper, are essential for the phagocytosis of *S. aureus* and apoptotic cells in *Drosophila*(Shiratsuchi et al., 2012 ; Kocks et al., 2005). Additionally, SIMU and Draper bind to phosphatidylserine on apoptotic cells, a

well-known "eat-me" signal, initiating signalling pathways that alter the migration and expression profile of haemocytes, further modulating the immune response(Savill and Gregory, 2007 ; Roddie et al., 2019 ; MacDonald et al., 2006). The function of various phagocytic receptors in apoptotic cell and bacterial clearance is depicted in Fig 1.4, highlighting the distinct receptors involved in recognizing and internalizing different targets. These receptors operate by binding to pathogen-associated molecular patterns (PAMPs), triggering actin cytoskeletal rearrangement and the formation of a phagocytic cup that engulfs the pathogen. Once a pathogen or apoptotic cell is recognized by these phagocytic receptors, the process of engulfment begins. This involves actin cytoskeletal rearrangement that forms a phagocytic cup, which envelops the target. The internalized material is then enclosed in a phagosome, which undergoes maturation through fusion with lysosomes, forming a phagolysosome. Within this compartment, pathogens are degraded by hydrolytic enzymes and antimicrobial peptides, which are crucial for neutralizing the threat(Günther and Seyfert et al., 2018).

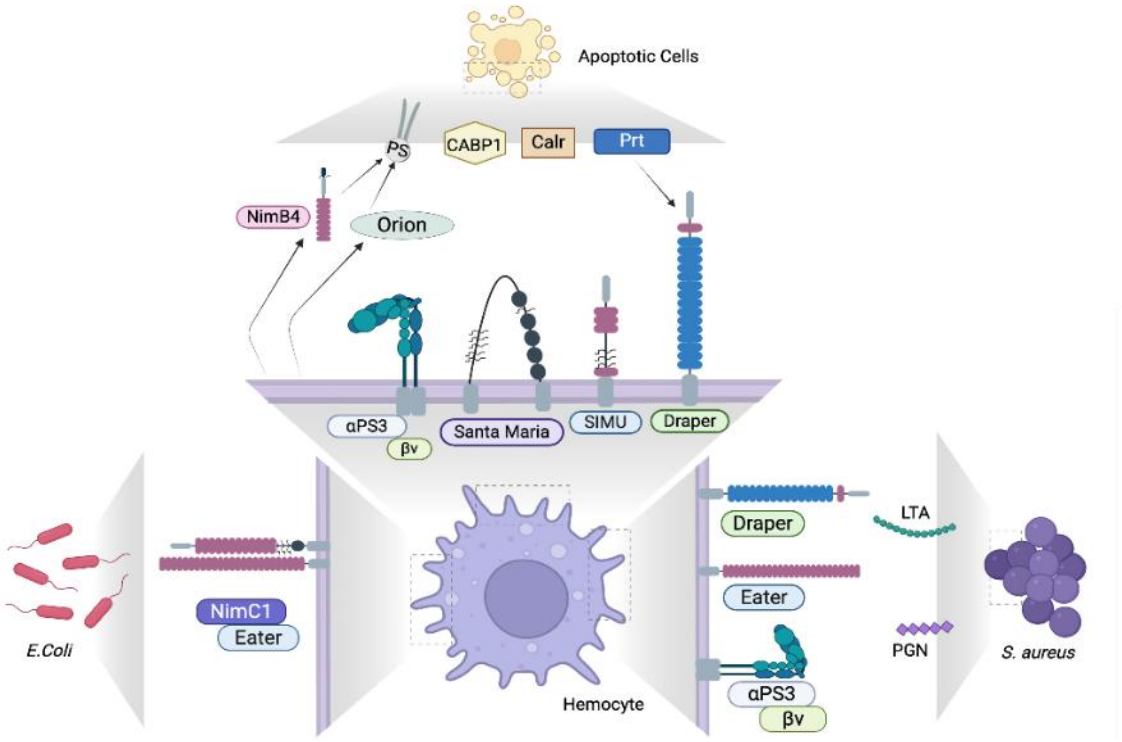

Figure 1.4: Phagocytic Receptors Involved in Apoptotic Cell and Bacterial Clearance. *This figure illustrates the key receptors mediating phagocytosis in Drosophila. Draper, SIMU, Santa Maria, and αPS3βv facilitate the recognition and ingestion of apoptotic cells. Additionally, NimB4 and Orion function as opsonin, enhancing phagocytosis. For bacterial clearance,*

Draper, Eater, and αPS3βv are involved in the phagocytosis of S. aureus, while NimC1, working alongside Eater, contributes to the phagocytosis of E. coli. Image derived from (Westlake et al., 2024).

Opsonins and bridging molecules, such as thioester-containing proteins(TEPs) and Orion, respectively, play a vital role in improving the efficiency of phagocytosis by marking pathogens and apoptotic cells for recognition by phagocytic receptors. These opsonin binds to specific molecular markers on the surface of pathogens or dying cells, like phosphatidylserine, and facilitate their recognition by receptors like Eater and Draper. This opsonization process significantly increases the likelihood of efficient pathogen uptake and internalization by plasmatocytes, as we observe with TEPs(Dostálová *et al.*, 2017). Orion bridges phosphatidylserine and glial Draper, contributing to the immune response(Ji *et al., 2023*). The cooperative action of opsonins and phagocytic receptors, therefore, enhances the immune response by promoting efficient clearance of pathogens and apoptotic cells, while also modulating immune signalling to ensure a balanced immune reaction. This interplay between opsonins and receptors is essential for maintaining immune homeostasis and pathogen defence in Drosophila.

The majority of phagocytic receptors are part of the Nimrod domain family(Kocks *et al.*, 2005 ; Shiratsuchi *et al.*, 2012 ; Melcarne *et al.*, 2019), which is characterized by the presence of Nimrod (NIM) repeats, a distinct type of epidermal growth factor (EGF) repeat typically linked to adhesion, coagulation, and receptor-ligand interactions. (Kurucz *et al.*, 2007 ; Callebaut *et al.*, 2003). These proteins possess a signal peptide followed by N-terminal motifs, with the first NIM repeat typically positioned immediately after a CCxGY amino acid motif. The Nimrod gene family is categorized into three distinct types based on their domain structure. Draper-type proteins, such as NimA and Draper, contain a single NIM domain followed by a variable number of EGF repeats and an Emilin (EMI) domain at the N-terminal end. Other members, known as poly-NIM proteins, are further divided into two subtypes: Nimrod C-type and Nimrod B-type. Nimrod C-type proteins are transmembrane proteins with multiple NIM repeats but no EGF domains, while Nimrod B-type proteins have similar characteristics but lack the transmembrane domain.(Kurucz *et al.*, 2007).

The Nimrod B proteins, comprising five members (NimB1–NimB5), are of particular interest due to their possible involvement in *Drosophila's* immune system and efferocytosis. Studies in other insects suggest that orthologs of *Drosophila* NimB proteins play an important role in immune responses, particularly in phagocytosis(Estévez-Lao and Hillyer *et al.*, 2014; Ju *et al.*, 2006). NimB1 and NimB4 play distinct roles in phagocytosis as an opsonin, specifically in the context of apoptotic cell clearance. NimB1 and NimB4 are essential regulators in the phagocytosis of apoptotic cells, but they function in opposing ways. NimB1, secreted by macrophages, inhibits the recognition and engulfment of apoptotic cells, thereby restricting their internalization and controlling the early phases of efferocytosis.(Dolgikh *et al.*, 2025). On the other hand, NimB4 acts as an opsonin, enhancing the recognition and clearance of apoptotic cells by macrophages(Petrignani *et al.*, 2021). The balance between these two proteins helps maintain tissue homeostasis by ensuring optimal rates of apoptotic cell clearance, avoiding excessive impaired clearance. Among NimBs, Nimrod B2 (NimB2) stands out as the most conserved, exhibiting strong evolutionary conservation across species(Somogyi *et al.*, 2008). However, its precise function in immune defence remains largely unexplored.

In *Anopheles gambiae*, the NimB2 ortholog (AgNimB2) has been implicated in the phagocytosis of *Staphylococcus aureus*. Interestingly, its role appears to be indirect, functioning as an opsonin rather than directly binding to *S. aureus*, it binds to TEP protein. In contrast, AgNimB2 identifies host-derived proteins on the pathogen's surface, promoting its uptake by phagocytic cells. (Midega *et al.*, 2013). Given the central role of phagocytosis in *Drosophila's* cellular immune response, it is plausible that NimB2 contributes to pathogen recognition and clearance, potentially acting in coordination with other opsonins, such as thioester-containing proteins (TEPs) and Nimrod family members. Furthermore, opsonization is a crucial strategy across diverse insect species, suggesting that NimB2 may be part of a conserved immune mechanism that enhances pathogen uptake by phagocytic receptors. Investigating NimB2's role in *S. aureus* infection will provide critical insights into how the host neutralizes and eliminates the pathogen at the molecular level.

In this study, we explored the role of NimB2 in phagocytosis, aiming to elucidate its contribution to *Drosophila's* cellular immune response. Our findings demonstrate

that NimB2 is specifically required for the recognition and clearance of *Staphylococcus aureus*, but not other tested bacteria. The absence of NimB2 in mutant flies led to a significant defect in the phagocytosis of *S. aureus*, resulting in an increased bacterial load. Notably, in NimB2 mutant flies, the failure to efficiently clear *S. aureus* allowed the bacteria to proliferate unchecked, ultimately reaching lethal levels. This highlights the critical role of NimB2 in early infection containment. Given its function in facilitating bacterial recognition, we propose that NimB2 acts as an immune opsonin, potentially interacting with known phagocytic receptors to enhance pathogen uptake by haemocytes. These findings provide new insights into how secreted Nimrod proteins contribute to host defence mechanisms in *Drosophila*.

Chapter 2. Materials and Methods

2.1 Fly stocks and genetics

The flies were reared on a standard medium consisting of 6% cornmeal, 6% yeast, 0.62% agar, and 0.1% fruit juice, with the addition of 10.6 g/L mold inhibitors and 4.9 ml/L propionic acid to prevent fungal contamination. The stocks were maintained at 25°C under a 12-hour light/dark cycle. Wandering third instar larvae (110–120 hours after egg laying, AEL) and mid-L3 larvae (96–110 hours AEL) were selected for the experiments. Homozygous mutants were used in all experiments, with w^{1118} Drosdel (w^{iso}) flies as the control. A complete list of the fly lines utilized is provided in Table 2.1.

Table 2-1: Genotypes of the fly lines majorly used

Stock Genotype	Chromosome number	Source
NimB2$^{ds\text{-}red}$	2	This Study
Def(All_NimBs)$^{1\text{-}2}$	2	This Study
NimB2$^{\Delta 49}$/cyo	2	This Study
NimB2$^{\Delta 442}$/cyo	2	This Study
;Hml-Gal4, UAS-GFP;	2	(Sinenko and Mathey-Prevot, 2004)
;Hml-Gal4,UAS-GFP,NimB2$^{ds\text{-}red}$;	2	This Study
Act/cyo	2	Bloomington
Lpp-Gal4^{ts}/sb	3	Bloomington
NimB2-Gal4	2	(Diao *et al.*, 2015)
UAS-CAMPARI	2	(Moeyaert *et al.*, 2018)
Rel^{E20}	3	(Neyen *et al.*, 2014)
Bom$^{\Delta 55c}$	2	(Clemmons *et al.*, 2015)
PPO1$^{\Delta}$2$^{\Delta}$	3	(Binggeli *et al.*, 2014)

Spz^{rm7}	3	(Neyen *et al.*, 2014)
NimC1^{1};Eater^{1}	2,3	(Melcarne *et al.*, 2019b)

2.2 Microscopy

2.2.1 Haemocyte Microscopy

Third-instar larvae (n=5) were cleaned and dissected in 150 µL of Schneider's medium, ensuring that vital organs remained intact. The samples were incubated in a humidified chamber for 45 minutes to allow haemocytes to settle and adhere to slides. The cells were then fixed with 4% paraformaldehyde (PFA) and stained with phalloidin (1:200) for 1 hour at room temperature, followed by three washes with 1X PBS. DAPI (1:20,000) was added for 10 minutes at room temperature, and the samples were washed three more times with 1X PBS. Finally, the slides were mounted with a mounting medium and sealed using nail polish.

2.2.2 Imaging and Quantification

Haemocytes were imaged using an inverted Olympus confocal microscope equipped with a 60x oil immersion lens. Maximum Z-projections were created for analysis, and image quantification was carried out using FIJI/ImageJ software.

2.3 Haemocyte Counting by Flow Cytometry

Haemocytes from *HmlGal4>UAS-GFP* larvae were used to characterise the haemocyte population in flow cytometry analysis. GFP-expressing haemocytes were gated using the B525 channel to exclude debris and non-haemolytic cells. This gating strategy was then applied across all genotypes to quantify total haemocyte counts and assess the proportion of GFP-positive cells.

For each genotype, six wandering third-instar (L3) larvae were bled into 150 µL of Schneider's insect medium. Before bleeding, larvae were vortexed in 1X PBS for 1 minute to dislodge sessile hemocytes. Approximately 60 µL of the hemocyte suspension was then analyzed by flow cytometry.

2.4 Phagocytosis Assay

2.4.1 Ex vivo Phagocytosis Assay

For ex vivo phagocytosis assays, five third-instar larvae were surface-sterilized and bled into 120 µL of Schneider's medium within 2 minutes. The haemolymph was collected in Protein Lo-Bind Eppendorf tubes to minimize sample loss. Approximately 20,000 apoptotic bodies or 10^5 bacterial bioparticles were added to the haemocyte suspension and incubated at room temperature for 1 hour. After incubation, the samples were placed on ice, and the haemocytes were immediately analyzed using a CytoFLEX flow cytometer (Beckman Coulter).

The phagocytic index (PI) was calculated using the following equations:

$$f = \frac{\text{No. of hemocytes in fluorescence positive gate}}{\text{Total number of hemocytes}}$$

$$PI = \text{Mean fluorescence intensity of hemocytes in fluorescence positive gate} * \text{f}$$

2.4.2 In vivo Phagocytosis Assay

For in vivo phagocytosis assays, approximately 10^5 bacterial bioparticles or live bacterial cells were injected into wandering third-instar larvae using a Nanoject microinjector. Injections were performed laterally into the haemolymph at the posterior end of the larvae to ensure systemic distribution of the bacteria. After injection, larvae were maintained at 25°C for 2 hours to allow phagocytosis to occur under physiological conditions. Following incubation, larvae were washed in 1X PBS, dissected in Schneider's insect medium, and haemocytes were collected onto glass slides. The cells were then fixed with 4% paraformaldehyde (PFA) for 15 minutes at room temperature, following the procedure outlined in the Haemocyte Microscopy section. After fixation, haemocytes were washed three times with 1X PBS to remove unbound bacteria and debris. The internalization of bacterial particles within haemocytes was assessed using confocal microscopy. For quantitative analysis, at least 100 haemocytes per experimental condition were examined, and the percentage of haemocytes containing fluorescent bacterial particles was calculated.

2.5 Binding Assay

To evaluate bacterial or apoptotic cell binding, haemocytes were collected by bleeding wandering third-instar larvae into 120 µL of cold Schneider's insect medium on a pre-chilled glass slide to ensure the preservation of cell integrity. Following the collection of haemolymphs, approximately 10^5 live fluorescent bacteria or apoptotic cells were immediately added to the haemocyte suspension to allow for binding interactions. The suspension was incubated on ice for 60 minutes to promote the attachment of the bacteria or apoptotic cells to the haemocyte surface under controlled conditions. After the incubation period, the samples were fixed using paraformaldehyde and stained according to the procedure described in the haemocyte microscopy section, ensuring clear visualization of the bound particles. The number of bound bacteria or apoptotic cells per haemocyte was quantified using a confocal microscope. For each experimental condition, 50 haemocytes per genotype were analyzed, allowing for a detailed comparison of binding efficiency across different genotypes. This quantification provided insight into the interactions between haemocytes and pathogens or apoptotic cells, which are key to understanding immune responses.

2.6 Infection Assay

Bacterial cultures were prepared by growing the respective bacterial strains overnight in Luria Broth (LB) medium. The following bacterial strains were used for infection experiments, with their corresponding optical densities at 600 nm (OD_{600}): *Enterococcus faecalis* (OD_{600} = 5), *Escherichia coli* (OD_{600} = 200), *Erwinia carotovora carotovora* strain 15 (Ecc15, OD_{600} = 200), and *Staphylococcus aureus* (OD_{600} = 0.5). *E. faecalis* and *S. aureus* were cultured at 37°C, while *Ecc15* was grown at 29°C. After overnight cultivation, bacterial pellets were harvested by centrifugation and then resuspended in 1X PBS for subsequent use in infection assays.

For systemic infection, adult flies aged 5–10 days were anesthetized and pricked in the thorax using a fine needle (0.2 mm in diameter) that was pre-soaked in the concentrated bacterial suspension. This method ensures that the bacteria are introduced directly into the systemic circulation of the flies, allowing for an efficient infection model to assess immune responses.

2.6.1 Survival Assay

Survival experiments were conducted under the conditions specified in Table 2.2. To distinguish between mortality caused by infection and that caused by injury, control flies were pricked with a sterile needle to induce a clean injury without infection. Each experimental group consisted of 20 flies per genotype, with three biological replicates. Survival rates were monitored and recorded daily until all flies had succumbed to the experimental conditions. This approach allows for a clear assessment of the effect of bacterial infection on fly survival while accounting for potential injury-related mortality.

Table 2-2: Maintenance of flies post infection

Bacteria	Condition
E. faecalis	12 days at 25°C
Ecc15	12 days at 29°C
E. coli	12 days at 29°C
S. aureus	12 days at 25°C
Clean injury	12 days at 29°C

2.7 Quantitative PCR (RT-qPCR)

To quantify mRNA expression levels, whole flies were collected at the designated time points. Total RNA was extracted from 10–12 adult flies per sample using TRIzol reagent (Invitrogen), following the manufacturer's instructions. The RNA quality and integrity were assessed by measuring the A260/A280 ratio using a Nanodrop spectrophotometer (Thermo Fisher Scientific). For reverse transcription, 500 ng of RNA was used in a 10-µL reaction mixture, utilizing the PrimeScript RT reagent kit (TAKARA), which included a combination of oligo-dT and random hexamer primers.

Quantitative PCR was conducted using cDNA on a LightCycler 480 system (Roche) in 96-well plates. The reaction utilized the LightCycler 480 SYBR Green I Master Mix (Roche), with SYBR Green I serving as the dsDNA-binding dye. Relative gene expression levels were determined using the ΔΔCt method, with normalization to the housekeeping gene Rp49. Each sample was analyzed in technical duplicates, and at least three biological replicates were performed for each condition. The primer sequences used for qPCR analysis are provided in Table 2.3.

Table 2-3: Primer sequences used for qPCR

Name	Target	Sequence
NimB2 exon 1 F	NimB2 exon 1	5'-TAT GGA CAC TAC GTG CAG CC-3'
NimB2 exon 1 R	NimB2 exon 1	5'-TTG TAG CAC ACA CCA CTG GC-3'
NimB2 exon 2 F	NimB2 exon 2	5'-TCA GTT CGT TGG CAA TGG CA-3'
NimB2 exon 2 R	NimB2 exon 2	5'-GGC GGT AGA TGT GCG GAT TT-3'
NimB2 exon 3 F	NimB2 exon 3	5’-GAG TGT CTG CCG AAG TGT GA-3’
NimB2 exon 3 R	NimB2 exon 3	5’-TCA CAT ATC CGG TCT TGC AG-3’
Drs F	Drosomycin	5’-CGT GAG AAC CTT TTC CCA TAT GAT-3’
Drs R	Drosomycin	5’-TCC CAG GAC CAC CAG CAT-3’

2.8 Statistical Analysis

All experiments were performed in triplicate unless specified otherwise. The data are presented as mean ± standard deviation (SD), with error bars and individual data points shown. Statistical analyses were conducted using Microsoft Excel and GraphPad Prism v.9.

Each data point on the graphs represents a single biological replicate unless stated otherwise. Statistical significance was determined using an unpaired Student’s t-test for pairwise comparisons and a one-way ANOVA for comparisons involving multiple groups. The significance levels are indicated as follows: (ns = not significant, $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****)).

Table 2-4: Software used for data analysis and visualization

Software	Source
BioRender	BioRender

Fiji (Fiji is Just ImageJ)	Open source
GraphPad Prism v9	GraphPad Software Inc
SnapGene Viewer	Dotmatics
Leica Application Suite x (LAS X)	Leica Microsystems
IMARIS	Oxford Instruments

Chapter 3. Results

NimB2 is mainly expressed in the fat body

The fat body serves as a critical immune and metabolic organ in *Drosophila*, playing a central role in systemic immune responses. *NimB2* expression analysis, from FlyBase revealed that it is highly enriched in this tissue(Supplementary Fig. S-1)(FlyBase: The Drosophila Database, 1996). Using the *NimB2-Gal4* driver line from the Bloomington *Drosophila* Stock Center crossed with *UAS-CAMPARI*, a fluorescent reporter, we observed strong *NimB2* expression in the fat body of third-instar larvae (Fig. 3.1.A). To further confirm this, we dissected the fat body and visualized its fluorescence, verifying that *NimB2* transcription is primarily localized to this tissue (Fig. 3.1.B).

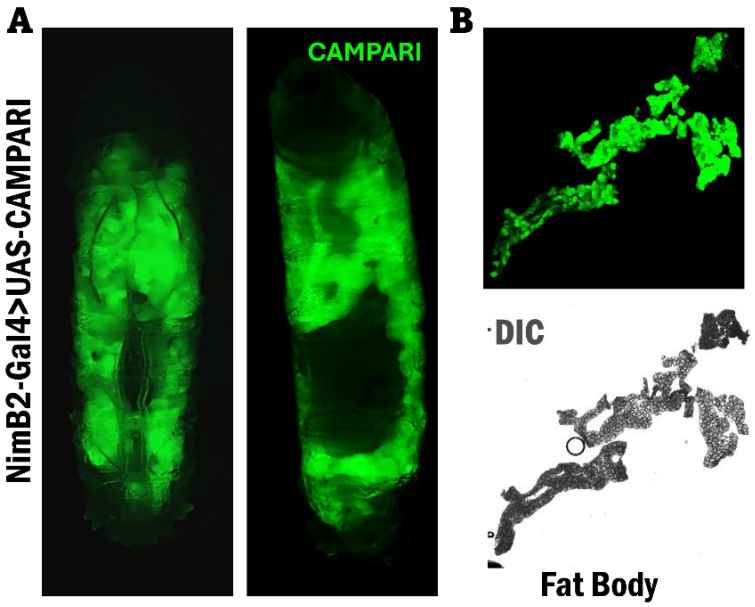

***Figure 3.1: Expression pattern of NimB2 in the fat body**. (A) NimB2-Gal4 was crossed with UAS-CAMPARI, a green fluorescent protein reporter, to visualize NimB2 expression in L3 larvae. (B) The dissected fat body from L3 larvae showed a CAMPARI signal in green fluorescence. The corresponding DIC image highlights the tissue structure.*

Additionally, quantitative PCR (qPCR) analysis further demonstrated that *NimB2* expression is enriched during the adult stage. Among the tissues examined in L3 larvae, the expression observed in the fat body was highest with qPCR, as shown in Supplemental Fig. S-1, highlighting its potential involvement in immune regulation during key developmental phases.

Generation of a *NimB2*$^{ds\text{-}red}$ null mutant by CRISPR-cas9

To explore the role of NimB2, we created a null mutation using CRISPR-Cas9. The NimB2 gene consists of three exons, and a targeted deletion of 290 bp was introduced in the second exon, leading to a premature stop codon and effectively disrupting *NimB2* function. Additionally, a *ds-red* selectable marker, driven by the 3xP3-hsp70 promoter, was inserted at the site of mutation, resulting in strong fluorescent expression in the larval and adult eyes. Fig. 3.2.A illustrates the specific region of the *NimB2* gene where the CRISPR-induced mutation was introduced, highlighting the deleted segment in exon 2.

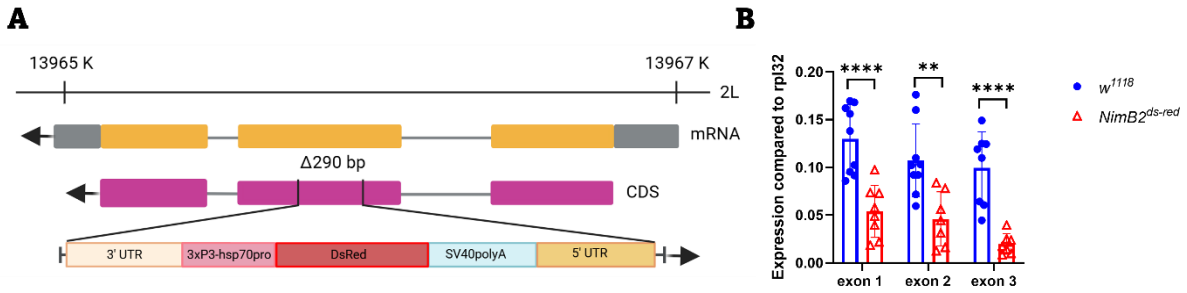

Figure 3.2: Gene targeting and deletion of NimB2. *(A) Schematic representation of the NimB2 gene structure, highlighting its three exons. The CRISPR-Cas9-induced 290 bp deletion in exon 2 is shown, along with the insertion of the ds-red selectable marker under the control of the 3xP3-hsp70 promoter. (B) Quantification of NimB2 expression across all three exons in w^{1118} (wild type) and NimB2$^{ds\text{-}red}$ mutants by qPCR, confirming the complete loss of NimB2 expression in the mutant line. The data are shown as mean ± SD from three independent experiments.*

To confirm that *NimB2*$^{ds\text{-}red}$ is a null allele, we performed a cDNA analysis targeting all three exons. qPCR analysis revealed a strong reduction in the expression across all exons in the mutant (Fig. 3.2.B), verifying that the mutation resulted in a complete loss of NimB2 function.

NimB2 Contributes to Resistance Against *S. aureus* Infection

To investigate the role of NimB2 in immune defence, we assessed the survival of *NimB2*$^{ds\text{-}red}$ and *Def(All_NimBs)*$^{1\text{-}2}$ flies following systemic bacterial infection.

Compared to wild-type controls, *NimB2*$^{ds\text{-}red}$ and *Def(All_NimBs)*$^{1\text{-}2}$ exhibited higher susceptibility to *S. aureus* infection, as indicated by their increased mortality rate (Fig. 3.3.A). This suggests that NimB2 plays a role in host defence against *S. aureus*. As a control for immune deficiency, *PPO1*$^{\Delta}$*2*$^{\Delta}$ mutant flies, which lack melanisation, displayed significantly reduced survival, consistent with previous findings.

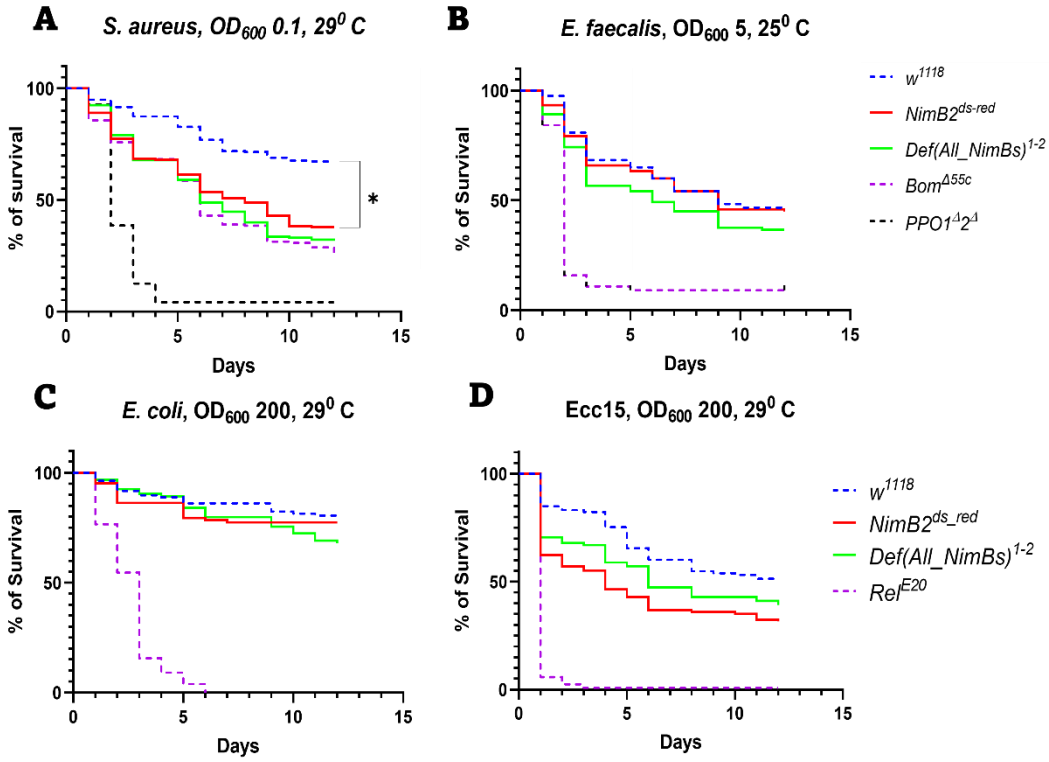

Figure 3.3: Survival analysis of flies upon bacterial infection*. Survival analysis of flies after systemic bacterial infection was conducted by pricking male flies in the thorax with a needle dipped in a concentrated bacterial culture. Survival was monitored over time, and data were analyzed using the log-rank test. Representative results from at least two independent biological replicates are shown (three replicates were conducted when significant differences were observed compared to control flies). The x-axis represents the time post-infection (days), while the y-axis shows the percentage of surviving flies. (a–b) Survival after septic injury with Gram-positive bacteria (E. faecalis, OD600 = 5, incubated at 25°C; S. aureus, OD600 = 0.1, incubated at 29°C). (c–d) Survival after septic injury with Gram-negative bacteria (E. coli and E. carotovora; OD600 = 200, incubated at 29°C).*

To determine whether this susceptibility was specific to *S. aureus*, we performed additional survival assays using the Gram-positive bacterium *E. faecalis* and the Gram-negative bacteria *E. coli* and *E. carotovora* (*Ecc15*). In these infections, *NimB2*$^{ds\text{-}red}$ and *Def(All_NimBs)*$^{1\text{-}2}$ showed no significant differences in survival compared to wild-type flies (Fig. 3.3.B-D). In contrast, *Rel*^{E20}, which lacks Imd pathway

activation, were highly susceptible to *E. coli* and *Ecc15*, while *Bom*$^{\Delta 55c}$ flies, which lack functional Toll pathway effectors, were highly susceptible to *E. faecalis*. These results suggest that while NimB2 does not appear to play a role in the immune response against these bacterial species, it may be selectively required for defence against *S. aureus*, potentially through mechanisms such as phagocytosis or antimicrobial peptide regulation.

To assess whether the increase susceptibility to *S. aureus* infection in *NimB2*$^{ds\text{-}red}$ and *Def(All_NimBs)*$^{1\text{-}2}$ could be linked to impaired bacterial clearance, we measured the bacterial load using colony-forming unit (CFU) counting. Flies were infected with *S. aureus* (OD_{600}= 0.1) and bacterial load was assessed 24 hours post-infection. As shown in Fig. 3.4, both *NimB2*$^{ds\text{-}red}$ and *Def(All_NimBs)*$^{1\text{-}2}$ exhibited significantly higher CFU counts compared to wild-type flies, indicating a substantially higher bacterial load in the mutants. This suggests that the increased susceptibility to *S. aureus* in these mutants could be attributed to impaired bacterial clearance, further supporting the role of NimB2 in the host defence mechanism against infection.

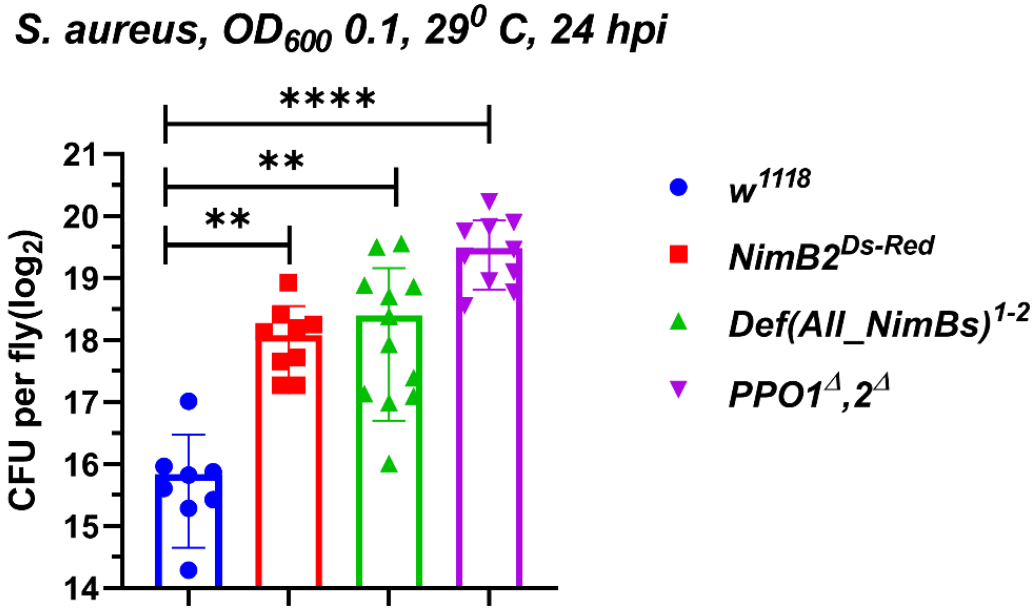

Figure 3.4: Increased bacterial load in NimB2$^{ds\text{-}red}$ flies following S. aureus infection. *Bacterial load was quantified in flies 24 hours post-infection with S. aureus incubated at 29°C. PPO1$^{\Delta}$2$^{\Delta}$ was used as a positive control for susceptibility to S. aureus infection. Data were from three repeat and are shown as mean ± SD.*

NimB2 null mutant flies have higher antimicrobial peptides against *S. aureus*

Following the survival assays, which indicated that *NimB2*$^{ds\text{-}red}$ and *Def(All_NimBs)*$^{1\text{-}2}$ mutants are highly susceptible to *S. aureus*, we aimed to determine whether this susceptibility could be attributed to deficiencies in antimicrobial peptide (AMP) production. To investigate this, we first examined expression levels of *Drosomycin*, an antifungal peptide gene used as a toll read-out. Quantitative-PCR(qPCR) was performed to measure *Drosomycin* expression at 6, 18, and 24 hours post-infection with *S. aureus* (OD = 0.1) across different genotypes. As shown in Fig. 3.5, the results revealed no defect in *Drosomycin* expression in either *NimB2*$^{ds\text{-}red}$ and *Def(All_NimBs)*$^{1\text{-}2}$ flies. Interestingly, we observed elevated levels of *Drosomycin* in these mutants after 18 and 24 hours post-infection, suggesting a potential overactivation of the immune response.

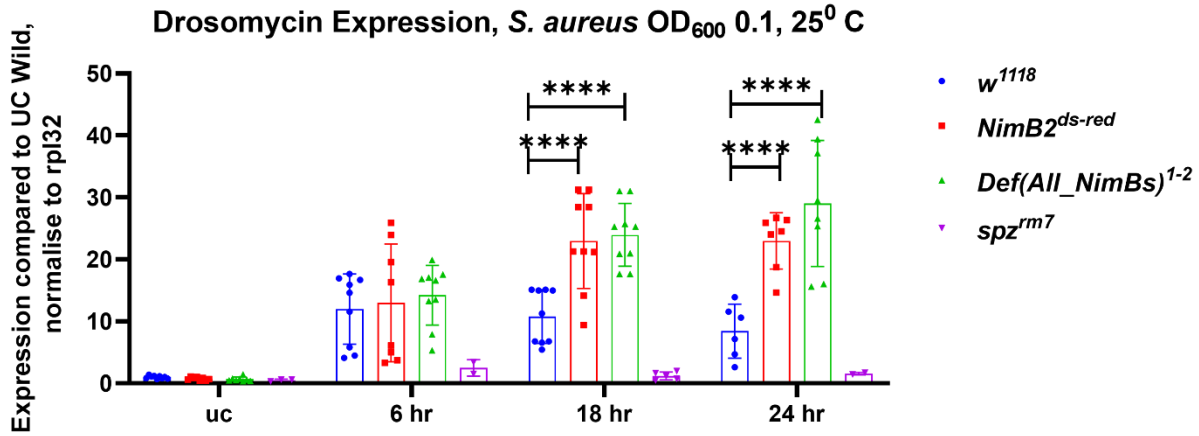

Figure 3.5: Higher drosomycin expression in NimB2$^{ds\text{-}red}$ upon S. aureus infection. *Drosomycin expression levels were quantified relative to the housekeeping gene RpL32 and normalized to wild-type unchallenged controls (w^{1118}). RNA was extracted from flies at 6, 18, 24, and 48 hours post-S. aureus septic injury. Unchallenged flies (UC) served as controls. The toll mutant spätzle (spz^{rm7}) was included as a control for immune signalling. The data are shown as the mean ± SD from three independent experiments.*

To assess whether this heightened AMP expression extended beyond *Drosomycin*, we also measured the expression of two additional AMPs, *Bomanin* and *Metchnikowin*, which play distinct roles in the immune response (Supplementary Fig. S-2). Similar to *Drosomycin*, both *Bomanin* and *Metchnikowin* showed no defect in the expression in

the mutant backgrounds, further confirming that AMP production is not impaired in *NimB2* or *NimB*-deficient mutants.

To determine whether the increased *Drosomycin* expression was specific to *S. aureus* infection, we also assessed its expression in NimB2 mutants flies infected with *M. luteus*. Interestingly, Drosomycin levels in the $NimB2^{ds\text{-}red}$ and $Def(All\_NimBs)^{1\text{-}2}$ were comparable to those in the wild-type upon *M. luteus* infection(Supplementary Fig. S-3). This suggests that the increased susceptibility of *NimB2* and *NimB*-deficient mutants to *S. aureus* is specifically related to their interaction with this pathogen rather than a general broad impact on Toll pathway activation. These findings indicate that the heightened susceptibility to *S. aureus* is not due to impaired AMP production.

NimB2-null larvae exhibit a higher number of haemocytes with a smaller surface area

To further explore the potential mechanism underlying the increased susceptibility of $NimB2^{ds\text{-}red}$ to *S. aureus*, we wanted to investigate the phagocytosis by the haemocytes(macrophages) from the $NimB2^{ds\text{-}red}$ mutant. So, we investigated the role of *NimB2* in haemocyte regulation by analysing haemocyte morphology, size, and number in $NimB2^{ds\text{-}red}$ mutants compared to w^{1118} controls. Confocal imaging of haemocytes stained with DAPI (nuclear marker) and phalloidin (actin cytoskeleton) revealed no significant morphological abnormalities in $NimB2^{ds\text{-}red}$ haemocytes (Fig. 3.6.A). However, quantification of the haemocyte area showed a significant reduction in cell size in $NimB2^{ds\text{-}red}$ mutants compared to wild-type(w^{1118}) controls (Fig. 3.6.B).

Next, we quantified the total number of haemocytes and observed a 3–4 fold increase in haemocyte numbers in $NimB2^{ds\text{-}red}$ mutants compared to w^{1118} (Fig. 3.6.C). To further visualize haemocyte distribution in vivo, we used the *HmlΔ-GAL4, UAS-GFP* system to label haemocytes in whole larvae. Fluorescence imaging confirmed that the sessile pattern of haemocytes remains intact in $NimB2^{ds\text{-}red}$ mutants, indicating no apparent disruption in their distribution (Fig. 3.6.D). These results imply that NimB2 is involved in the regulation of haemocyte number and size, which may have an impact on cellular immune responses in Drosophila.

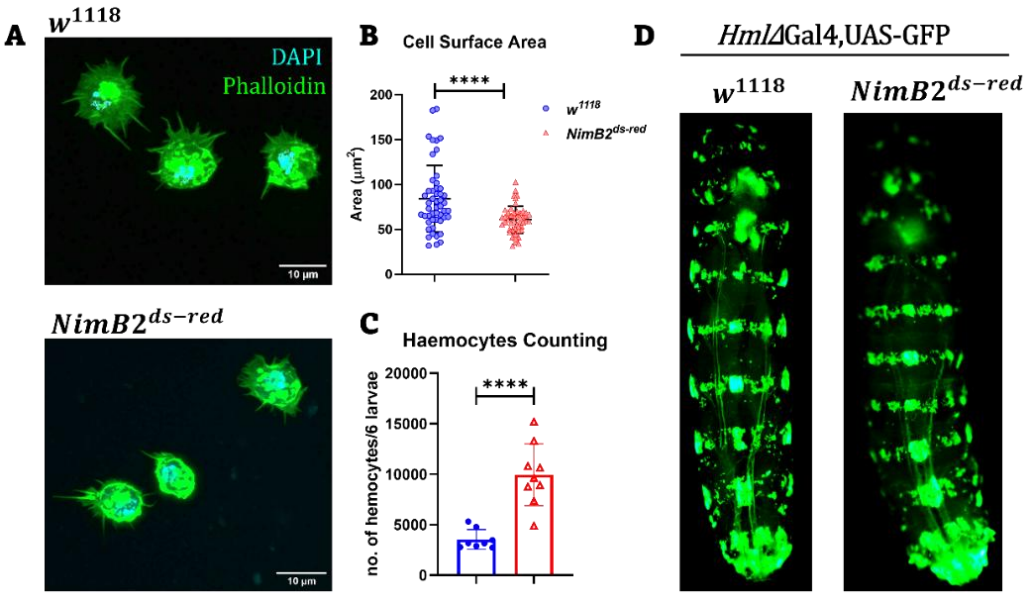

Figure 3.6: NimB2^{ds-red} exhibit reduced haemocyte surface area and increased haemocyte count. *(A) Representative confocal images of haemocytes stained with DAPI (blue, nuclear marker) and phalloidin (green, actin cytoskeleton) to visualize cellular morphology in w^{1118} and NimB2^{ds-red} mutants. (B) Quantification of mean cell area from fixed haemocytes which were spread on slides for 45 minutes and stained with AlexaFluor488-phalloidin. A total of 300 cells were analyzed using Fiji software. (C) The total number of singlet peripheral haemocytes per six third-instar wandering larvae (w^{1118} and NimB2^{ds-red}) was measured via flow cytometry. The data are shown as mean ± SD from three independent experiments. (D) Whole larval images of w^{1118} and NimB2^{ds-red} third-instar larvae, expressing UAS-GFP under HmlΔ-GAL4 to label plasmacytes. The dorsal side of the larvae is shown.*

NimB2 contributes to phagocytosis of *S. aureus*

We explored the role of NimB2 in phagocytosis, a key process in bacterial clearance, through ex vivo phagocytosis assays. We have *NimC1*^{1}*, eater*^{1} group as a positive control known to be unable to phagocytose bacteria as they lack important receptors for phagocytosis. Haemocytes were extracted from the different genotypes, and their ability to engulf *S. aureus* bioparticles was assessed. As shown in Fig. 3.7, haemocytes from both *NimB2*$^{ds-red}$ and *Def(All_NimBs)*$^{1-2}$ exhibited a slight defect in phagocytic activity at 1-hour post-incubation. This defect became more pronounced after 2 hours, with a significant difference observed between the wild-type and NimB2 mutant larvae.

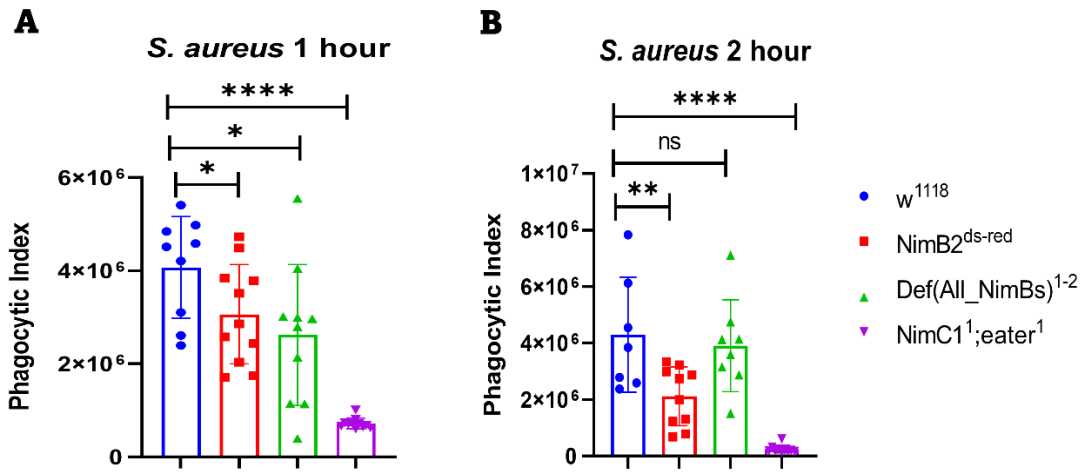

Figure 3.7: Phagocytic defect in NimB2$^{ds\text{-}red}$ haemocytes ex-vivo against S. aureus bioparticles. *Phagocytic index of S. aureus bioparticles labelled with AlexaFluor™ 488 and internalized by haemocytes following an incubation period of (A) 1 hour and (B) 2 hours at room temperature. Phagocytosis was quantified via flow cytometry. The data are shown as mean ± SD from three independent experiments.*

To determine whether this defect was specific to *S. aureus,* which we had observed with the survival assay, we also conducted phagocytosis assays with *E. coli* and apoptotic cells, as shown in Supplementary Fig. S-4. No defects in phagocytosis were observed in these cases, suggesting that the impairment in phagocytosis is specific to *S. aureus* infection. These findings suggest that NimB2 is involved in the phagocytic response to S. aureus, potentially explaining the heightened susceptibility observed in the mutants.

To further investigate the phagocytic activity of haemocytes in vivo, we developed an in vivo phagocytosis assay in L3 stage larvae to assess the ability of haemocytes from different genotypes to internalize bioparticles. We used phRodo-labeled *S. aureus* bioparticles, which fluoresce upon internalization by haemocytes, allowing us to track the phagocytosis process. Haemocytes were isolated from different genotypes, and the percentage of haemocytes capable of internalizing the fluorescent bioparticles was calculated. The microscopy images of haemocytes, shown in Fig. 3.8, clearly demonstrate the internalization of phRodo bioparticles. The quantification revealed that both *NimB2$^{ds\text{-}red}$* and *Def(All_NimBs)$^{1\text{-}2}$* exhibited a significantly lower percentage of haemocytes performing phagocytosis compared to wild-type controls. This suggests that the loss of NimB2 function leads to a reduced ability of haemocytes to internalize *S. aureus* particles in vivo.

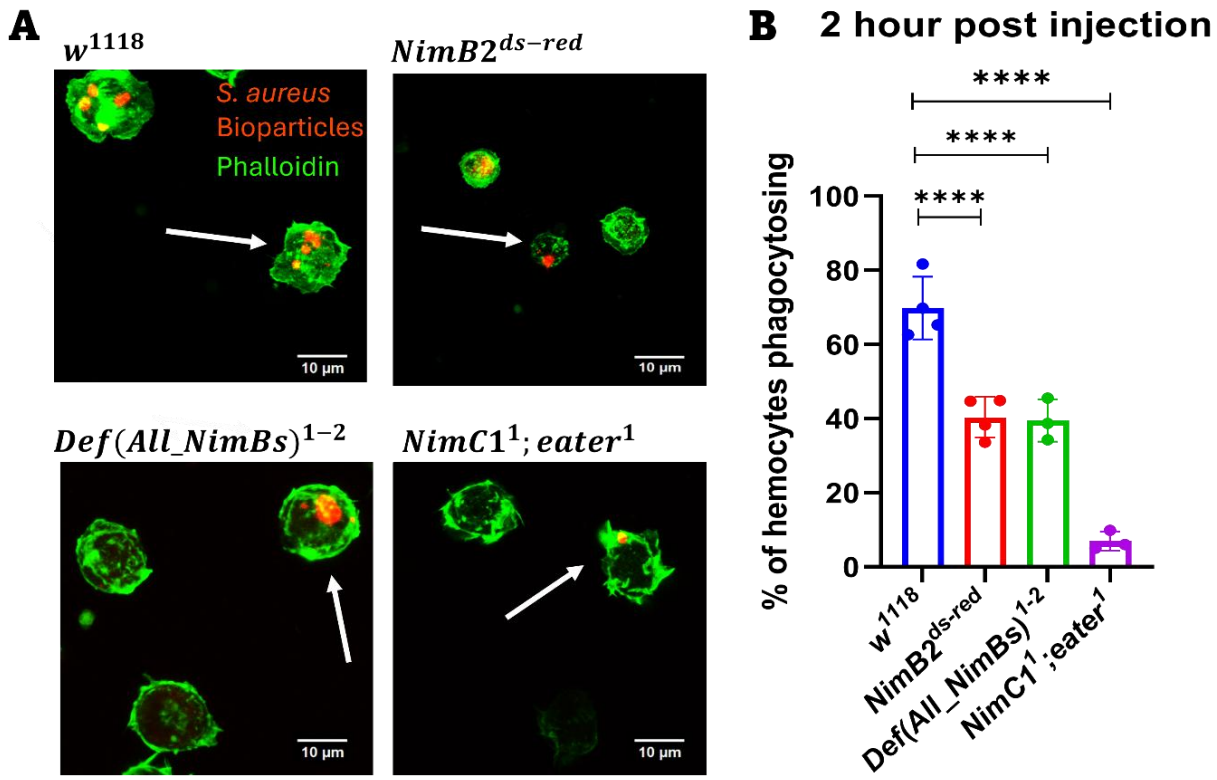

Figure 3.8: Phagocytic defect in NimB2^{ds-red} haemocytes in-vivo against S. aureus bioparticles*. (A) Representative microscopy images of haemocytes stained with Phalloidin (marking the actin cytoskeleton) showing the internalization of pHrodo-S. aureus bioparticles across different genotypes. The white arrow indicates active phagocytosis, as evidenced by the increased fluorescence intensity of pHrodo-S. aureus upon internalization. (B) Quantification of the percentage of haemocytes that successfully internalized S. aureus bioparticles. The data were obtained by analysing the fluorescence intensity of individual haemocytes, and the percentage of haemocytes with positive internalization was calculated. Results were repeated four times and are shown as the mean ± SD from independent experiments.*

To investigate the in vivo interaction between haemocytes and *S. aureus*, we performed a phagocytosis assay using *S. aureus* labelled with GFP (green fluorescent protein). This allowed us to observe and quantify the attachment and internalization of *S. aureus* bacteria by haemocytes from different genotypes. As shown in Fig. 3.9, *S. aureus* bacteria were visibly attached to and internalized by haemocytes in all genotypes. We quantified the percentage of haemocytes with attached and internalized *S. aureus* bacteria using a 3D reconstruction of z-stack images, which provided a more accurate assessment of phagocytic activity. Our results showed that both *NimB2^{ds-red}* and *Def(All_NimBs)$^{1-2}$* exhibited a significantly lower percentage of haemocytes with internalized bacteria compared to wild-type controls.

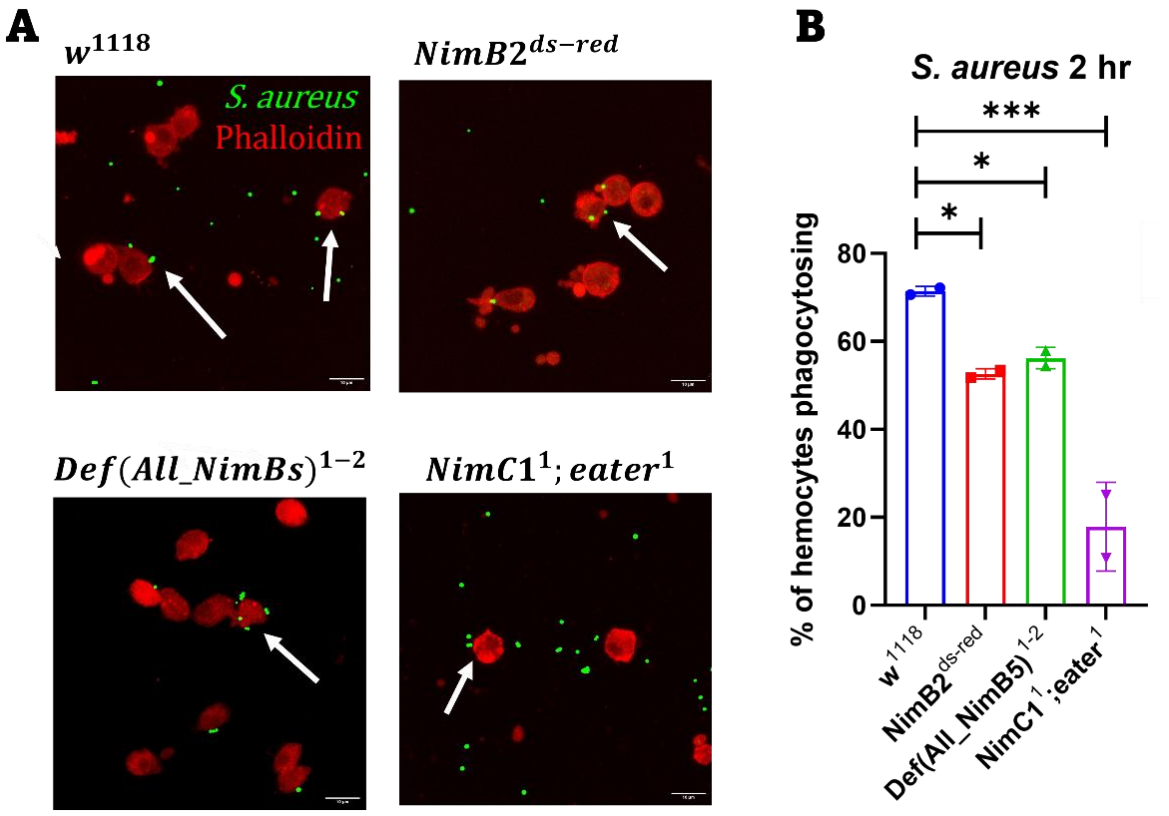

Figure 3.9: Phagocytic defect in NimB2^{ds-red} haemocytes in-vivo against S. aureus bacteria. *(A) Representative microscopy images of haemocytes stained with Rhodamine Phalloidin (marking the actin cytoskeleton), showing the attachment and internalization of S. aureus bacteria (GFP) across different genotypes. The attachment and internalization of bacteria are confirmed by 3D reconstruction of confocal z-stack images. (B) Quantification of the percentage of haemocytes that attached to or internalized S. aureus bacteria. The data were obtained by analysing the fluorescence intensity of individual haemocytes, and the percentage of haemocytes with positive attachment or internalization was calculated. Results were repeated two times and are shown as the mean ± SD from independent experiments.*

To confirm that the observed phagocytic defect in *NimB2^{ds-red}* mutants was indeed due to the absence of functional NimB2, and not caused by other factors, we investigated phagocytosis in different combinations of NimB2 mutants. Specifically, we used two non-viable NimB2 mutants, *NimB2$^{\Delta 49}$* and *NimB2$^{\Delta 422}$*, to ensure that the effects were due to the loss of NimB2 function and not other genetic factors. Since homozygous mutants of *NimB2$^{\Delta 49}$* and *NimB2$^{\Delta 422}$* are non-viable, we generated viable trans-homozygous combinations: *NimB2^{ds-red}/NimB2$^{\Delta 49}$*, *NimB2^{ds-red}/NimB2$^{\Delta 422}$*, *and NimB2$^{\Delta 49}$/NimB2$^{\Delta 49}$*.

Phagocytosis assays were then performed with *S. aureus* bioparticles ex vivo, as shown in Fig. 3.10. After 2 hours, haemocytes from all combinations of NimB2 mutants exhibited a significant phagocytic defect when compared to wild-type and heterozygous controls. This confirms that the impairment in phagocytosis is specifically associated with the loss of NimB2 function, as no such defect was observed in the wild-type or heterozygous control lines.

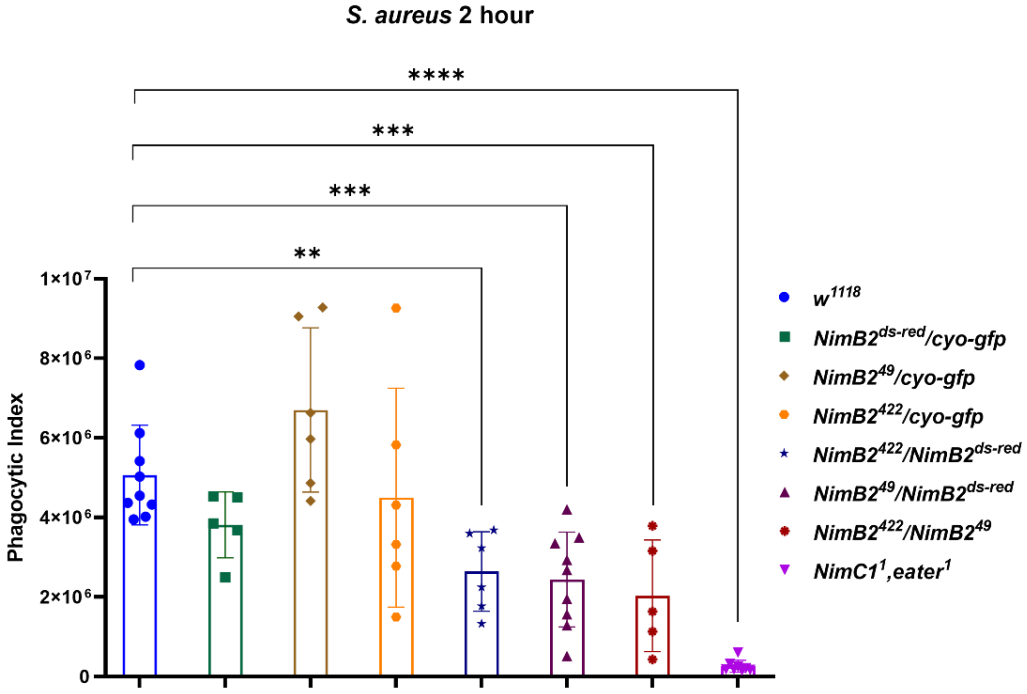

Figure 3.10: Phagocytic defect in multiple NimB2 mutant haemocytes ex-vivo against S. aureus bioparticles. *The phagocytic index of S. aureus bioparticles was labelled with Alexa-488 and internalized by haemocytes after an incubation of 2 hours at room temperature, which was measured via flow cytometry. Haemocytes from different NimB2 mutant combinations (NimB2$^{ds\text{-}red}$/NimB2$^{\Delta 49}$, NimB2$^{ds\text{-}red}$/NimB2$^{\Delta 422}$, and NimB2$^{\Delta 49}$/NimB2$^{\Delta 49}$) were assessed for their phagocytic activity in comparison to wild-type haemocytes. The data are shown as mean ± SD from three independent experiments.*

These findings suggest that the loss of NimB2 function impairs the ability of haemocytes to efficiently phagocytose *S. aureus*, contributing to the increased susceptibility observed in these mutants.

NimB2 facilitates *S. aureus* recognition and binding

To assess whether NimB2 acts as an opsonin involved in bacterial recognition, we conducted cold-binding assays with *S. aureus* bioparticles. Our findings demonstrated a marked decrease in the binding of *S. aureus* bioparticles to haemocytes from the *NimB2*$^{ds\text{-}red}$ and *Def(All_NimBs)*$^{1\text{-}2}$ genotypes. (Fig. 3.11). This indicates that NimB2 has a role in facilitating the recognition and binding of *S. aureus* by haemocytes. In contrast, the binding of apoptotic cells was not significantly affected in these mutants (Supplement Fig. 5), suggesting that NimB2's role is specific to pathogen interactions, particularly with *S. aureus*, rather than a general cell recognition function. These findings show that NimB2 fulfils the function of an opsonin, which helps in the phagocytosis of *S. aureus* bacteria.

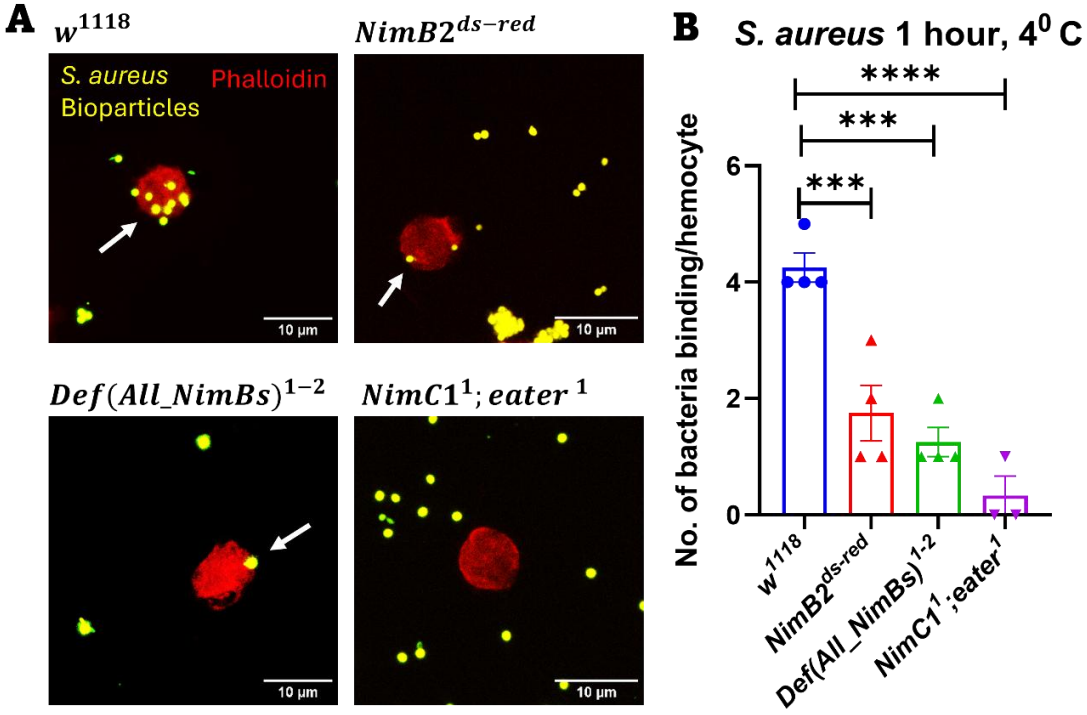

Figure 3.11: Bacterial adhesion defects in NimB2$^{ds\text{-}red}$ haemocytes against S. aureus. *(A) Haemocytes from corresponding genotypes were kept on ice for 1 hour with S. aureus bioparticles labelled with AlexaFluor™ 488. Following fixation with 4% paraformaldehyde, haemocytes were stained with Rhodamine Phalloidin (red) to visualize the actin cytoskeleton. Representative images are shown. (B) Quantification of the number of bacteria bound per haemocyte from different genotypes. Data were obtained by counting the number of bacteria*

bound to individual haemocytes. Each experiment was repeated four times, and the results are shown as the median ± SD from independent experiments.

Chapter 4. Discussion

Our study provides significant insights into the role of *NimB2* in the immune response of *Drosophila melanogaster*, particularly in the recognition and clearance of *Staphylococcus aureus* infections. By employing a combination of ex vivo and in vivo assays, we demonstrate that *NimB2* mutants exhibit phagocytosis and pathogen-binding defects. As a result, it underscores its role in haemocyte-mediated bacterial clearance. Furthermore, our results indicate that *NimB2* mutants exhibit an upregulation of antimicrobial peptides (AMPs) exclusively in response to *S. aureus,* which is a consequence of bacterial growth. These findings suggest that *NimB2* is a critical component of the immune response against *S. aureus*, demanding further investigation into its molecular function.

One of the most striking observations in our study is that NimB2 mutants show a markedly reduced ability to recognize and internalize *S. aureus*, despite exhibiting normal phagocytosis of apoptotic cells and gram-negative bacteria such as *Escherichia coli(E. coli)*. Cold binding assays revealed that haemocytes from NimB2 mutant flies and all NimB family-deficient backgrounds exhibit impaired bacterial recognition, suggesting that NimB2 is crucial for facilitating pathogen binding. One possible explanation for this defect is that NimB2, secreted from the fat body, functions as an opsonin, enhancing the recognition of *S. aureus* by haemocytes. This hypothesis is supported by our survival assays, which show that NimB2 mutant flies die more rapidly to *S. aureus* infection, while their survival remains unaffected when challenged with other bacteria such as *Enterococcus faecalis*, *E. coli*, or *Erwinia carotovora* (Ecc15). These findings underscore the specificity of NimB2's role in targeting *S. aureus* and suggest that it may be acting as a key immune factor required for the efficient clearance of this pathogen.

To further explore how NimB2 contributes to immune regulation, we examined the expression of antimicrobial peptides (AMPs), key effectors of the Toll pathway, which are upregulated in response to Gram-positive bacterial and fungal infections. Interestingly, we observed that NimB2 mutant flies exhibit a significant increase in the expression of *drosomycin, metchnikowin, bomanin* following *S. aureus* infection. One possible explanation for this heightened AMP response is the impaired phagocytosis

in NimB2 mutants, which leads to an increased bacterial load, as confirmed by CFU assays. This elevated bacterial burden could, in turn, trigger stronger activation of the Toll pathway, resulting in enhanced drosomycin production.

Alternatively, another possible explanation is the existence of a compensatory mechanism, where the loss of NimB2-mediated phagocytosis triggers an enhanced humoral immune response as a way to counteract the defect in bacterial clearance. To investigate this further, we analysed *Spätzle* mutant flies, which lack Toll signalling and fail to produce AMPs in response to S. aureus infection. Interestingly, *Spätzle* mutants displayed higher phagocytic activity than wild-type flies (Supplementary Fig. S-6), supporting the idea of a dynamic interplay between cellular and humoral immunity. A similar reciprocal relationship has been reported in in vitro studies, where haemocytes that efficiently phagocytose *E. coli* exhibit lower expression of Diptericin, an AMP regulated by the IMD pathway (Johansson et al., 2006). These findings suggest that phagocytosis and AMP regulation are intricately linked, with potential compensatory mechanisms ensuring immune homeostasis in response to bacterial infections

Our proposed model, as illustrated in Fig. 4.1, provides a mechanistic framework for how NimB2 orchestrates this immune response. We hypothesize that NimB2 is secreted by the fat body and binds to *S. aureus*, acting as a molecular bridge between the bacteria and haemocytes. This interaction enhances bacterial recognition, leading to efficient internalization and subsequent killing of the pathogen. In the absence of NimB2, haemocytes struggle to bind and engulf S. aureus, resulting in increased bacterial persistence. The immune system amplifies AMP production to counterbalance this deficiency, which might serve as an alternative defence strategy to contain the infection. This model provides a compelling explanation for the phagocytic defect observed in the NimB2 mutants.

In conclusion, our study establishes NimB2 as a crucial immune regulator required for the recognition and clearance of *S. aureus* in *Drosophila*. By elucidating its role in linking pathogen recognition to phagocytosis and AMP regulation, we provide a new perspective on how the immune system coordinates multiple defence mechanisms to maintain homeostasis. Future studies will be necessary to uncover the precise

molecular interactions underlying NimB2-mediated bacterial binding and to determine whether similar mechanisms operate in other infection contexts. Understanding these processes may ultimately contribute to the development of novel antimicrobial strategies by targeting host immune factors that enhance pathogen clearance.

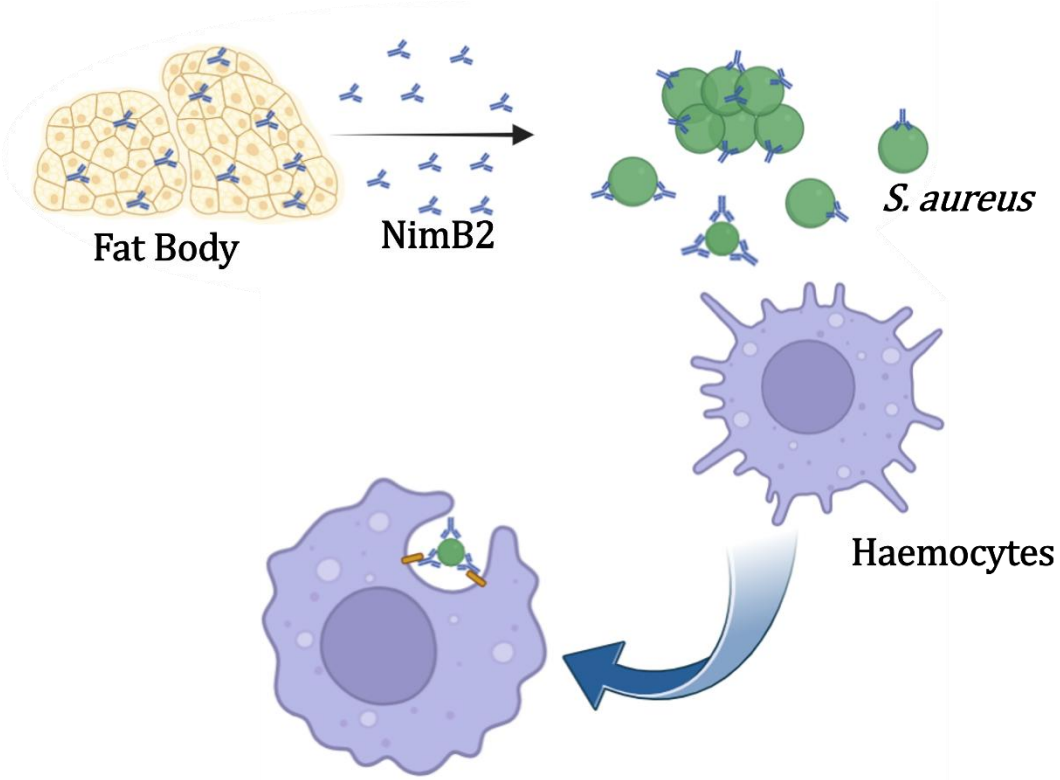

Figure 4.1: Proposed model of NimB2 function in immune response. *NimB2 is secreted from the fat body and binds to S. aureus, enhancing haemocyte recognition. This promotes efficient phagocytosis, which contributes to pathogen clearance.*

These findings open several avenues for further research. Does NimB2 interact with specific haemocyte surface receptors to facilitate pathogen binding? Are there additional factors that compensate for its function under certain conditions? Investigating these questions will expand our understanding of Drosophila immunity and provide insights into conserved immune mechanisms that extend to higher organisms. By delineating the role of NimB2 in immune homeostasis, this study lays the groundwork for exploring new paradigms in host defence strategies.

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Appendix

Figure S-1: Expression of NimB2: Data from FlyBase (A) and qPCR analysis (B). (qPCR performed by Asya Dolgikh)

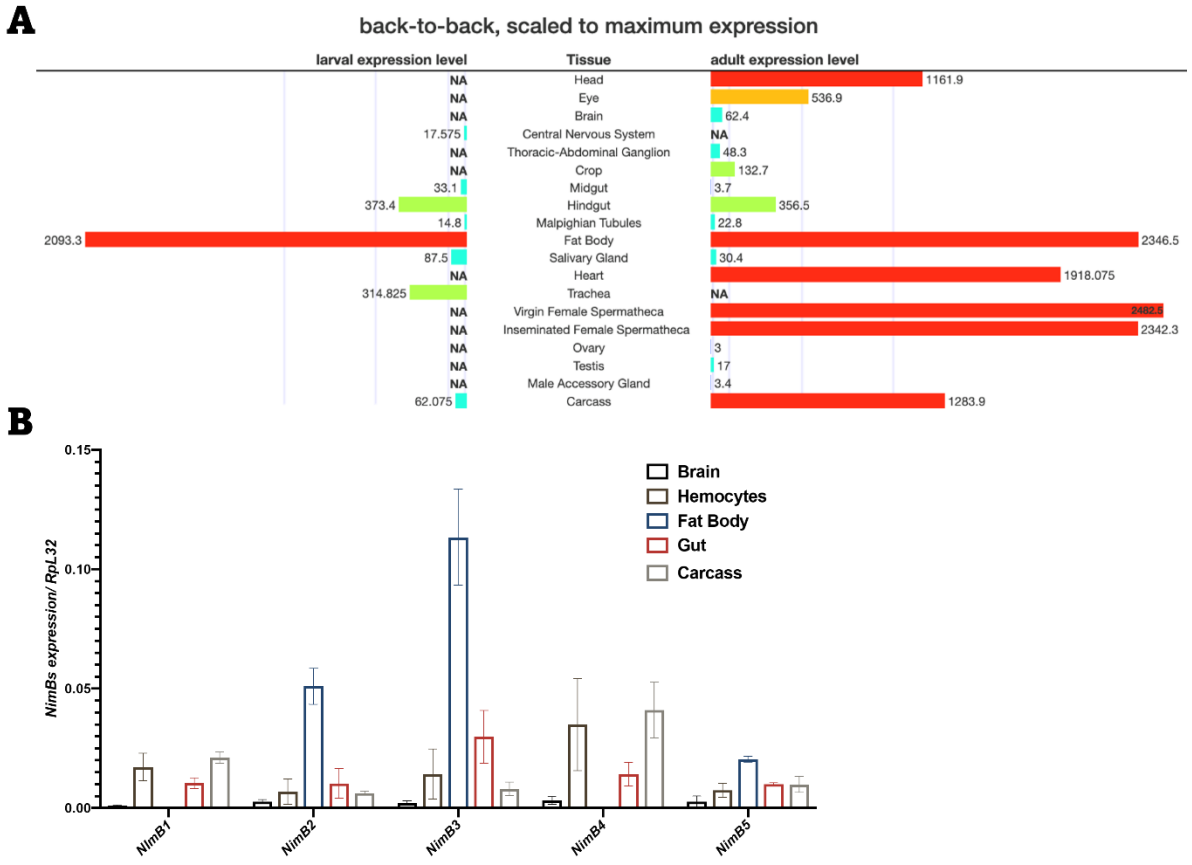

Figure S-2: Expression of *Bomanin* and *Metchnikowin* after *S. aureus* infection.

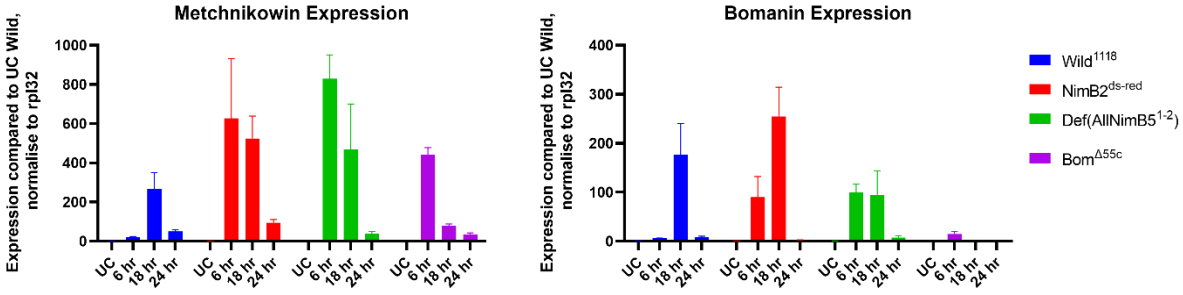

Figure S-3: Expression of *Drosomycin* 24 hours post-infection with *S. aureus*.

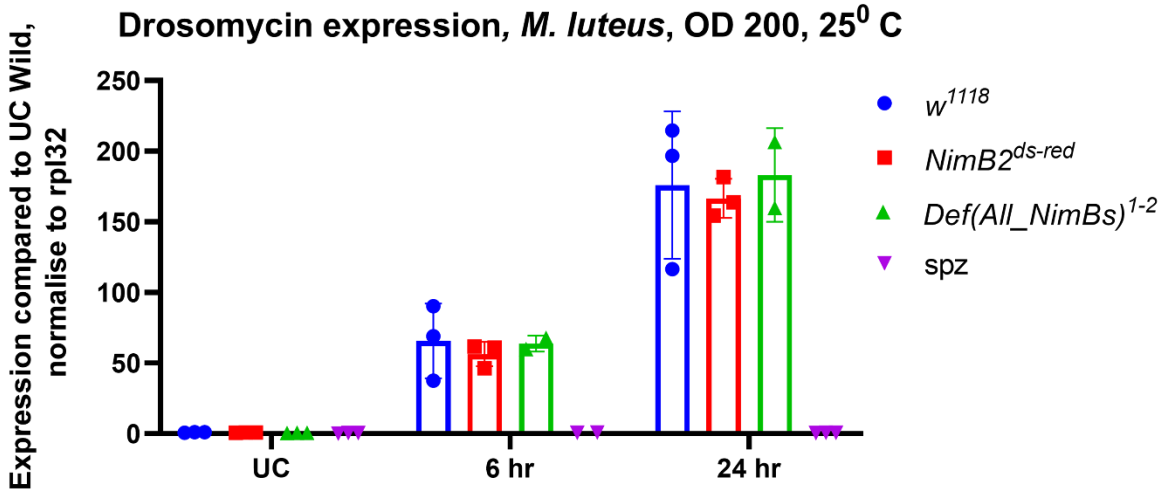

Figure S-4: Phagocytosis Assay with *E. coli* bioparticles and Apoptotic cells.

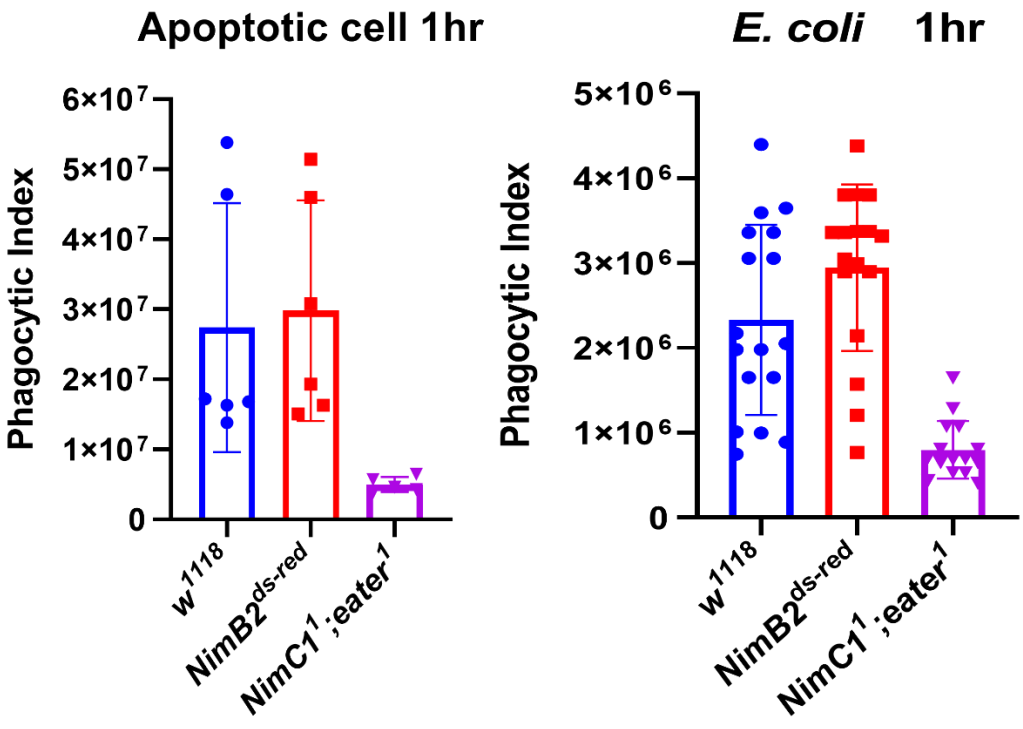

Figure S-5: Binding Assay of Haemocytes with Apoptotic cells.

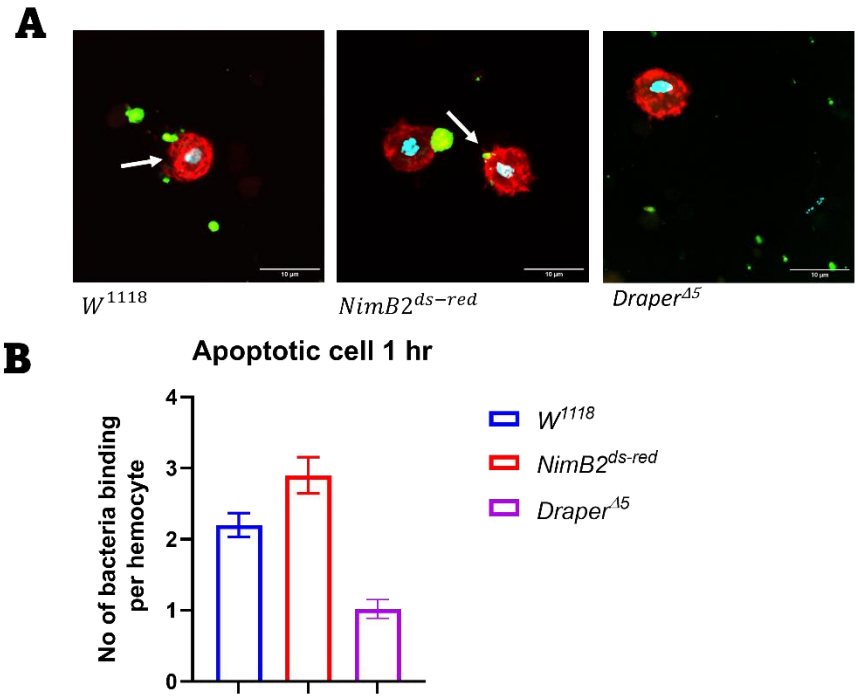

Figure S-6: Phagocytosis Assay with *spätzle* mutant with *S. aureus* bioparticles.