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Unexpected role of the IMD pathway in *Drosophila* gut defense against *Staphylococcus aureus*

Aki Hori [a, b], Shoichiro Kurata [b, *], Takayuki Kuraishi [b, c, d, e, **]

[a] Graduate School of Medical Sciences, Kanazawa University, Kanazawa, Ishikawa, Japan
[b] Department of Molecular Biopharmacy and Genetics, Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Japan
[c] Faculty of Pharmacy, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Kanazawa, Ishikawa, Japan
[d] Department of Microbiology and Immunology, Keio University School of Medicine, Tokyo, Japan
[e] PRESTO, Japan Science and Technology Agency, Tokyo, Japan

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ABSTRACT

In this study, fruit fly of the genus *Drosophila* is utilized as a suitable model animal to investigate the molecular mechanisms of innate immunity. To combat orally transmitted pathogenic Gram-negative bacteria, the *Drosophila* gut is armed with the peritrophic matrix, which is a physical barrier composed of chitin and glycoproteins: the Duox system that produces reactive oxygen species (ROS), which in turn sterilize infected microbes, and the IMD pathway that regulates the expression of antimicrobial peptides (AMPs), which in turn control ROS-resistant pathogens. However, little is known about the defense mechanisms against Gram-positive bacteria in the fly gut. Here, we show that the peritrophic matrix protects *Drosophila* against Gram-positive bacteria *S. aureus*. We also define the few roles of ROS in response to the infection and show that the IMD pathway is required for the clearance of ingested microbes, possibly independently from AMP expression. These findings provide a new aspect of the gut defense system of *Drosophila*, and helps to elucidate the processes of gut-microbe symbiosis and pathogenesis.

1. Introduction

The insect intestinal epithelium is frequently exposed to harmful pathogens, and thus, must be armed with an efficient and powerful gut defense system to protect itself from infection [1,2]. Studies in *Drosophila* adults have revealed the mechanisms that regulate gut defense against microbial infection by using Gram-negative bacteria, which comprise the following components: (i) physical barriers, such as the peritrophic matrix and epithelial integrity [3,4]; (ii) reactive oxygen species (ROS) production [5] and epithelium renewal in response to ROS-induced gut damage [6]; and (iii) the secretion of antimicrobial peptides (AMP) through the IMD pathway [7,8].

The peritrophic matrix is an acellular structure that forms a layer comprising chitin polymers and glycoproteins. In *Drosophila* adults, Drosocrystallin (Dcy) protein, crosslinked by Transglutaminase [9], is a structural component of the peritrophic matrix. A loss-of-function mutation in *dcy* reduces the thickness of the peritrophic matrix and causes higher permeability, which is associated with a greater susceptibility to infection from ingested pathogenic bacteria and toxins [3]. ROS is produced in response to oral infection with entomopathogenic bacteria, such as *Erwinia carotovora* subsp. *carotovora 15* (*Ecc15*) via Dual oxidase (Duox), a plasma membrane-expressing NADPH oxidase [10]. Pathogenic bacteria release uracil, which activates an as-yet-unidentified G protein-coupled receptor [11], leading to the enzymatic activation of Duox through the G protein alpha subunit q protein (Gαq)–phospholipase Cβ (PLCβ) signaling pathway [12]. ROS eliminate infected bacteria as well as injure epithelia [13]. To repair the epithelial damage and maintain gut homeostasis, intestinal stem cells (ISC) are activated to proliferate and differentiate into new enterocytes. The JAK-STAT pathway, activated by *upd3* produced by damaged cells, is crucial to epithelial renewal, and a complex network of signaling pathways are involved in gut repair [6]. Antimicrobial peptides (AMP) serve as an immune response in the gut to control Gram-negative bacteria [7,8]. Pattern recognition

* Corresponding author.
** Corresponding author. Faculty of Pharmacy, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Kanazawa, Ishikawa, Japan.
E-mail addresses: kurata@m.tohoku.ac.jp (S. Kurata), tkuraishi@staff.kanazawa-u.ac.jp (T. Kuraishi).

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receptors peptidoglycan recognition protein (PGRP)-LE and PGRP-LC sense Gram-negative bacteria [14,15], thereby activating the IMD pathway, comprised of the signaling components IMD, Dredd, dTAK1, and the IKK complex. This activation finally induces nuclear translocation of the NF-κB transcription factor Relish, which is responsible for the expression of antimicrobial peptide genes [16].

In contrast to the above mechanisms controlling Gram-negative bacteria in the gut, defense mechanisms against Gram-positive bacteria remain uncharacterized in the gut of *Drosophila* adults. Indeed, in the midgut, a main part of the *Drosophila* intestine, the Toll pathway, a key activator of NF-κB pathway upon systemic infection [17], is considered to be non-functional [18]. Therefore, in this study, we seek to investigate gut defense responses against Gram-positive bacteria, mainly focusing on the role for the peritrophic matrix, ROS, and the IMD pathway.

2. Materials and methods

2.1. Fly stocks

Drosophila stocks were maintained in standard cornmeal–yeast–agar medium vials at 18 °C or 25 °C. Oregon-R and w^{1118} flies were used as wild-type controls. The dcy^{1} (#26106) flies were obtained from the Bloomington Drosophila Stock Center. The imd^{NP1182} (#112555) and *Df(2R)PCG* (#106715) flies were obtained from the Drosophila Genetic Resource Center (Kyoto Institute of Technology, Japan). The *Gαq* mutant flies were kindly provided by Dr. W.J. Lee. The imd^{1} and $Relish^{E20}$ mutants and *delta-Gal4; UAS-nlsGFP* were kindly provided by Dr. B. Lemaitre. The $dredd^{B118}$ and $dTAK1^{2}$ flies were a kind gift from Dr. F. Leulier.

2.2. Microbial infection

The *Ecc15* and *Ecc15-GFP* strains were described previously by Basset et al. [19], and were grown in Luria-Bertani broth (LB) at 29–30 °C for all experiments. The *E. coli K12* strains was grown at 37 °C in LB. The *S. aureus* strain (ATCC 10801) and *S. aureus-GFP* strain (received from Dr. K. Sekimizu) were grown at 37 °C in nutrient broth (NB) and were allowed to reach the stationary phase. Cells were then concentrated to OD600 = 200. For oral infection, flies were starved for 2 h at 29 °C and then placed in a fly vial with food solution. The food solution was made by mixing a pellet of bacteria and adding it to a filter disk, which completely covered the surface of the standard fly medium. Flies were maintained at 29 °C and survival was monitored at different time points. Assays were performed with more than 90 of 7 to10 days old male flies for each genotype.

For colony forming unit (CFU) assays, 15 flies subjected to oral infection were collected, washed with 70% EtOH, and rinsed with MilliQ water. The flies were crashed with a bead mill homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) in an LB or NB medium, the mediums were serially diluted and spread onto agar medium plates, and the number of colonies were counted to calculate the CFU.

2.3. Total RNA isolation and real-time qPCR

Real-time qPCR was performed as previously described by Kanoh et al. [20]. Briefly, total RNA (1 μg) was extracted from dissected adult midguts by using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA), and used for cDNA synthesis with ReverTra Ace reverse transcriptase (Toyobo Ltd., Osaka, Japan) and oligo(dT) 15 primers (Promega, Madison, WI, USA). Using first-strand cDNA (0.5 μL), real-time qPCR was performed using a LightCycler (Roche Diagnostics, Roswell, GA, USA), and *rpL32* was used as an internal control. The following primers were used for real-time qPCR (F = forward, R = reverse): rpL32: 5′-AGATCGTGAAGAAGCGCACCAAG-3′ (F), 5′-CACCAGGAACTTCTTGAATCCGG-3′ (R); *Dpt*: 5′-GTTCACCATTGCCGTCGCCTTAC-3′ (F), 5′-CCAAGTGCTGTCCATATCCTCC-3′ (R).

2.4. ROS measurement

ROS were detected using dimethyl cyanine 3, which is essentially described in Kundu et al. [21]. Then, 2 μL of 50 mM dimethyl cyanine 3 (Lumiprobe) was added into 50 μL of phosphate-buffered saline (PBS). A total of 500 mM of sodium borohydride (Lumiprobe) was gradually added until the solution became clear. This solution was designated as Hydro Cy3, and 60 μL of 5% sucrose solution, 200 μM of paraquat solution, or 200 μM of bacterial solution were mixed with 60 μL of 200 μM Hydro Cy3. Flies were starved for 2 h at 29 °C, and fed with this mixed solution at 25 °C for 30 min. For quantification, the guts were dissected after feeding and observed under a conventional fluorescence microscope, using 20 to 30 flies, to see red Cy3 signals that were oxidized by the ROS from Hydro Cy3.

2.5. Immunohistochemistry

Antibody stainings were performed as previously described by Kenmoku et al. [22,23] with 1:500 mouse anti-GFP (Invitrogen, Carlsbad, CA, USA), 1:500 rabbit anti-PH3 9701 (Cell Signaling, Danvers, MA, USA), and 1:500 Alexa 488-coupled and Alexa 546-coupled secondary antibodies (Invitrogen), using the guts of 7 to10 days old female flies. The samples were visualized with a Leica TCS-SPE confocal microscope (Leica Microsystems, Mannheim, Germany), and observed at the posterior midgut, and images were reconstructed using Photoshop (Adobe).

2.6. Collection of hemolymph and defecation assay

Hemolymph samples from adult flies were collected as essentially described in MacMillan et al. [24], except for the airflow to the flies was manually delivered using an insect aspirator.

To quantify the amount of excreted bacteria, GFP proteins expressed in the bacteria were measured. For this, 180 (30 flies × 6 vials) male adult flies were fed with *Ecc15-GFP* or *S. aureus-GFP*. After 30 min, flies were observed under the stereo-fluorescent

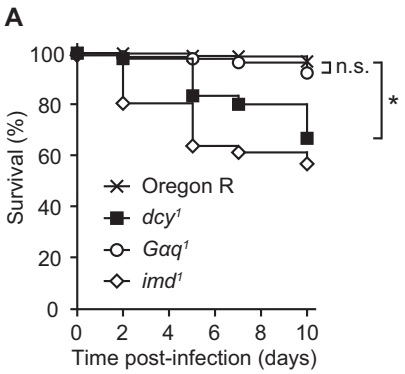

Fig. 1. Survival analysis of *dcy* mutant flies, mutant flies with defective reactive oxygen species production, and *imd* mutant flies upon oral infection with *S. aureus*. Each survival curve is representative of more than three independent experiments, each with 30 adult flies. *P* values were calculated with the log-rank test (*$P < 0.0001$).

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microscope to select flies with GFP. The selected flies were put into a 50 mL conical tube to collect the feces. The tube was changed every 30 min. The inside of the tubes were washed extensively with PBS and centrifuged to collect the feces. The feces were then subjected to an enzyme-linked immunosorbent assay for GFP by using a GFP SimpleStep ELISA kit (Abcam #ab171581, Cambridge, UK).

2.7. Statistical analysis

Statistical analyses were performed by Student's *t*-test, log-rank test, or Mann–Whitney U test, and *P* values < 0.05 were considered significant.

3. Results

3.1. Requirement of dcy for gut defense against S. aureus

We first examined the requirement of the peritrophic matrix for survival against oral infection with Gram-positive bacteria by using *dcy* mutant flies. As a representative of Gram-positive bacterium, we employed *S. aureus*, a bacterium commonly used for systemic infection in *Drosophila* adults [25]. Fig. 1 shows that *dcy* mutant flies succumbed to the oral infection, while wild-type flies exhibited almost no death upon the infection. This result indicates that the *dcy* gene is required for gut defense against oral infection with *S. aureus*, and suggests that the peritrophic matrix, a physical

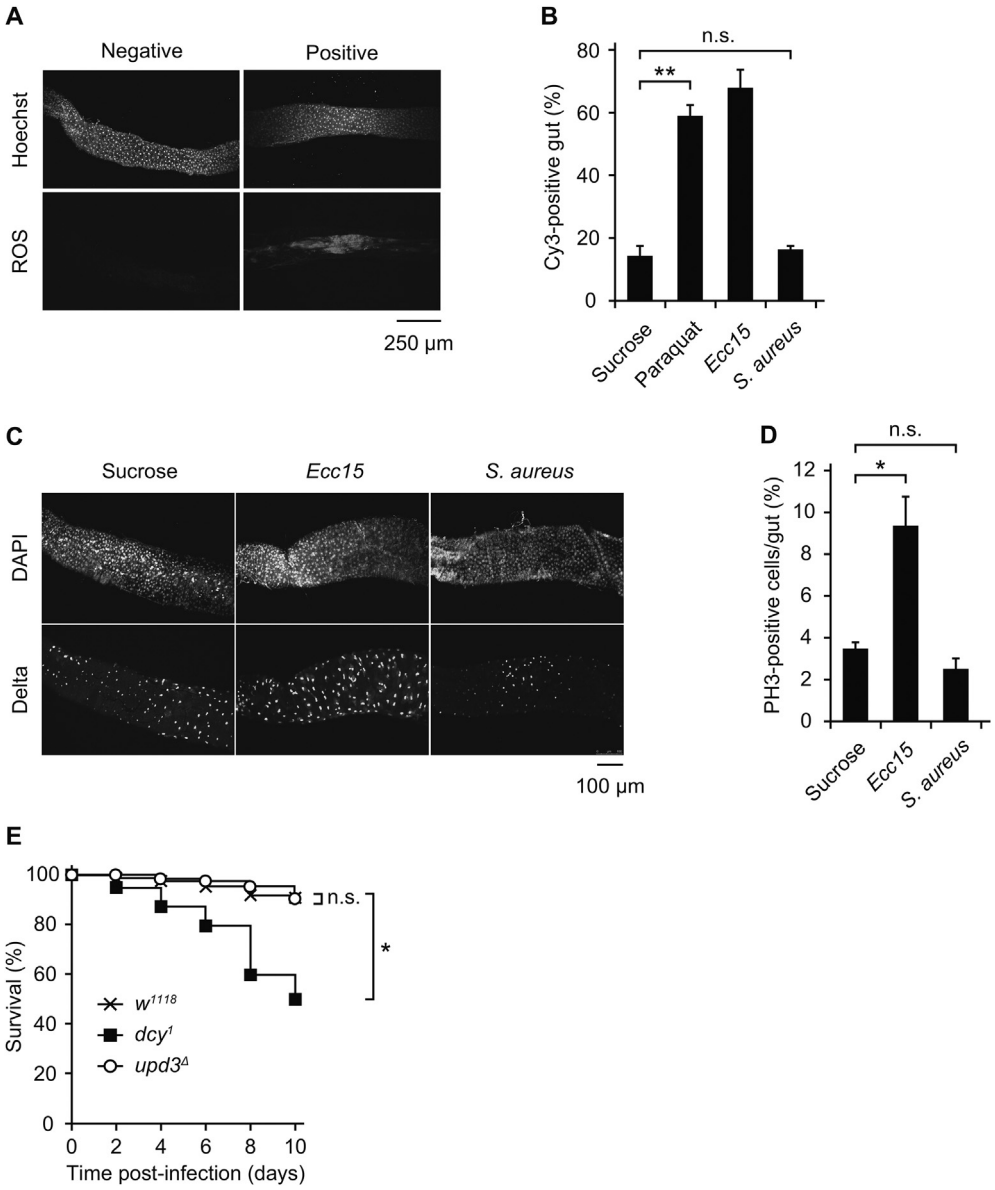

Fig. 2. **(A)** ROS were measured with dimethyl cyanine 3 (Cy3). Nuclei were stained with Hoechest33342 (bar, 250 μm). Negative indicates representative of Cy3-negative gut, and positive means representative of Cy3-positive gut.**(B)** Quantification of Cy3-positive guts following oral infection. The number of Cy3-positive guts in **(A)** was counted by observing around 20 dissected guts, and is shown as a percentage. Values represent the mean ± SE of two independent experiments, each with 10–20 guts. **(C)** Immunohistochemistry with anti-GFP antibody using the *delta-Gal4; UAS-nlsGFP* strain. Sixteen hours after ingestion with sucrose, *Ecc15,* or *S. aureus*, the dissected guts were stained with anti-GFP antibodies and DAPI. (bar: 100 μm). **(D)** The mitotic index of intestinal stem cells was quantified with Oregon R flies by counting PH3 positive cells. Data were analyzed by Student's *t*-test (*$P < 0.0001$, **$P < 0.01$), and values represent the mean ± SE of three independent experiments with 10–15 guts each. **(E)**. Survival analysis of *upd3* mutant flies. Each survival curve represents more than three independent experiments, each with 30 adult flies. *P* values were calculated with the log-rank test (*$P < 0.0001$).

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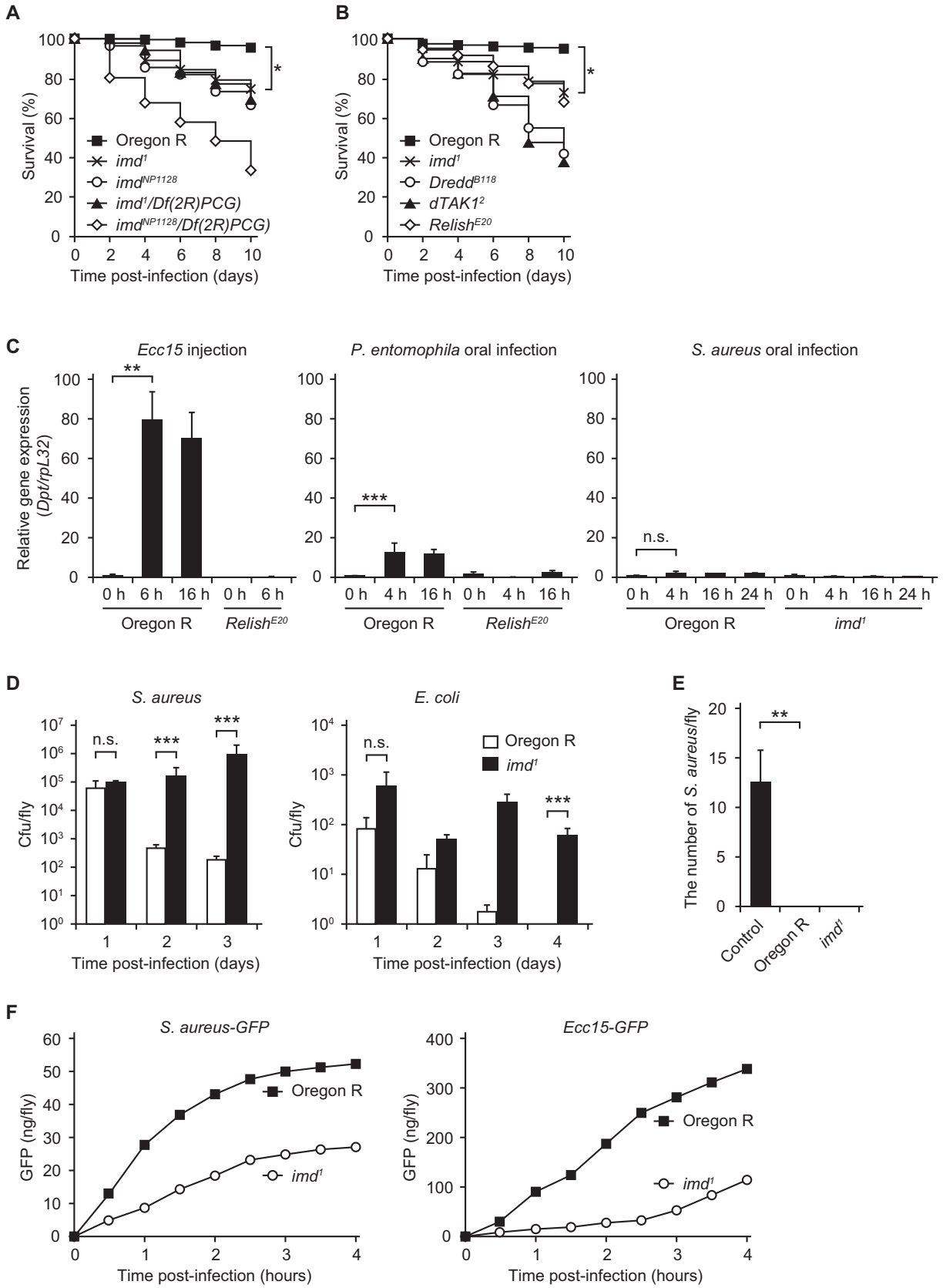

Fig. 3. **(A)** Survival analysis of *imd* mutant flies after oral infection with *S. aureus*. **(B)** Survival analysis of IMD pathway mutant flies after oral infection with *S. aureus*. Each survival curve represents more than three independent experiments, each with 30 adult flies. *P* values were calculated with the log-rank test (*$P < 0.0001$). **(C)** RT-qPCR analysis of *Diptericin (Dpt)* expression. The whole body 6 and 16 h after septic injury with *Ecc15*; the intestine 4 and 16 h after oral infection with *P. entomophila*; and the intestine 4, 16, and 24 h after oral infection with *S. aureus* were used. The mRNA abundance in uninfected Oregon R in each experiment was set to one. Data were analyzed by Student's *t*-test (**$P < 0.01$, ***$P < 0.05$) and values represent the mean ± SE of three independent experiments, each with 10–20 flies or guts. **(D)** Colony forming unit (CFU) assay following 1, 2, 3, and 4 d after infection.

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barrier for the gut epithelium, is a prerequisite for protection against ingested Gram-positive bacteria.

3.2. Role of reactive oxygen species for oral infection with S. aureus

Next, we investigated whether ROS are involved in gut defense against *S. aureus*. Survival analysis showed that *Gαq* mutant flies were not susceptible to oral infection with *S. aureus* (Fig. 1), suggesting that ROS produced by the Duox pathway is dispensable for defense against *S. aureus* in the gut. We then examined whether ROS were induced upon the oral infection but not pivotal to the survival, or were not induced in the first place. ROS were measured by reduced dimethyl cyanine 3 (Hydro Cy3), and the observation that the oral infection with Gram-negative bacteria *Ecc15* resulted in the emission of fluorescence from.

Hydro Cy3 provides the validation of this assay (Fig. 2A). More than 65% of the *Ecc15*-infected guts became Cy3 positive, while the number of Cy3-positive guts in *S. aureus*-infected flies remained the same as in sucrose-fed flies (Fig. 2B). This result indicates that *S. aureus* infection does not trigger ROS production in the gut. To further support this observation, we monitored stem cell proliferation, which is a response used to repair the epithelium after gut damage caused by ROS. Immunohistochemistry for Delta, a marker for stem cells, showed that the apparent number of Delta-positive cells in *S. aureus*-infected guts were as same as in sucrose-fed guts, while *Ecc15*-infected guts showed an increased number of positive cells (Fig. 2C). Similarly, the amount of PH3-positive cells, the cells under mitosis, was increased with *Ecc15* oral infection, but not with *S. aureus* oral infection (Fig. 2D). Consistent with these results, mutant flies for *upd3*, which is required for gut repair after ROS-induced damage [26], were not sensitive to oral infection with *S. aureus* (Fig. 2E). These results collectively suggest that ROS have no defensive role during oral infection with *S. aureus*.

3.3. Role of the IMD pathway in the clearance of S. aureus from the gut

We next examined the role of the IMD pathway for gut defense against *S. aureus*. We noticed that imd^{1} mutants were sensitive to the infection (Fig. 1), and after further analysis of other *imd* alleles, we found that imd^{NP1182}, $imd^{1}/Df(2R)PCG$, and $imd^{NP1182}/Df(2R)PCG$ flies were susceptible to oral infection with *S. aureus* (Fig. 3A), suggesting that the *imd* gene is important for gut defense against *S. aureus*. We then tested survival upon oral infection with *S. aureus* by using other mutants of the IMD pathway. In addition to *imd* mutant flies, *dredd*, *dTAK1*, and *Relish* mutant flies were susceptible to oral infection with *S. aureus* (Fig. 3B), suggesting that the IMD pathway is required for controlling infection of the gut with Gram-positive bacteria. Despite the clear role of the IMD pathway in the protection against oral infection with Gram-positive bacteria, real-time qPCR analysis indicated that the level of induction of *Diptericin*, an AMP whose expression is solely controlled by the IMD pathway, in wild-type guts is comparable to that in *imd*-mutant guts (Fig. 3C). Intriguingly, however, the number of *S. aureus* was not decreased in *imd* mutant flies, while the bacteria in wild-type flies were cleared from the body (Fig. 3D, left), in a response similar to that of oral infection with *E. coli* (Fig. 3D, right). Fig. 3E demonstrates that a lethal amount of GFP-labeled *S. aureus* had not entered the hemolymph following oral infection. These results suggest that the IMD pathway is involved in the removal of ingested *S. aureus* from the gut, with mechanism(s) independent from AMP expression. Since gut peristalsis could be such an effector mechanism, we monitored the amount of excreted *S. aureus* by using GFP-labeled bacteria, and the GFP was quantified by enzyme-linked immunosorbent assay. We found that the amount of defecated *S. aureus-GFP* was lower in *imd* mutant flies than in wild-type flies during the four hours after oral infection (Fig. 3F, left). Notably, the amount of expelled GFP derived from *Ecc15* was also lower in *imd* mutant flies (Fig. 3F, right). These results suggest that excretion of ingested bacteria from the *imd* mutant gut is compromised in some way, and we infer that the IMD pathway is associated with the excretion of bacteria infecting the gut.

4. Discussion

We have demonstrated in this study that *Drosophila* adults employ the peritrophic matrix and the IMD pathway to combat oral infection with *S. aureus*, a Gram-positive bacteria in the gut. Notably, the induction level of AMP upon oral infection with *S. aureus* remained low, suggesting that other gut resistance mechanisms [27], rather than AMP expression conferred by the IMD pathway, could be present. In fact, we showed that the excretion of orally ingested bacteria in the *imd* mutant flies was compromised, which might be a defense mechanism controlled by the IMD pathway. On the other hand, the production of ROS, an important effector mechanism for protection against pathogenic bacteria in the intestine, was not observed after *S. aureus* ingestion. Considering that no deaths were observed in wild-type flies following oral infection with *S. aureus*, this bacterium would not be pathogenic to normal flies and may not secrete uracil that would activate the Duox pathway [11]. From this point of view, gut defense mechanisms against *S. aureus* shown in this study would provide clues as to how *Drosophila* handle non-pathogenic or symbiotic gut microbes.

All of the intracellular signaling components of the IMD pathway that we examined involved sensitivity to oral infection with *S. aureus*. Concerning the upstream of *imd*, PGRP-LE is known to recognize meso-diaminopimelic acid (DAP)-type peptidoglycan that Gram-negative bacteria possess [16]. On the other hand, lysine-type peptidoglycan, a component of the Gram-positive bacterial cell wall, is recognized by PGRP-SA. Thus, we tested *pgrp-sa* mutant flies after oral infection with *S. aureus*, but the mutants did not exhibit increased susceptibility (data not shown). Then, how is the IMD pathway activated by oral infection with *S. aureus*? One possibility is that crosstalk with other immune-related signaling pathways, such as TNF-α or neuropeptide signaling, would induce moderate activation of the IMD pathway [28]. Another possibility is that the IMD pathway would not be activated upon oral infection, but that basal or developmental activity of the IMD pathway is required for bacterial resistance.

We demonstrated that defecation of the ingested *S. aureus*, as well as Gram-negative bacteria *Ecc15*, was compromised in *imd* mutant flies, although the degree of its contribution to survival after the infection was not investigated in the present study. Peristaltic movement of the gut or intestinal fluid balance that might control defecation [2] could be undermined by the loss of IMD

The y-axis indicates the number of colonies per fly. Data were analyzed by Mann–Whitney U test (***$P < 0.05$) and values represent the mean ± SE of four independent experiments, each with 15 flies. **(E)** Quantification of the number of GFP-labeled *S. aureus* in the hemolymph collected from one fly after injection or oral infection with *S. aureus-GFP*. At 3 or 5 d post ingestion, hemolymph samples were observed to count the number of GFP signals. "Control" indicates hemolymph samples from flies that received direct injection of *S. aureus-GFP*. Data were analyzed by Student's *t*-test (**$P < 0.01$) and values represent the mean ± SE of three independent experiments. **(F)** Quantification of excreted bacteria by measuring labeled GFP. Feces from the flies were collected every 0.5 h (from 0.5 to 4 h after ingestion) and GFP was quantified by enzyme-linked immunosorbent assay, and the amount per one fly is shown. Values are representative of two independent experiments.

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signaling. Indeed, neuroenteric peptides, such as NPLP-1 and ion-transport peptide, modulate NF-κB signaling via cGMP, which, in flies, acts against salt stress in the Malpighian tubules where epithelial fluid transport occurs [29]. *Drosophila* may also utilize similar tactics to control enteric infection or non-pathogenic microbe numbers in the intestine. In this study, we did not evaluate the role of the Toll pathway, because the Toll pathway is not considered to be the main immune response mechanism in the midgut. However, it is suggested that the Toll pathway is functional in the foregut and hindgut [6]. Thus, future studies could examine intestinal control of Gram-positive bacteria by the Toll pathway.

In conclusion, we showed an unexpected role of the IMD pathway in defense against gut infection with *S. aureus*. The IMD pathway controls the defecation of ingested bacteria, which may function as an important mechanism to remove microbes from the intestine of the fly. Further research is needed to clarify the underlying molecular connection between innate immune signaling and the gut excretory function.

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Author contributions

A.H. and T.K. conceived this study, designed and performed the experiments, analyzed the data, and wrote the draft. All authors finalized the manuscript, and S.K. and T.K. oversaw the study.

Conflicts of interest

The authors declare no conflicts of interest.

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