

## Sterile induction of innate immunity in *Drosophila melanogaster*

Rangga Meidianto Asri<sup>1,2</sup>, Emil Salim<sup>1,3</sup>, Firzan Nainu<sup>2</sup>, Aki Hori<sup>1</sup>, Takayuki Kuraishi<sup>1</sup>

<sup>1</sup>Faculty of Pharmacy, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Kanazawa, Ishikawa, Japan, <sup>2</sup>Faculty of Pharmacy, Universitas Hasanuddin, Makassar, Indonesia, <sup>3</sup>Faculty of Pharmacy, Universitas Sumatera Utara, Medan, North Sumatera, Indonesia

### TABLE OF CONTENTS

1. Abstract
2. Introduction
  - 2.1. Sterile inflammation
  - 2.2. Innate immune responses in *Drosophila*
  - 2.3. Factors that induce sterile inflammation in *Drosophila*
3. Pinching-induced humoral innate immune response in larvae
  - 3.1. Induction of antimicrobial peptides by pinching with forceps
  - 3.2. Signaling pathways involved in pinching-induced *Drosomycin*
  - 3.3. Tissues responsible for pinching-induced *Drosomycin*
4. Novel ligand for Toll receptor
  - 4.1. Novel ligand activity for Toll receptor in larval extract
  - 4.2. *Spz5* as a Toll ligand
  - 4.3. No requirement of proteolytic processing of *spz5* as a Toll ligand
5. Summary and perspective
6. Acknowledgements
7. References

### 1. ABSTRACT

In the past decades, much has been learned about the protective signatures of innate immune responses during the course of infections. However, it is now evident that induction of immune effectors also commonly occurs in the absence of pathogenic cues. Such an event, termed sterile inflammation, has been linked to several debilitating acute and chronic host conditions. Using *Drosophila melanogaster* as a simple yet powerful model organism, identification of diverse sets of damage-associated molecular patterns and their corresponding surface and/or inside pattern recognition receptors on the cells, as well as elucidation of their significant roles in the host physiology and pathological conditions related to sterile inflammation, have been continuously reported. In addition, revelation of non-pathogenic molecular triggers leading to the orchestration of unnecessary activation of inflammatory responses has been a subject of interest. Here, we review decades of efforts to elucidate the molecular mechanisms responsible in the emergence of sterile inflammation. The characterization of the respective contributing factors, including recent demonstration

of pinching as a novel sterile-stimuli in *Drosophila*, is also discussed.

### 2. INTRODUCTION

#### 2.1. Sterile inflammation

Innate immune system can be activated under certain circumstances without infection by microorganisms. However, aseptic activation of innate immunity remains poorly understood. Last decade, researchers became enthusiastic to demystify how the sterile inflammation process occurs because if this process is uncontrolled, it can cause serious damage or diseases in humans. Several studies have noted that many pathological conditions or diseases related to inflammatory responses in the absence of pathogens, including myocardial infarction and stroke (1), drug induced liver injury (2), and Systemic Inflammatory Response Syndrome (SIRS) (3).

Sterile inflammation can be triggered by aseptic stimuli, such as mechanical trauma,

radiation, ischemia, toxins, minerals, crystals, chemicals, autoimmune conditions, and antigens (4). Based on past findings, sterile inflammation is initiated by two factors: dead (necrotic) cells or a variety of particulates from an intracellular source or extracellularly located molecules (5). Necrotic cells lose their plasma membrane integrity after dying and release or expose their intracellular matrix containing some endogenous materials originated from cytosol, nucleus, and mitochondria, which can be recognized as pro-inflammatory molecules (1). Those factors are collectively called alarmins, damage (danger)-associated molecular patterns (DAMPs) or cell-death associated molecular patterns (CDAMPs). Inflammatory particles are able to stimulate the damaged or diseased tissue in which the particles have accumulated to a sufficient level. Those include inorganic materials (e.g., silica (6), iron oxide (7), calcium pyrophosphate (8)) and organic compounds (e.g., monosodium urate and cholesterol crystal) (8). Those particles are also regarded as alarmins that ultimately contribute to the downstream cellular and vascular manifestations of inflammation as in microorganism infections (9,10).

For the induction of sterile inflammation in mammals, inflammasomes play a central role. Inflammasomes are large multiple cytoplasmic protein complexes, which are particularly structured by Nod-Like Receptors (NLRs) as a sensor, apoptosis-associated speck-like protein containing a caspase recruitment domain or CARD (ASC) as an adaptor, and caspase-1 as a catalyst of pro-IL-1 $\beta$  processing (11-13). The principal function of inflammasome complexes is the cleavage of pro-IL-1 $\beta$  into its active form, i.e., IL-1 $\beta$ , as an important cytokine in inflammatory responses (14,15). Several different NLRs can compose various inflammasome complexes. For instance, the complex of NACHT, LRR, and PYD domains-containing protein 3 (NLRP3), ASC, and caspase-1 are called NLRP3 inflammasome (11-13).

Inflammasome(s) are activated by infectious stimuli known as pathogen-associated molecular patterns (PAMPs), but a number of endogenous and exogenous non-infectious substances or DAMPs can also induce their activation. NLRP3 inflammasome could be activated by various DAMPs, such as ATP, monosodium uric (MSU) crystals, cholesterol crystal, silica, and other particulate matters (14-16). After conditions caused by infection or sterile factors, the release of IL-1 $\beta$  is regulated by two-steps: a priming step and pro-IL-1 $\beta$  processing step by inflammasome(s). In a priming step, PAMPs or DAMPs may activate Toll-like receptors (TLRs) or other innate immune receptors, which induce the nuclear localization of NF- $\kappa$ B that transactivates gene expressions of pro-IL-1 $\beta$  and the components of the inflammasome. Then, inflammasomes are activated by several

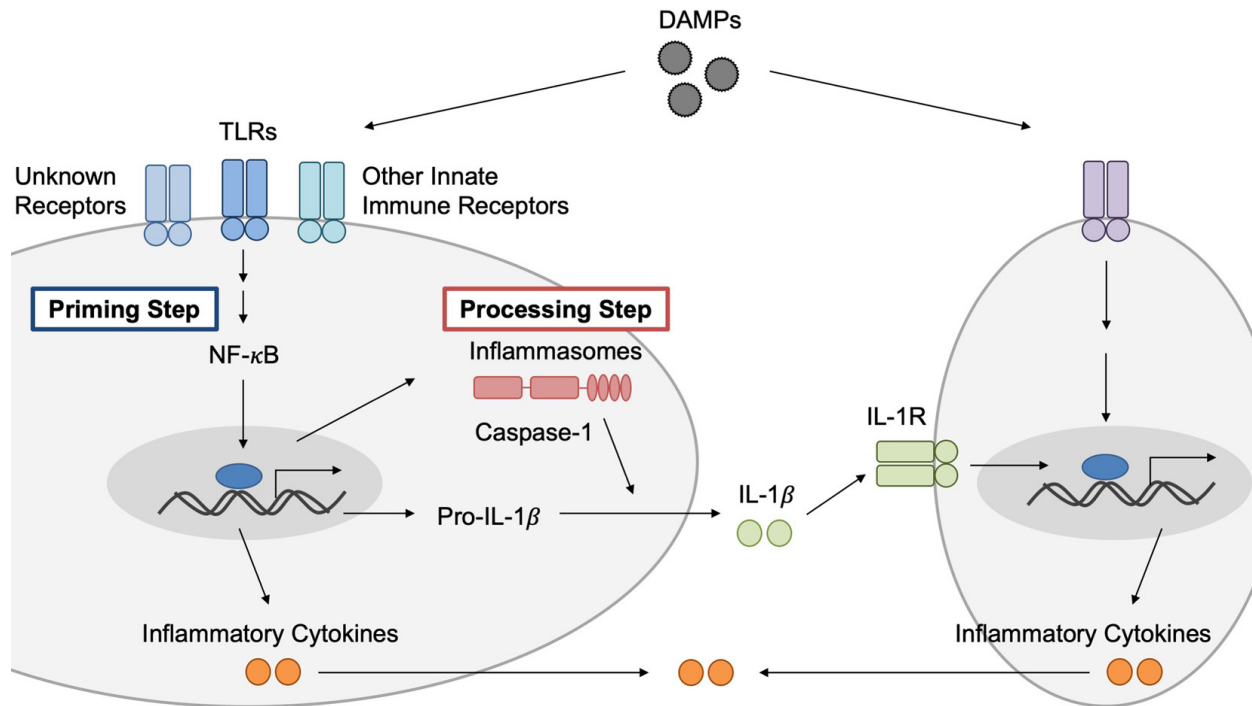
signaling mechanisms, resulting in the processing of pro-IL-1 $\beta$  into its mature form (11,16). In sterile inflammatory responses, the NLRP3 inflammasome plays a particularly important role for the cleavage of accumulated cytosolic pro-IL-1 $\beta$ . The activation of NLRP3 inflammasome likely collaborates with other surface pattern recognition receptors (PRRs) like TLRs or unknown upstream signaling processes that involve NF- $\kappa$ B induction as a priming step (16) (Figure 1). However, the molecular mechanisms of the priming step are still unclear.

To precisely reveal the processes by which inflammation is activated in a sterile milieu, in particular how innate immunity is regulated and activated, many experiments must be established since it seems to be involved in multiple and complex pathways. Therefore, the utilization of animal models, such as insects and mammals, is useful. The fruit fly *Drosophila melanogaster* has been used as a leading model to study several molecular mechanisms of innate immunity. *Drosophila* has innate immune receptors to facilitate downstream signaling to activate NF- $\kappa$ B that drives the expression of antimicrobial peptides (AMPs) as their immune responses (17,18). Lacking the NLR gene family and thus inflammasomes, fruit flies are an excellent model to identify mechanisms involved in the priming step, as discussed above. This article will review our current knowledge and our own studies, focusing on how the sterile stimuli induce inflammation, especially the expression of AMPs in *Drosophila*.

## 2.2. Innate immune responses in *Drosophila*

Metazoan animals have many mechanisms that allow them to protect themselves against threats caused by infection, parasitism, and neoplasia (19). The study of *D. melanogaster* is highly relevant to mammalian innate defense mechanisms particularly in signaling processes and transduction pathways. Without an adaptive immune response, *Drosophila* provides a powerful model for studying the aspects of the innate immune system (20). There are two main responses in *Drosophila* innate immunity: the humoral and cellular systems. The humoral responses are induced in an abundant tissue, particularly in larvae, known as the fat body. The fat body can produce and secrete large amounts of AMPs into the hemolymph, while cellular responses involve defense mechanisms, such as phagocytosis and encapsulation of invading pathogens (17).

The humoral innate immunity against bacterial and fungal infection is governed largely by two NF- $\kappa$ B signaling pathways, Toll and IMD (19). TLRs, named after the discovery of the Toll receptor in fly immunity, are evolutionally conserved receptors required for innate immunity as well as developmental processes. *Drosophila* Toll receptors are composed of a protein



**Figure 1.** Model of Sterile Inflammation in Mammals. (1) Priming Step: damage-associated molecular patterns (DAMPs) activate pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), other immune receptors, and unknown receptors, leading to the production of pro-IL-1β, components of inflammasomes, and other inflammatory cytokines. (2) Processing Step: DAMPs also activate inflammasomes, which result in the cleavage of pro-caspase-1 to its active form caspase-1, which in turn, cleaves pro-IL-1β into its active form, IL-1β. IL-1β then binds to its receptor in other immune cells leading to the generation of inflammatory cytokines.

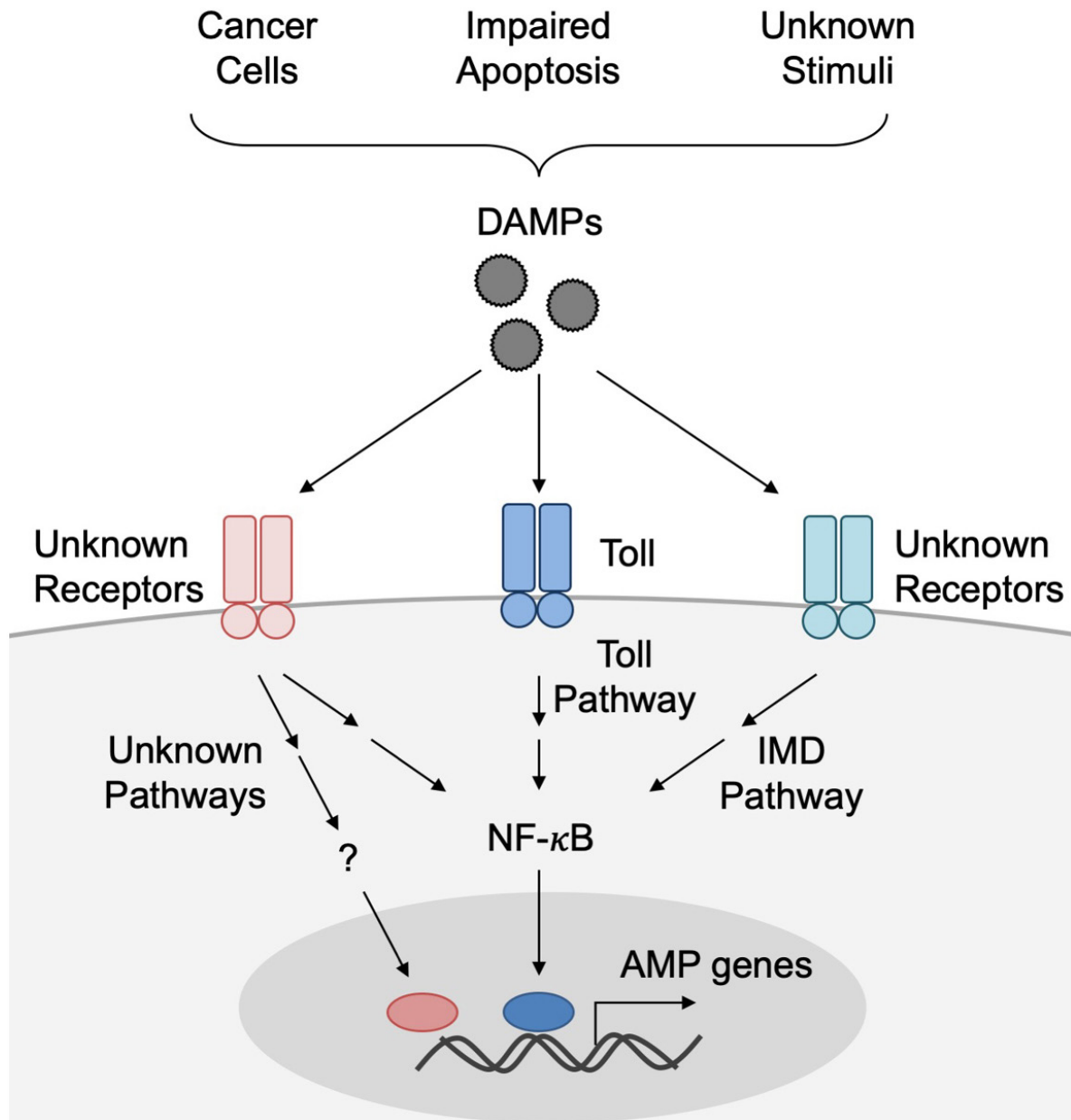
family, similar to mammalian TLRs. The *Drosophila* genome encodes nine Toll receptors, some of which have been functionally characterized, and Toll-1 has an important innate immune role (21). The Toll pathway, the downstream pathway of the Toll receptor, is responsible for resistance to fungal and Gram-positive bacterial infections. Unlike vertebrate TLRs, the fly Toll receptor does not bind directly to pathogens. Instead, it is activated by the endogenous protein Spätzle (Spz), a cysteine-knot protein with structural similarities to nerve growth factor in mammals. Toll activation by the binding of Spz stimulates the recruitment of a protein complex called Myddosome, composed of dMyd88, Tube, and Pelle kinase, via E3 ligase Sherpa (22). The complex around the plasma membrane with Toll leads to destabilization of the IκB protein Cactus with uncharacterized mechanisms, resulting in the activation of the NF-κB proteins Dorsal and Dif, which are responsible for the expression of antimicrobial genes, such as *Drosomycin* (23).

The IMD pathway is required for defense against infection by Gram-negative bacteria in adult flies. Diaminopimelic acid (DAP)-type peptidoglycans released from Gram-negative bacteria activate peptidoglycan recognition protein LC (PGRP-LC) or PGRP-LE, the transmembrane or intracellular pattern recognition receptors, inducing the recruitment of an

adaptor protein IMD. Then, the caspase-like protein Dredd cleaves the IMD protein to form a signaling complex containing the E3 ubiquitin ligase Diap2, which in turn activates TAK1 and then the IκB kinase (IKK) complex. The IKK complex then phosphorylates and activates Relish, a NF-κB to induce the expression of genes that encode antimicrobial proteins including *Diptericin* (17,19).

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling is one of the evolutionary conserved signaling pathways involved in immunity (24). In *Drosophila*, the JAK/STAT pathway is important for defense against viral infection, regeneration of the midgut after bacterial infection, hematopoiesis, and lamellocyte differentiation in response to a parasitic infestation (19). The ligand of a receptor for the JAK/STAT pathway consists of three proteins called unpaired (upd), upd2, and upd3. These upd proteins could bind a single receptor, Domeless (Dome), and activate JAK, hopscotch (hop), and STAT transcriptional factor, Stat92E (17,19).

The cellular immune response consists of circulating immune surveillance cells (hemocytes) in the hemolymph. In *Drosophila* larvae, three classes of hemocytes can be identified: plasmatocytes, crystal cells, and lamellocytes. Plasmatocytes are professional



**Figure 2.** Model of Sterile Inflammation in *Drosophila*. Cancer cells, impaired apoptosis, and unknown stimuli are presumed to produce DAMPs, which stimulate signaling pathways including Toll, IMD, and unknown pathways that result in the activation of NF- $\kappa$ B or other unknown transcription factor(s) that lead to the production of AMPs.

phagocytes most similar to the mammalian monocyte/macrophage lineage and comprise up to 95% of the circulating and sessile population (25). Phagocytosis is a crucial defense mechanism for invading pathogens as well as internally emerged altered-self, such as apoptotic cells. Plasmatocytes utilize several types of receptor proteins for recognizing non-self. These include members of the scavenger receptor family proteins dSR-CI and Croquemort and the EGF-domain proteins Eater and Draper (26,27). Crystal cells secrete components of the phenol oxidase cascade and are

involved in the melanization of invading organisms and in wound repair. Lamellocytes are involved in the encapsulation of invading pathogens, such as wasp eggs, that are too large to be phagocytosed (17).

### 2.3. Factors that induce sterile inflammation in *Drosophila*

To date, elucidation of numerous signaling pathways responsible in the coordination of innate immune system in *Drosophila* has shown that

conserved mechanisms exist from fruit fly to humans and such beautifully orchestrated events are critical in the maintenance of homeostasis during the life of metazoan species (17, 28). It is not surprising that loss of control in such tightly coordinated processes can cause massive havoc. This is what we are now seeing in the form of sterile inflammation. In the absence of an adaptive immune system, the fruit fly has a layer of innate immune responses as the primary defense mechanism against PAMPs and DAMPs that exist in abnormal circumstances; therefore, they are an excellent model because of the many functional and molecular similarities to mammals (17, 20).

In general, sterile inflammation occurs as a result of unexpected activation of the immune system due to the presence of aseptic triggering signals, either produced intracellularly or of extracellular origin (1, 4, 5, 20). Induction of inflammation by the sterile stimuli, may occur as a result of impairment in tight-coordinated daily routines. One example is the abrupt evolutionally conserved silent elimination of normally present yet potentially harmful apoptotic cells by phagocytes. It is apparent that successful phagocytosis will prevent the activation of inflammatory response (29). However, in the case of patients with impaired phagocytic activities, apoptotic cells are not properly processed thus leading to the activation of a necrotic mechanism where cells die and release their intracellular contents to the extracellular area (20). This in turn abrogates the delicate process of phagocytic elimination of apoptotic cells and creates a chance to activate a sterile inflammatory response. This conceptual framework has been experimentally observed in the case of apoptosis deficiency due to mutation in the caspase genes and a DNase gene.

Experiments by Ming *et al.* (2014) successfully demonstrated the link between apoptosis deficiency and activation of the Toll signaling pathway. In their study, it was shown that deficiency of initiator caspase Dronc led to the active processing of Spz in a Persephone-dependent manner, which further activated the canonical Toll signaling pathway (30). The physiological role of such an event was then subsequently revealed by Obata *et al.* (2014). In the later study, the authors demonstrated the relationship of apoptosis deficiency, dFoxO hyperactivation, and Toll activation to the upregulation of glycine N-methyltransferase (Gnmt) that leads to lipolysis and the energy-wasting phenotype in *Drosophila* (31). In addition to those two studies, an early study by Nagata's group using a loss-of-function fly mutant that failed to express DNase II demonstrated that the accumulation of endogenous DNA due to DNase II deficiency leads to the expression of *Diptericin* and *Attacin*, two well-known AMPs that are under the control of the IMD pathway in *Drosophila* (32). This suggests that the absence of DNase II resulted in

the abruption of apoptotic DNA fragmentation, one observable phenotype in cells undergoing complete apoptosis, and such an event further stimulates the activation of the humoral innate immune system in an IMD-dependent manner. However, it is interesting to note that undigested DNA-mediated sterile inflammation did not occur in a Toll-dependent manner as DNase II lacking *Drosophila* did not experience an increased production of Toll-dependent AMPs, such as *Drosomycin* (32). Subsequently, by the same group it was revealed that the activation of the IMD signaling pathway by undigested DNA was associated with the specific role of the threonine phosphatase domain of EYA in *Drosophila* (33). Collectively, findings reported in those studies strongly suggest that an impaired process of apoptosis could provoke an unexpected sterile inflammatory response in a Toll- or an IMD-dependent manner, supporting the pathophysiological role of DAMPs, such as undigested endogenous DNA, in the induction of sterile inflammation in *Drosophila*. Undoubtedly, further assessment of the mechanism(s) responsible in the induction of "impaired apoptosis"-mediated sterile inflammation might provide important pharmaceutical insights.

As described, the presence of inflammatory factors is an important trigger of noninfectious sterile inflammation in *D. melanogaster*. In addition to apoptosis dysfunction, it is important to note that cells with tumor/cancer-like properties play a conserved and central role in the induction of sterile inflammation across metazoan species. Thus, providing readers with a description of such entities will be an important achievement. Innate defense mechanisms are selectively reactive to foreign materials, particularly to imminent threats to host survival, such as pathogenic microorganisms and tumor/cancer cells (17, 20, 28). Like its mammalian counterpart, *Drosophila* performs routine and timely scans using an array of innate immune sensors and effectors to detect and if possible, eliminate cells with tumor-like properties that might become potential threats. Once such cells are discovered, a tightly controlled disposal procedure is activated (34).

Recent findings in *Drosophila* confirmed that the presence of long-range, inter-tissue communication between fat bodies and adipocytes plays a role in the hemocytes-mediated activation of the Toll/NF-KB signaling pathway to manage tumor growth via a non-cell autonomous mechanism in *Drosophila* (35). Tumor cells stimulate the activation of signaling pathways associated with hemocyte proliferation, such as Pvf1/Pvr, Spz/Toll, and Eiger/TNF (35), which have essential roles in triggering systemic immune responses that are important for the initiation of tumor cell death. This occurs in the absence of a pathogen, which recapitulates a hallmark of sterile inflammation. In addition to the stimulation



of cellular innate immunity, increased expression of antimicrobial peptide genes, such as *Drosomycin*, *Cecropin A1*, and *Attacin*, have been reported in mutant flies with *RAS<sup>v12</sup>* (36), suggesting a bipartite link between the induction of Toll- and IMD-mediated activation of humoral immune responses and the rate of tumor progress. Although the precise mechanistic explanation to these events remains elusive, to this end, it is high likely that sterile signals elicited from cells with tumor-like properties can interact with cellular and humoral components of innate immune responses in *Drosophila*.

### 3. PINCHING-INDUCED HUMORAL INNATE IMMUNE RESPONSE IN LARVAE

#### 3.1. Induction of antimicrobial peptides by pinching with forceps

Previous studies from several groups have reported that sterile wounding in *Drosophila* adults induces the expression of AMPs, although the intensity of the response seems to be less than that upon infection. In contrast, Kenmoku *et al.* (2017) demonstrated that aseptic injury with tungsten needles strongly induced the expression of AMPs in larvae, the level of which is comparable to septic injury with bacteria (37). Intriguingly, they realized that even pinching with forceps, the standard handling method for collecting larvae, can trigger AMP expression powerfully. They noticed that AMP expression was induced not only by stimulus with forceps, but by just touching larvae with almost any material, such as toothpicks or brushes. Notably, induced genes include several AMPs, IM genes, and stress response genes, indicating that pinching by forceps can induce humoral innate immunity. They confirmed that this induction is infection independent by using germ-free larvae. Approximately 15% of pinched larvae showed small melanization spots inside their bodies, suggesting that pinching by forceps causes tissue injury. As with the regular systemic induction of AMPs, pinching-induced humoral immunity occurs at fat bodies. In sum, they revealed that larvae are surprisingly susceptible to mechanical stimuli in terms of the induction of innate immunity. The physiological significance of this phenomenon is unclear, although it may be an innately imprinted protective measure against expected infection, since larvae are frequently attached by a variety of predators, such as parasitoid wasps.

#### 3.2. Signaling pathways involved in pinching-induced *Drosomycin*

Next, they examined which signaling pathways are involved in pinching-induced *Drosomycin* (*Drs*) expression in larvae. As mentioned earlier, *Drs* induction is totally dependent on the Toll pathway

upon systemic infection with Gram-positive bacteria. Thus, they first examined the Toll pathway by using *psh*, *modSP*, *spz*, *dMyd88*, and *Dif* mutant animals. Pinching-induced *Drs* expression is partly dependent, though the dependency is not high, on *spz* and *dMyd88*. Remarkably, the induction is totally independent from *psh*, *modSP*, and *Dif*. These results indicate that the canonical Toll pathway is partially involved in the mechanically induced *Drs*, and the protease cascade that is required for the activation of Spz upon adult infection is not engaged in larvae. Note that their preliminary experiment implies that the processing of Spz is SPE independent (data not shown). They next assessed the involvement of the IMD pathway using *imd* and *relish* mutant larvae and indicated that there was no requirement of the IMD pathway for the induction of *Drs* upon pinching. Furthermore, the double mutant larvae of the Toll and IMD pathways still exhibited the substantial induction of *Drs*, indicating that large part of the pinching-induced *Drs* utilizes conventional innate immune pathways in adult flies. They additionally tested the involvement of the JNK, p38, PPO, and insulin signaling by using a mutant strain of the corresponding pathways, but no mutants showed decreased expression of *Drs* upon pinching, suggesting the requirement of the unknown signaling mechanism for the sterile inflammation is induced by mechanical stimuli.

#### 3.3. Tissues responsible for pinching-induced *Drosomycin*

Though pinching by forceps is performed only on part of a larval body, *Drs* expression is induced in the whole fat body. This suggests that some signaling factors would be released and circulated to activate the fat body. They first wondered whether sensory neurons under the cuticle could recognize and send signals to induce *Drs* in the fat body. However, apparently no involvement of sensory neurons was demonstrated by their experiments. First, the induction of *Drs* was not affected by the removal or dysfunction of sensory neurons, using the overexpression of apoptosis-inducing genes *reaper* and *hid*, or of the dominant negative *shibire*. Second, artificial activation of sensory neurons by expressing the dTrpA1 ion channel without pinching did not activate the expression of *Drs*. They next investigated the role of hemocytes for pinching-induced *Drs* expression. By using hemocyte-deficient larvae, which were generated again with a hemocyte-specific overexpression of *reaper* and *hid*, they showed that no difference could be detected between wild-type and hemocyte-ablated larvae with respect to the level of *Drs* induction. So far, they have not succeeded in the determination of responsible cells or tissues that signal from pinching stimuli to the fat body, which is a key challenge to be tackled in future studies.

## 4. NOVEL LIGAND FOR TOLL RECEPTOR

### 4.1. Novel ligand activity for Toll receptor in larval extract

To analyze sterile inflammation of *Drosophila*, Kanoh *et al.* attempted to identify DAMPs that activate innate immune signaling. Since it is generally believed in vertebrates that tissue damage could activate an inflammatory signal immediately after the damage, they reasoned that DAMPs may already exist inside the cells in *Drosophila*. To test this idea, they prepared tissue extracts of *Drosophila* by several extraction methods and found that the larval tissue extract prepared using an organic solvent strongly potentiated the expression of *Drs* in DL1 cultured cells (38). They did not detect such activity in cell lines or adult flies (data not shown).

As a next step, the authors examined whether the tissue extract activity that induces *Drs* is already a known Toll ligand, Spz. They prepared a tissue extract from the strong hypomorphic spz mutant *spz<sup>rm7</sup>* as well as from the null mutant *spz<sup>Δ8</sup>* (data not shown), and tested *Drs* inducing activity. They found that those extracts possessed equal *Drs*-inducing activity, suggesting that the activity from the larval tissue extract is not derived from the Toll ligand Spz. In addition, they prepared the tissue extract from germ-free larvae to exclude the possibility that the *Drs*-inducing activity is derived from bacterial components or pathogen-associated molecular patterns. This extract also contained comparable activity to the normal tissue extract. These results collectively indicate that larval tissue extract contains novel *Drs*-inducing activity (38).

Next, they performed RNAi experiments to investigate whether the Toll pathway is involved in *Drs* induction by larval tissue extract. Inhibition of expression for all the canonical Toll pathway components, *Toll*, *dMyd88*, *Tube*, *Pelle*, and *Dif* by RNAi resulted in the reduction of the expression level of *Drs* stimulated with the extract, indicating that the Toll pathway is required for this *Drs* expression. Of note, knockdown of the Toll receptor also exhibited the effect, suggesting that a novel *Drs*-inducing activity in the extract is an unknown ligand for the Toll receptor.

### 4.2. Spz5 as a Toll ligand

The novel ligand activity for Toll is sensitive to protease treatment, suggesting that the molecular nature of the activity is composed of protein. In their following study, Nonaka *et al.* used the candidate gene approach to identify the novel ligand (39). They expressed six Spz family of proteins, from Spz to Spz6, in S2 cells by the standard transfection method and prepared the cell extract to test *Drs*-inducing activity. This experiment revealed that the extract from

Spz5-expressing cells contained strong activity. RNAi experiments showed that *Drs* induction by Spz5 is *Toll*- and *dMyd88*-dependent. These results suggest that Spz5 could be a ligand for the Toll receptor. To prove this possibility, they generated spz5-deficient flies using the CRISPR/Cas9 technique. They successfully obtained a null mutant for *spz5* and prepared the larval extracts from the mutant larvae. The *Drs* reporter assay with DL1 cells clearly revealed that the *spz5*-deficient larval extract lost the activity almost completely, indicating that Spz5 is the responsible protein of the ligand activity for Toll receptor. Then, they examined whether Spz5 could function as a DAMP in terms of sterile induction of *Drs* in larvae with pinching by forceps. Unfortunately, *Drs* induction upon pinching of larvae with the *spz5* null mutant indicated that Spz5 is dispensable for pinching-induced sterile immunity. Future studies are necessary to reveal *in vivo* roles of Spz5, particularly its role, if any, as a DAMP.

### 4.3. No requirement of proteolytic processing of spz5 as a Toll ligand

The Spz family of proteins is known to become active by proteolytic cleavage. For example, Spz is cleaved by Easter or SPE (40,41), Spz3 by Easter (42), and Spz5 by Furin proteases (43). The cleaved form of Spz functions as a Toll ligand, and cleaved Spz5 works as a neurotrophin through Toll-6 (44). Furin proteases are evolutionally conserved proteases that process pro-neurotrophins, such as brain-derived neurotrophic factor (45,46). Foldi *et al.* (43) showed that the Furin cleavage site at R284 in Spz5 is a functional target site and is the predominant cleavage site of Spz5. Therefore, the authors introduced mutations into Spz5 at the R284 to make a Furin-resistant form, and then, these constructs were transfected into S2 cells and cell extracts were prepared from the cells. They confirmed that wild-type Spz5 were cleaved and the mutated Spz5R284G were not. By using these forms of Spz5, they performed the *Drs* reporter assay and showed that the R284G mutant form of Spz5 can activate Toll signaling. These results suggest that protease cleavage of Spz5 is unnecessary for its activity as a Toll receptor ligand. This implies that Spz5 might function as a DAMP that is released by cell damage or necrotic cell death since proteolytic processing is required for secretion of Spz5 from cells. But again, this is just a possibility that should be pursued in future studies.

## 5. SUMMARY AND PERSPECTIVE

As a defense mechanism in metazoan species, inflammation is vital in the daily skirmish against pathogenic microorganisms. Inflammatory reaction promotes the recruitment and activation of downstream innate immune effector mechanisms that are necessary to resolve cases of infection. However,

despite the importance of a prudently orchestrated reaction to host survival during infection, a prolonged and unnecessary inflammatory response is detrimental to the host (5).

Emerging evidence has demonstrated the existence of an enigmatic form of inflammation, termed sterile inflammation. In general, sterile inflammation is induced in the absence of infections or pathogens. With such an aseptic nature, sterile inflammation is thought to be caused by the involvement of multiple stimuli that are either present intracellularly or extracellularly as a response to mechanical and non-mechanical trauma. The activation of such an undesirable response often leads to tissue damage and, in some cases, recapitulates as acute diseases that could develop into chronic ones if the sterile inflammation is not resolved (4,5).

To date, there has been much progress in the exploration of potential causes and the elucidation of possible mechanisms of sterile inflammation (1,4,5). Multiple stimuli, both inorganic or organic materials, that are collectively characterized as DAMPs, have been reported to induce sterile inflammation in both vertebrate (5) and invertebrate (19) model organisms. One commonly used invertebrate in sterile inflammation-related studies is the fruit fly (*D. melanogaster*). Equipped with innate immune responses including the Toll and IMD signaling pathways that closely resemble those in humans, *D. melanogaster* has been experimentally proven as a suitable model organism to investigate diverse aspects of sterile inflammation; it has been used to elucidate the cause(s) of sterile inflammation and signaling pathways responsible in the aseptic activation of innate immune responses.

In addition to the role of multiple stimuli that can induce sterile inflammation (which have been described in refs 1,4,5), this review emphasizes pinching-related stimuli, a novel subset of sterile stimuli that can induce inflammation in *D. melanogaster* larvae. Pinching is a common method used by *Drosophilist* to collect larvae at all stages. Interestingly, a study by Kenmoku *et al.* (2017) demonstrated the unexpected induction of *Drosomycin* (*Drs*) upon pinching, which is regarded as a case of sterile inflammation. However, the upstream signal(s), including ligand(s) responsible for the induction of *Drs* by pinching remain elusive. A further attempt to elucidate the mechanism of such events has demonstrated that a canonical Toll signaling pathway is partially involved in pinching-induced sterile inflammation, but it is not in a manner dependent on a newly established Spz5-Toll-1 receptor axis (28).

It is presently unclear whether aseptic induction of *Drs* in *D. melanogaster* is clinically relevant to human conditions. Nevertheless, this

important finding shows that sterile inflammation is far more common in occurrence; for example, in the case of mechanical trauma caused by pinching. With this in mind, it is important to be cautious, especially in the field of immunology, when interpreting data of AMPs induction obtained from larvae handled with such method.

Despite our advances on this particular topic, a great deal of mystery remains. For example, how is *Drs* induced and what are the physiological and pathological roles of such event in the sterile inflammation scenario in *Drosophila*? In addition, is Toll a sole signaling pathway playing a role in the induction of *Drs* upon pinching or are there any non-canonical pathways responsible in the aseptic induction of *Drs* that are distinct to the canonical ones described to date? Perhaps even simple questions like what causes *Drs* induction in the first place and whether *Drs* will activate other downstream molecules will provide insight beyond our current knowledge.

In humans, sterile inflammatory response has been linked to the pathogenesis of several debilitating diseases including life-threatening cardiovascular disorders, such as atherosclerosis and ischemia (4). Based on the importance of this topic, especially in the medical setting, further studies are required to improve our understanding of how sterile inflammation is induced and orchestrated. Devising a strategic plan to address the remaining key questions described above will provide a better way to develop a comprehensive understanding of the processes regulating sterile inflammation, which is vital for the formulation of new treatment approaches for therapeutic purposes.

## 6. ACKNOWLEDGEMENTS

This work was supported by grants from the Japan Science and Technology Agency (JST) for T.K.; the Ministry of Education, Culture, Sports, Science, and Technology of Japan (MEXT) for R.M.A and T.K. ; Indonesia Endowment Fund for Education (LPDP) and Ministry of Research, Technology and Higher Education of the Republic of Indonesia (BUDI-LN Scholarship) for E.S. The authors declare no conflict of interest.

## 7. REFERENCES

1. H. Shen, D. Kreisel, D. R. Goldstein. Processes of Sterile Inflammation NIH Public Access. *J Immunol* 191, 2857–2863 (2013)
2. Y. Chen, M.N. Yousaf, W. Z. Mehal. Role of sterile inflammation in fatty liver diseases. *Liver Res* 2, 21–29 (2018)  
DOI: 10.1016/j.livres.2018.02.003



3. L. Duprez, N. Takahashi, F. Van Hauwermeiren, B. Vandendriessche, V. Goossens, T. Vanden Berghe, W. Declercq, C. Libert, A. Cauwels, P. Vandenabeele. RIP Kinase-Dependent Necrosis Drives Lethal Systemic Inflammatory Response Syndrome. *Immunity* 35, 908–918 (2011)  
DOI: 10.1016/j.immuni.2011.09.020
4. K.L. Rock, E. Latz, F. Ontiveros, H. Kono. The Sterile Inflammatory Response. *Annu Rev Immunol* 28, 321–342 (2010)  
DOI:10.1146/annurev-immunol-030409-101311
5. G.Y. Chen, G. Nuñez. Sterile inflammation: sensing and reacting to damage. *Nat Rev Immunol* 10, 826–37 (2010)  
DOI: 10.1038/nri2873
6. L. Franchi, T. Eigenbrod, G. Núñez. Cutting edge: TNF- $\alpha$  mediates sensitization to ATP and silica via the NLRP3 inflammasome in the absence of microbial stimulation. *J Immunol* 183, 792–6 (2009)  
DOI: 10.4049/jimmunol.0900173
7. C.G. Billings, P. Howard. Occupational siderosis and welders' lung: a review. *Monaldi Arch chest Dis = Arch Monaldi per le Mal del torace* 48, 304–14 (1993)
8. H-K Ea & F. Lioté. Calcium pyrophosphate dihydrate and basic calcium phosphate crystal-induced arthropathies: update on pathogenesis, clinical features, and therapy. *Curr Rheumatol Rep* 6, 221–7 (2004)  
DOI: 10.1007/s11926-004-0072-6
9. M.E. Bianchi. DAMPs, PAMPs and alarmins: all we need to know about danger. *J Leukoc Biol* 81, 1–5 (2007)  
DOI: 10.1189/jlb.0306164
10. B. Sangiuliano, NM Pérez, DF Moreira, JE Belizário. Cell death-associated molecular-pattern molecules: Inflammatory signaling and control. *Mediators Inflamm* 2014 (2014)  
DOI: 10.1155/2014/821043
11. H. Guo, J. B. Callaway, JP-Y Ting. Inflammasomes: mechanism of action, role in disease and therapeutics. *Nat Med* 21, 677–687 (2015)  
DOI: 10.1038/nm.3893
12. S. Kesavardhana, T-D. Kanneganti. Mechanisms governing inflammasome activation, assembly and pyroptosis induction. *Int Immunol* 29, 201–210 (2017)  
DOI: 10.1093/intimm/dxx018
13. M. Lamkanfi, V. M. Dixit. Mechanisms and Functions of Inflammasomes. *Cell* 157, 1013–1022 (2014)  
DOI: 10.1016/j.cell.2014.04.007
14. F. Martinon, K Burns, J. Tschopp. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL- $\beta$ . *Mol Cell* 10, 417–26 (2002)  
DOI: 10.3410/f.1008963.115857
15. B. K. Davis, H. Wen, J. P-Y Ting. The inflammasome NLRs in immunity, inflammation, and associated diseases. *Annu Rev Immunol* 29, 707–35 (2011)  
DOI: 10.1146/annurev-immunol-031210-101405
16. T. Karasawa, M. Takahashi. The crystal-induced activation of NLRP3 inflammasomes in atherosclerosis. *Inflamm Regen* 37, 18 (2017)  
DOI: 10.1186/s41232-017-0050-9
17. B. Lemaitre, J. A. Hoffmann. The Host Defense of *Drosophila melanogaster*. *Annu Rev Immunol* 25, 697–743 (2007)  
DOI: 10.1146/annurev.immunol.25.022106.141615
18. C. Hetru, J. A. Hoffmann. NF- $\kappa$ B in the immune response of *Drosophila*. *Cold Spring Harb Perspect Biol* 1, a000232 (2009)  
DOI: 10.1101/cshperspect.a000232
19. S. Govind. Innate immunity in *Drosophila*: Pathogens and pathways. *Insect Sci* 15, 29–43 (2008)  
DOI: 10.1111/j.1744-7917.2008.00185.x
20. Z. Shaukat, D. Liu, S. Gregory. Sterile inflammation in *Drosophila*. *Mediators Inflamm* 2015, 369286 (2015)
21. F. Leulier, B. Lemaitre. Toll-like receptors — taking an evolutionary approach. *Nat Rev Genet* 9, 165–178 (2008)  
DOI: 10.1038/nrg2303
22. H. Kanoh, L. L. Tong, T. Kuraishi, Y. Suda, Y. Momoiuchi, F. Shishido; S. Kurata. Genome-wide RNAi screening implicates the E3 ubiquitin ligase ShpA in mediating innate immune signaling by Toll in *Drosophila* adults. *Sci Signal* 8, 1–15 (2015)  
DOI: 10.1126/scisignal.2005971
23. C. Hetru, J. A. Hoffmann. NF- $\kappa$ B in the immune response of *Drosophila*. *Cold Spring*

- Harb Perspect Biol* 1, a000232 (2009)  
DOI: 10.1101/cshperspect.a000232
24. C. Dostert, E. Jouanguy, P. Irving, L. Troxler, D. Galiana-Arnoux, C. Hetru, J. A. Hoffmann, J.-L. Imler. The Jak-STAT signaling pathway is required but not sufficient for the antiviral response of *Drosophila*. *Nat Immunol* 6 (2005)  
DOI: 10.1038/ni1237
25. M. J. Williams. Immunity Hemopoiesis and Cellular *Drosophila*. *J Immunol Ref* 178, 4711–4716 (2007)
26. A.J. Bretscher, V. Honti, O. Binggeli, O. Burri, M. Poidevin, E. Kurucz, J. Zsomboki, I. Ando, B. Lemaître. The Nimrod transmembrane receptor Eater is required for hemocyte attachment to the sessile compartment in *Drosophila melanogaster*. *Biol Open* 4, 355–363 (2015)  
DOI: 10.1242/bio.201410595
27. Y. Hashimoto, Y. Tabuchi, K. Sakurai, M. Kutsuna, K. Kurokawa, T. Awasaki, K. Sekimizu, Y. Nakanishi, A. Shiratsuchi. Identification of Lipoteichoic Acid as a Ligand for Draper in the Phagocytosis of *Staphylococcus aureus* by *Drosophila* Hemocytes. *J Immunol* 183, 7451–7460 (2009)  
DOI: 10.4049/jimmunol.0901032
28. N. Buchon, N. Silverman, S. Cherry. Immunity in *Drosophila melanogaster* — from microbial recognition to whole-organism physiology. *Nat Rev Immunol* 14, 796–810 (2014)  
DOI: 10.1038/nri3763
29. Y. Nakanishi, K. Nagaosa, A. Shiratsuchi. Phagocytic removal of cells that have become unwanted: Implications for animal development and tissue homeostasis. *Dev Growth Differ* 53, 149–160 (2011)  
DOI: 10.1111/j.1440-169x.2010.01224.x
30. M. Ming, F. Obata, E. Kuranaga, M. Miura. Persephone/Spätzle pathogen sensors mediate the activation of Toll receptor signaling in response to endogenous danger signals in apoptosis-deficient *Drosophila*. *J Biol Chem* 289, 7558–68 (2014)  
DOI: 10.1074/jbc.m113.543884
31. F. Obata, E. Kuranaga, K. Tomioka, M. Ming, A. Takeishi, C-H Chen, T. Soga, M. Miura. Necrosis-Driven Systemic Immune Response Alters SAM Metabolism through the FOXO-GNMT Axis. *Cell Rep* 7, 821–833 (2014)  
DOI: 10.1016/j.celrep.2014.03.046
32. N. Mukae, H. Yokoyama, T. Yokokura, Y. Sakoyama, S. Nagata. Activation of the innate immunity in *Drosophila* by endogenous chromosomal DNA that escaped apoptotic degradation. *Genes Dev* 16, 2662–71 (2002)  
DOI: 10.1101/gad.1022802
33. X. Liu, T. Sano, Y. Guan, S. Nagata, J. A. Hoffmann, H. Fukuyama. *Drosophila* EYA Regulates the Immune Response against DNA through an Evolutionarily Conserved Threonine Phosphatase Motif. *PLoS One* 7, e42725 (2012)  
DOI: 10.1371/journal.pone.0042725
34. J. C. Pastor-Pareja, M. Wu, T. Xu. An innate immune response of blood cells to tumors and tissue damage in *Drosophila*. *Dis Model Mech* 1, 144–154 (2008)
35. F. Parisi, R. K. Stefanatos, K. Strathdee, Y. Yu, M. Vidal. Transformed Epithelia Trigger Non-Tissue-Autonomous Tumor Suppressor Response by Adipocytes via Activation of Toll and Eiger/TNF Signaling. *Cell Rep* 6, 855–867 (2014)  
DOI: 10.1016/j.celrep.2014.01.039
36. T. Hauling, R. Krautz, R. Markus, A. Volkenhoff, L. Kucerova, U. Theopold. A *Drosophila* immune response against Ras-induced overgrowth. *Biol Open* 3, 250–60 (2014)  
DOI: 10.1242/bio.20146494
37. H. Kenmoku, A. Hori, T. Kuraishi, S. Kurata. A novel mode of induction of the humoral innate immune response in *Drosophila* larvae. *Dis Model Mech* 10, 271–281 (2017)  
DOI: 10.1242/dmm.027102
38. H. Kanoh, T. Kuraishi, L. L. Tong, R. Watanabe, S. Nagata, S. Kurata. Ex vivo genome-wide RNAi screening of the *Drosophila* Toll signaling pathway elicited by a larva-derived tissue extract. *Biochem Biophys Res Commun* 467, 400–406 (2015)  
DOI: 10.1016/j.bbrc.2015.09.138
39. S. Nonaka, K. Kawamura, A. Hori, E. Salim, K. Fukushima, Y. Nakanishi, T. Kuraishi. Characterization of Spz5 as a novel ligand for *Drosophila* Toll-1 receptor. *Biochem Biophys Res Commun* 506, 510–515 (2018)  
DOI: 10.1016/j.bbrc.2018.10.096

40. A. N. R. Weber, S. Tauszig-Delamasure, J. A. Hoffmann, E. Lelièvre, H. Gascan, K. P. Ray, MA Morse, J-L. Imler, NJ Gay. Binding of the *Drosophila* cytokine Spätzle to Toll is direct and establishes signaling. *Nat Immunol* 4, 794–800 (2003)  
DOI: 10.1038/ni955
41. B. Moussian, S. Roth. Dorsoventral Axis Formation in the *Drosophila* Embryo—Shaping and Transducing a Morphogen Gradient. *Curr Biol* 15, R887–R899 (2005)  
DOI: 10.1016/j.cub.2005.10.026
42. S. L. Ballard, D. L. Miller, B. Ganetzky. Retrograde neurotrophin signaling through Tollo regulates synaptic growth in *Drosophila*. *J Cell Biol* 204, 1157–1172 (2014)  
DOI: 10.1083/jcb.201308115
43. I. Foldi, N. Anthoney, N. Harrison, M. Gangloff, B. Verstak, M. P. Nallasivan, S. Al Ahmed, B. Zhu, M. Phizacklea, M. Losada-Perez, M. Moreira, N. J. Gay, A. Hidalgo. Three-tier regulation of cell number plasticity by neurotrophins and Tolls in *Drosophila*. *J Cell Biol* 216, 1421–1438 (2017)  
DOI: 10.1083/jcb.201607098
44. G. McIlroy, I. Foldi, J. Aurikko, J. S. Wentzell, M. A. Lim, J. C. Fenton, N. J. Gay, A. Hidalgo. Toll-6 and Toll-7 function as neurotrophin receptors in the *Drosophila melanogaster* CNS. *Nat Neurosci* 16, 1248–1256 (2013)  
DOI: 10.1038/nn.3474
45. Y. Rouillé, S. J. Duguay, K. Lund, M. Furuta, Q. Gong, G. Lipkind, A. A. Oliva, S. J. Chan, D. F. Steiner. Proteolytic processing mechanisms in the biosynthesis of neuroendocrine peptides: the subtilisin-like proprotein convertases. *Front Neuroendocrinol* 16, 322–361 (1995)  
DOI: 10.1006/frne.1995.1012
46. N.G. Seidah, S. Benjannet, S. Pareek, M. Chrétien, R. A. Murphy. Cellular processing of the neurotrophin precursors of NT3 and BDNF by the mammalian proprotein convertases. *FEBS Lett* 379, 247–50 (1996)  
DOI: 10.1016/0014-5793(95)01520-5

**Abbreviations:** SIRS: Systemic Inflammatory Response Syndrome; DAMPSs: damage (danger)-associated molecular patterns; CDAMPs: cell-death associated molecular patterns; NLRs: Nod-Like Receptors; ASC: apoptosis-associated speck-like protein containing a caspase recruitment domain or CARD; PAMPs: pathogen-associated molecular

patterns; MSU: monosodium uric; TLRs: Toll-like receptors; PRRs: Pattern recognition receptors; AMPs: antimicrobial peptides; Spz: Spätzle; Imd: immune deficiency; JAK/STAT: Janus kinase/signal transducer and activator of transcription; Dome: Domeless; hop: hopscotch; Stat92E: STAT transcriptional factor; PGRP-LC: peptidoglycan recognition protein LC; IKK: IκB kinase; upd: unpaired; GnmT: glycine N-methyltransferase; Drs: *Drosomycin*

**Key Words:** *Drosophila*, Innate Immunity, Sterile Inflammation, antimicrobial peptides, Review

**Send correspondence to:** Takayuki Kuraishi, Shizenken, Kakuma-machi, Kanazawa, Ishikawa 920-1192, Japan, Tel: +81-76-264-6204, E-mail: tkuraishi@staff.kanazawa-u.ac.jp