Review

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Studies of host-pathogen interactions and immune-related drug development using the silkworm: interdisciplinary immunology, microbiology, and pharmacology studies

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Summary **Innate immunity acts as a front-line barrier against invading pathogens, and the majority of the components are widely conserved among species. Regulation of innate immunity is important for overcoming infections and preventing self-damaging sepsis. Using the silkworm (*Bombyx mori*) as an animal model, we elucidated the activation processes of innate immunity with emphasis on a multifunctional insect cytokine called paralytic peptide. Moreover, we established an ex vivo system using silkworm larval specimens to quantitatively evaluate the immunostimulatory activity of natural compounds. We observed that overactivation of innate immunity in silkworms induces tissue damage followed by host death, resembling sepsis-induced multi-organ failure in humans. Here, we summarize our recent findings and propose the usefulness of the silkworm as an animal model for studying immune regulation and for evaluating compounds with the potential to regulate innate immunity.**

Keywords: Innate immunity, insect cytokine, sepsis, virulence factors, antibiotics

1. Insect innate immunity

Animals continuously encounter pathogenic microorganisms throughout life. The immune system has thus evolved to overcome infection and maintain health. The immune system in higher animals is categorized as either innate or acquired. Innate immunity acts as the first-line barrier at an early stage of infection and sends signals to alert the acquired immune system, which produces specific antibodies. Rapid and coordinated activation of innate immunity is vital to prevent the growth of microorganisms. Therefore, understanding the regulatory mechanisms underlying innate immunity is crucial in terms of medical treatment and drug development for infectious diseases in humans.

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Recent studies revealed that most components of innate immunity are widely conserved from mammals to insects (*1*). Insects possess relatively simple physiologic systems and, because they lack antibody-producing organs, rely solely on innate immunity. This makes insects a suitable model for studying the basis of innate immunity. A representative insect-oriented immunologic study is the discovery of Toll in *Drosophila*, which led to the identification of mammalian Toll-like receptors. Insect Toll functions as a receptor for an endogenous ligand called Spaetzle and relays signals to transcription factors that produce antimicrobial peptides (AMPs) (*2*). Genomic studies revealed that insects possess several isotypes of the toll gene, and the repertoire and activation pattern varies among species. The silkworm (*Bombyx mori*) has 12 toll isotypes, some of which are expressed several hours after pathogen infection (*3,4*). Batteries of signaling molecules mediate the Toll pathway, which are also self-induced together with AMPs (*5-9*). In addition, infectious stimuli activate other innate immune pathways, such as IMD (*10*), JNK (*11*), and JAK/STAT (*12*), to act in concert with the Toll pathway (*13*). The production processes of AMPs and other toxic substances in the cell-free system (*e.g.*,

melanization) are referred to as humoral immunity, whereas immunosurveillence hemocytes are involved in cellular immunity, such as engulfment and clearance of non-self agents (*e.g.*, phagocytosis and encapsulation) (*14*). Appropriate regulation of the time and degree of immunoactivation is crucial to achieve efficient host protection against infectious pathogens. Our understanding of the regulatory mechanisms in insect innate immunity, however, remains insufficient at the molecular level, relative to that of individual responses.

2. Activation of innate immunity by the insect cytokine, paralytic peptide, in silkworms

As described below, we established an alternative infection model using the silkworm as a host animal to evaluate the effectiveness of antibiotics (*15*) and to analyze the virulence mechanisms of pathogenic bacteria (*16*). In the course of our studies using the silkworm infection model, we observed that bacteria-infected larvae were paralyzed, accompanied by slow muscle contraction (*17*). This paralysis was apparently different from the rapid and direct stimulation of muscle contraction by neurotransmitter-like substances (*18*), but very similar to that caused by an endogenous cytokine-like factor named paralytic peptide (PP). PP was originally identified as the factor responsible for paralysis caused by "blood donation" between silkworms; larvae injected with hemolymph isolated from another silkworm exhibit slow paralysis (*19*). PP was initially transcribed as a 14-kDa inactive precursor, and the N-terminus 23 amino acid residues are cleaved by proteolysis to generate the active form of PP in the stimulated hemolymph (*20*). Although the physiologic relevance of PP-induced paralysis was unknown at that time, we considered that PP was involved in bacteria-induced paralysis in silkworms (Figure 1A).

To test the above hypothesis, we first established an *ex vivo* assay system to quantitate the degree of paralysis by measuring the length of the larval muscle specimen after sample injection (Figure 1B). We confirmed using this system that injecting both heat-killed bacteria and a chemically synthesized active form of PP induces slow muscle contraction, which reaches a maximum after 5 to 10 min and then recovers to the basal level within 30 min (*17*). Among the microorganism-derived components, we found that peptidoglycan (PG) and beta-glucan (BG), major cell wall components of bacteria and fungi respectively, induce muscle contraction in a dose-dependent manner (*17*). In contrast, lipopolysaccharide (LPS), another bacterial component that is a potent immunostimulant, is ineffective even at high concentrations (*21*), suggesting ligand specificity. When the specimens were treated with anti-PP serum that specifically binds to PP and inhibits its biologic activities (*22*), muscle contraction caused by the bacterial PG and BG, as well as that induced by active PP, is completely suppressed (*17*). Moreover, we observed the rapid generation of active PP in the hemolymph of silkworms injected with PG and BG (*17*). These findings suggest a causative relationship between pathogen-induced paralysis and PP activation in silkworm hemolymph (Figure 1A).

The findings that bacterial and fungal cell wall components induce PP activation raise the following questions: *1*) how is PP activated? *2*) what happens after PP activation? and *3*) what is the biologic significance of the bacteria-induced PP activation? In the following

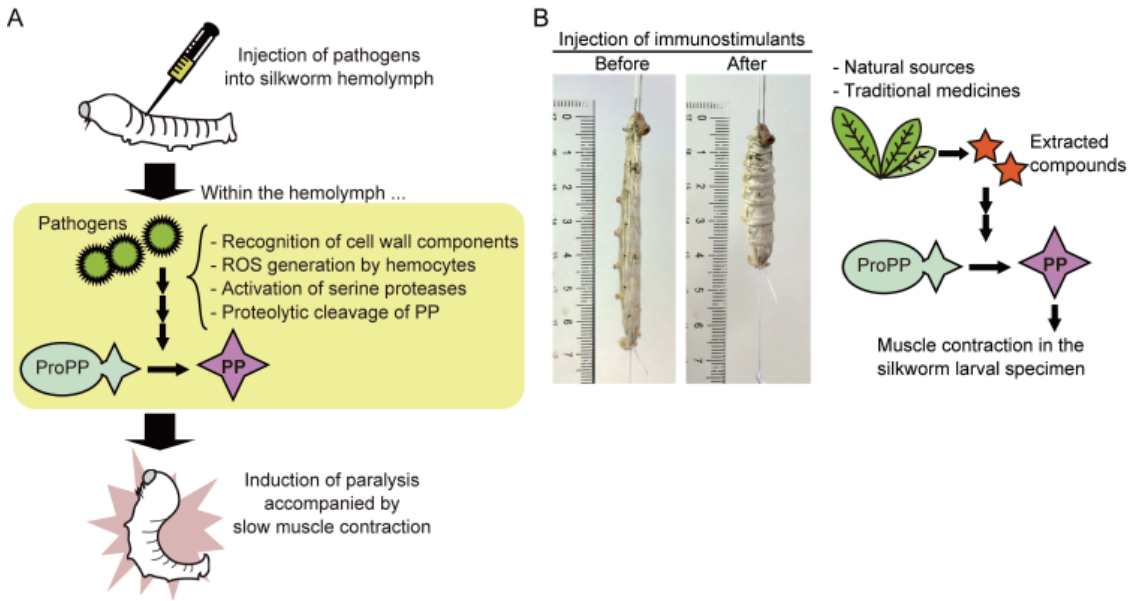

Figure 1. Activation of the insect cytokine paralytic peptide (PP) induced by pathogens. (A) PP activation processes induced by pathogens in the silkworm hemolymph. **(B)** Silkworm larval muscle contraction assay and its application for evaluation of immune-activating compounds. Abbreviations: AMP, antimicrobial peptides; MAPK, mitogen-activated protein kinase; NO, nitric oxide; ROS, reactive oxygen species.

subsections, we summarize our recent findings regarding these issues.

2.1. *Upstream processes of PP activation*

For the upstream processes of PP activation, we focused on reactive oxygen species (ROS). Using specific pharmacologic inhibitors, we found that the presence of live hemocytes, ROS, and activated serine proteases are required for pathogen-induced PP activation (*17*). We considered that live hemocytes recognize the pathogen-derived components and generate ROS (*i.e.*, a process called oxidative burst), followed by the onset of a putative serine-protease cascade that cleaves the PP precursor (Figure 1A). Specification of ROS types, hemocyte populations, pathogen-recognition receptors, and proteases will be examined in future studies. As serine proteases are involved in other immune responses, such as melanization (*23-25*) and Spaeztle activation (*26-28*), their crosstalk and role in PP activation will be interesting to evaluate in future studies.

2.2. *Downstream processes induced by active paralytic peptide*

Based on our findings that PP activation is triggered by pathogen components, PP might mediate defensive responses against infectious pathogens. To gain insight into the downstream events induced by PP, we performed a genome-wide microarray analysis of the hemocytes and fat body, the main immune organs in the silkworm larvae. Among a number of genes with altered expression patterns after PP injection, several genes encoding cytokine-like factors were upregulated, suggesting the existence of a complex network of insect cytokines (*29*).

We further studied the effect of PP on defensive responses in each organ. Active PP induced the expression of phagocytosis-related genes in hemocytes and promoted the engulfment of bacteria (Figure 2). In addition, the amount of AMP mRNA expressed in the fat body increased after PP injection, and activation of p38 mitogen-activated protein kinase (MAPK) mediated this PP-dependent AMP production (Figure 2). In addition to the well-known role of p38 MAPK in stress responses, recent studies demonstrated its contribution to insect immunity (*30*). Moreover, we found that PP induces the expression of nitric oxide (NO) synthase in the fat body, and NO production is required for both p38 MAPK activation and AMP expression (*31*). Although previous reports revealed that NO functions as a messenger molecule in insect immunity (*32*), but the pathway responsible for NO production has remained unknown (*33*). Our findings are thus the first to reveal a regulatory axis comprising insect cytokine PP, NO, and p38 MAPK. Further studies

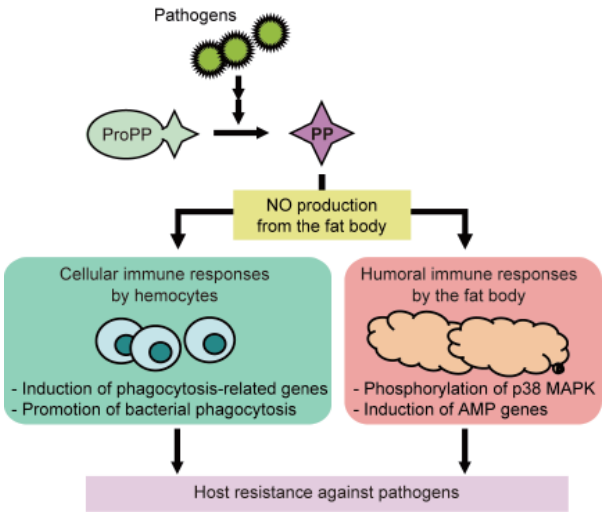

Figure 2. Immune responses induced by active paralytic peptide (PP). Abbreviations: LPS, lipopolysaccharides.

of signal mediators may reveal the connection between the extracellular cytokine network and intracellular signaling matrices that lead to regulated immune-gene expression.

PP and its Lepidoptera homologs belong to the ENF peptide family (*34*), named after their conserved C-terminal amino acid residues (glutamic acid, asparagine, and phenylalanine). The PP homologs have various biologic activities, which are reflected by the names given to these homologs in other Lepidoptera species: growth-blocking peptide, plasmatocyte-spreading peptide, and cardioactive peptide (*34*). We demonstrated that the C-terminus ENF residues are required for the immunostimulating activity of PP (*31*), as observed in PP homologs with other activities (*35,36*). The above signaling pathways mediating the PP-dependent immune responses might also be involved in different biologic events, such as development, downstream of other PP homologs. In addition to Lepidoptera insects, a *Drosophila* ENF peptide regulates the switching of humoral and cellular immunity (*37,38*). Moreover, PP homologs share similarity in their tertiary structure with mammalian epidermal growth factor and interact with mammalian epidermal growth factor receptors (*39*). We expect that conserved counterparts of the PP pathway exist in vertebrates and that studies of insect cytokines will shed light on the basis of innate immune regulation.

2.3. *Biologic relevance of paralytic peptide activation in host-pathogen interactions*

The biologic significance of PP activation has been demonstrated in the silkworm infection model using several pathogenic bacteria. Given that PP activation is triggered by pathogen components and that the active form of PP induces gene expression in immune tissues, we examined the protective role of PP in

infected host insects. The death of silkworms infected with *Staphylococcus aureus*, a human opportunistic pathogen, is delayed by injecting an excess amount of active PP. In contrast, the bacteria-dependent silkworm killing effects are accelerated when the larvae are treated with an anti-PP serum that specifically inhibits the biologic functions of PP. These findings suggest that PP acts as an insect cytokine that confers host protection against pathogens (*17*).

Using the silkworm infection model, we demonstrated that most human pathogens have silkworm-killing effects (*40,41*). Among them, *Serratia marcescens*, another pathogenic bacterium that infects infants and immunocompromised patients, kills silkworms with an extremely small number of cells (*42,43*). Silkworms infected with *S. marcescens* have unique phenotypes that are not induced by other pathogens, reduced hemocyte viability (*42*) and an increased number of freely circulating hemocytes in the hemolymph (*44*). While the hemocyte killing effects are induced by live *S. marcescens* cells and not the culture supernatant, the hemocyte number is increased by injecting either live bacteria or the supernatant, suggesting that the two phenotypes are caused by different mechanisms.

By screening the *S. marcescens* transposon mutant library, we found that mutants defective in the biosynthesis of LPS O-antigen and flagella, cell surface components required for bacterial motility, exhibit attenuated hemocyte-killing ability (*42*). This LPS- and flagella-dependent hemocyte killing by *S. marcescens* impairs glucan-induced muscle contraction in the muscle contraction assay (*42*), further supporting our previous notion that the presence of live hemocytes is required for PP activation (*17*). Isolated LPS and flagella proteins themselves, however, fail to kill hemocytes; thus, it is likely that other uncharacterized factors are directly involved in the apoptosis induction. Nevertheless, mutants lacking LPS O-antigen and flagella have much lower virulence in silkworm larvae compared with the parent strain (*42*), suggesting that suppression of host immunity *via* hemocyte killing largely contributes to *S. marcescens* pathogenicity (Figure 3A).

Another phenotype observed in silkworms injected with the culture supernatant of *S. marcescens*, but not other bacterial species, is the transient increase in the number of freely circulating hemocytes. We purified the factor responsible for the hemocyte-increasing activity of the culture supernatant through biochemical approaches and identified serralysin metalloprotease (*44*). We demonstrated that serralysin degrades the adhesive molecules at the hemocyte surface, resulting in cell detachment from the internal body cavity and an increase in freely circulating cells. Consistent with previous reports, PP induces adhesive molecules in hemocytes thereby increasing cell adhesiveness (*29*). On the other hand, the PP-dependent increase in hemocyte adhesiveness is blocked by serralysin treatment, without

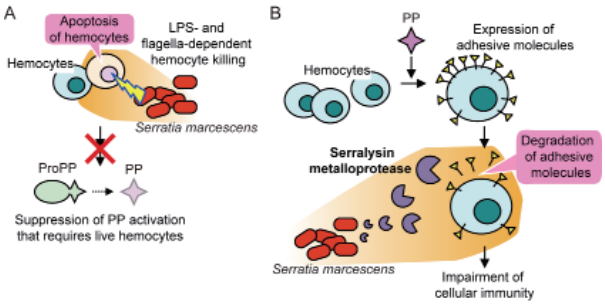

Figure 3. Immune-evading mechanisms of the pathogenic bacterium *S. marcescens*. (A) Suppression of paralytic peptide (PP) activation *via* hemocyte killing by *S. marcescens*. **(B)** Impairment of cellular immunity *via* production of serralysin metalloprotease by *S. marcescens*. Abbreviations: LPS, lipopolysaccharides; ROS, reactive oxygen species.

direct degradation of PP itself. Moreover, serralysin suppresses immunologic activities, such as phagocytosis, by hemocytes and bacterial clearance within the silkworm hemolymph (*44*). Together, these findings suggest that *S. marcescens* impairs host immunity, including the PP pathway, *via* two distinct mechanisms: direct hemocyte-killing mediated by bacterial surface components (Figure 3A), and degradation of adhesive molecules by the secretion of serralysin metalloprotease (Figure 3B). The significance of PP in self-defense is highlighted through analyses of these battles between host animals and pathogens.

3. Evaluation of immunostimulatory compounds using silkworms

As described above, the silkworm muscle contraction assay is suitable for evaluating stimulatory effects of test samples on the PP pathway (Figure 1). The silkworm *ex vivo* system has several advantages compared to other immunologic assays, such as interleukin production using mammalian macrophages. Because bacterial LPS, a contaminant frequently present in natural sources, fails to trigger PP activation (*21*), the activity measured by the silkworm contraction assay is free from false-positives due to environmental LPS. This is presumably due to the presence of LPS-absorbing proteins in the silkworm hemolymph (*45*). Moreover, samples injected into the hemolymph of the larval specimen are subjected to drug-metabolic processes. Therefore, we consider that the silkworm muscle contraction assay is applicable for evaluating the immunostimulatory effects of compounds. In this section, we present the practical use of the silkworm muscle contraction assay.

3.1. *Purification of immunostimulatory polysaccharides from green tea leaves*

Tea leaves contain various compounds, such as polyphenol, that affect human health. The factors responsible for the bioactivities reported in tea remain largely unknown at the molecular level. A hot water

extract of green tea (*Camellia sinensis*) leaves exhibits potent activity in the silkworm muscle contraction assay (*46*). On the other hand, known components of green tea leaves, such as catechin, polyphenon, and cellulose, fail to induce muscle contraction, suggesting that the leaves contain uncharacterized substances that stimulate innate immunity. We purified the active component from the tea extract by successive chromatography steps. Based on the physical properties of the active fraction, we assumed that it contained polysaccharides. Monosaccharide analysis and nuclear magnetic resonance revealed that the polysaccharide structure contains D-galacturonic acid and methyl ester residues (*46*). Moreover, the activity of the purified fraction is sensitive to enzymatic treatment by beta-glucanase, suggesting the presence of a beta-glucan structure connected with a polygalacturonic acid backbone (*46*). We further verified the immunostimulatory effect of this polysaccharide in a conventional interleukin-6 production assay using mouse peritoneal macrophages (*46*). These findings support our statement that the silkworm muscle contraction assay is useful for identifying novel substances with immunostimulatory activity.

3.2. *Characterization of immunostimulatory glucans from rock tripe*

Rock tripe, a group of lichens that grow on rocks, is used as a traditional medicine in Eastern countries (called "iwatake" in Japanese and "seogi" in Korea). These lichens produce various polysaccharides, some of which have stimulatory effects on mammalian immune cells (*47*). By using the silkworm contraction assay, we evaluated the immunostimulatory effects of these rock tripe-derived polysaccharides. We found that GE-3, a beta-1,6 glucan from *Gyrophora esculenta*, induces muscle contraction in larval specimens (*21*). Moreover, GE-3 promotes PP activation within the silkworm hemolymph in a dose-dependent manner (21). A previous microarray analysis revealed that PP injection upregulates immune genes that are also induced in virus-infected insects (*48,49*). Using the *Bombyx mori* nucleopolyhedrosis virus silkworm infection model (*50*), we demonstrated that GE-3 has a host protective effect (*21*).

In contrast to GE-3, some of the plant-derived beta-glucans (*e.g.*, laminaran, lentinan, schizophyllan, and ukonan) fail to induce muscle contraction (*21*), suggesting that the host system recognizes specific structural patterns of beta-glucans that lead to PP activation and host protection. The precise mechanism of ligand recognition and the downstream anti-viral immune reactions, however, require further studies.

4. Overactivation of innate immunity in silkworms

The immune system is a "double-edged sword", and overactivation of immune reactions damages the host animal itself. In humans, severe inflammatory states are due mainly to the dysregulation of innate immunity, rather than acquired immunity, and are related to numerous diseases; either local or systemic, acute or chronic. In mammals, pathogen-recognizing receptors such as Toll-like receptors trigger exaggerated responses called cytokine storms, in which overproduced cytokines amplify the inflammatory signal. A striking example is Toll-like receptor 4-dependent septic shock in mice injected with bacterial LPS; death caused by LPS treatment is clearly suppressed in *tlr4* gene-knock out mice (*51*). In this sense, studies of the mechanisms that modulate immune responses are crucial to understand and overcome the "negative" side of immunity. Regardless of the basic conservation in innate immunity, however, there has been no reported model of sepsis in invertebrates that includes immune system overactivation.

Among the human pathogens with lethal effects in silkworms, we focused on *Porphyromonas gingivalis*, a Gram-negative bacterium causative for periodontal disease. Larval death caused by this specific bacterium has distinct features not observed in silkworms infected with other model pathogens. First, the silkworms infected with *P. gingivalis* are not cured by antibiotic administration, despite inhibition of the *in vitro* growth of *P. gingivalis* and the finding of therapeutic effects on other pathogenic bacteria. While viable bacterial cells within the silkworm hemolymph decrease soon after infection, *P. gingivalis* continues to kill silkworms after several days. Moreover, heat-killed *P. gingivalis* cells are as toxic to silkworms as live bacteria. Second, the hemolymph of *P. gingivalis*-infected larvae show intense blackening, a defensive reaction called melanization, several hours after injection. The degree of hemolymph blackening is much higher after *P. gingivalis* infection than infection by other pathogens. We considered from these findings that *P. gingivalis* surface components kill silkworms by excess activation of melanization (*52*).

We further assessed the killing mechanism by *P. gingivalis*. Both hemolymph blackening and host-killing by *P. gingivalis* are suppressed by 1-phenyl-2-thiourea and serine protease inhibitors, agents that inhibit insect melanization. As melanin itself is not toxic to silkworms, we shifted our focus to ROS generated as side-products during melanin polymerization. ROS are a well-known trigger of cell-death signals and are thus implicated in a wide range of stress-related diseases and inflammation. We found that radical scavengers and pharmacologic inhibitors of cell-death signals exhibited therapeutic effects in silkworms infected with *P. gingivalis* (Figure 4). Although *P. gingivalis* also induced potent muscle contraction, inhibition of the PP pathway by PP-antiserum did not affect silkworm killing, suggesting that melanization is specifically involved in the host damaging process. Together, these

findings suggest that *P. gingivalis*-induced excess ROS production during melanization is followed by activation of cell death signals and organ failure. We proposed this as the first insect model of bacteria-induced immune overactivation causing severe damage against the host (*52*).

How does *P. gingivalis* induce excess melanization? We only know that PG, a major cell wall component in bacteria, is responsible for the onset of melanization. The composition and structure of PG vary among bacterial species. In general, Gram-negative or Gram-positive bacteria possess either diaminopimelic acid - or Lys-type PG, followed by more detailed classifications. Comparison with the toxic effects of PG from other bacteria, however, fails to reveal a clear relationship between the PG type and immunoreactivity (*52*). Furthermore, *P. gingivalis* PG-induced host killing is not observed in two Diptera species (*D. melanogaster* and *Sarcophaga peregrina*), whereas other Lepidopteran and Coleopteran insects are killed and their hemolymph blackened (*52*). Identification of the precise PG structure and the host PG-receptor involved in the excess melanization may provide reasonable explanations for the above comparative study, and further elucidate the evolutionary diversification of immunomodulation.

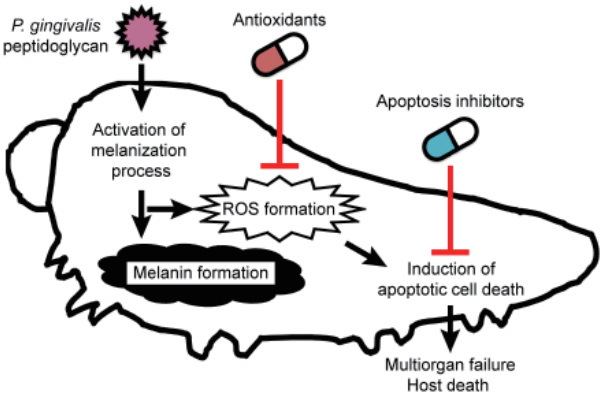

Figure 4. Overactivation of immune responses by *P. gingivalis* peptidoglycan in silkworms.

5. Perspectives for using silkworms as a model animal for the development of immuno-regulatory drugs

As mentioned above, we initially established a pathogen infection model using the silkworm as a host animal. The lower rearing costs and fewer ethical issues associated with using a large number of silkworms for drug screening and development are advantageous compared with mammalian models. The pharmacokinetic parameters and toxicity of most drugs evaluated in silkworms are comparable to those reported in mammals, suggesting that both models possess similar drug metabolism systems (*15,53,54*). Using the *S. aureus*-silkworm infection model, we recently identified a novel antibiotic named lysocin E (*55*). During the purification procedure of therapeutically active substances from the *Lysobacter* culture supernatant, we observed that the *in vitro* antibacterial activity does not increase in proportion with the *in vivo* therapeutic activity (*55*), indicating the importance of evaluating *in vivo* activity using the whole animal, and not only in *in vitro* assays, to screen and identify therapeutically active compounds (Figure 5).

As exemplified by the antibiotic lysocin E, this silkworm infection model allows us to determine compounds that modulate the immune system using silkworms. As presented in the above sections, the silkworm muscle contraction assay is suitable for screening substances, such as polysaccharides, contained in natural sources that stimulate innate immunity. The combination of an *ex vivo* muscle contraction assay and the silkworm infection model may lead to the identification of compounds with therapeutic effects through immune system activation rather than direct toxicity to pathogens. Because these compounds recruit host immune systems to suppress pathogen activity, the development of drug resistance may be limited, as discussed in the case of host AMPs used for infection control (*56*). In addition, these compounds could be effective against a wide range of pathogens, and may also be applicable for cancer therapy (*56*).

On the other hand, the *P. gingivalis*-silkworm

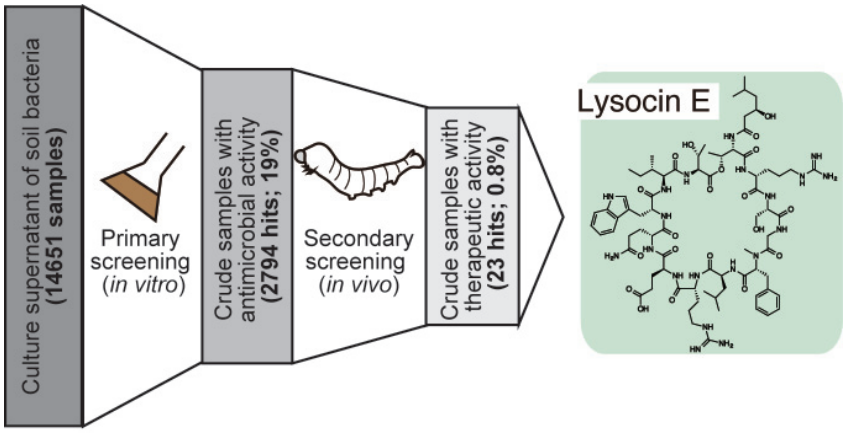

Figure 5. Identification of a novel antibiotic, lysocin E, through *in vivo* drug screening using the silkworm infection model.

infection model could be used to evaluate compounds that attenuate the overactivated state of the host immune system. Because excess ROS production is widely considered to cause cell death, radical scavengers and apoptosis inhibitors are expected to prevent tissue damage at the inflammatory site. We demonstrated that *N*-acetyl-L-cysteine and glutathione, antioxidants with therapeutic effects in mammalian sepsis (*57*), suppress silkworm killing by the *P. gingivalis* peptidoglycan (*52*). While antioxidant activity is reported in a number of natural origins and traditional medicines, such as ginseng (*58*), in most cases the active substances responsible for the activity are yet to be determined. We consider that the silkworm model is suitable for searching for compounds from these potential sources with protective effects against sepsis that accompanies immune overactivation.

6. Concluding remarks

Insects are simple and useful models for analyzing the basis of regulatory mechanisms in innate immunity. Recent findings regarding the multifunctional insect cytokine PP in silkworms provide novel insights into the communication network among immune tissues and host-pathogen interactions. In contrast to mammalian models, insects are currently rarely applied to human drug development. The established silkworm assays evaluating both "positive" (cytokine activation) and "negative" (excess inflammation) aspects of immunity have potential applications in the development of therapeutic agents that affect host immunologic states. The silkworm is thus revolutionizing basic science by opening new fields of interdisciplinary research that include immunology, microbiology, and pharmacology.

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