Full title: **The *Drosophila* Toll pathway in innate immunity: from the core pathway toward effector functions**[1]

Running title: **The *Drosophila* Toll pathway in innate immunity**

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Non-standard abbreviations used in this article: DAMP, damage- or danger-associated molecular pattern; Dif, Dorsal-related immunity factor; Dl, Dorsal; Drs, Drosomycin; IM, immune-induced molecule; Pli, Pellino; Psh, Persephone; SPE, Spätzle processing enzyme, Spz, Spätzle

Abstract

The *Drosophila melanogaster* Toll signaling pathway has an evolutionarily conserved role in controlling immune responses. Whereas the microbial recognition mechanisms and the core-signaling pathway leading to activation of the humoral immune response via the nuclear factor κB (NF-κB) transcription factors have been well established for many years, the mechanistic understanding of the effector functions at the molecular level is currently rapidly evolving. Here we review the current developments in elucidating the role of the *Drosophila* Toll signaling pathway in immunity. We will discuss the emerging role of Toll in viral infections and sex-specific differences in immunity. Mainly, we will focus on Toll pathway regulation, the effector molecules, and cellular immunity.

Introduction

In 2011, the importance of innate immunity was recognized by awarding the Nobel Prize in Physiology or Medicine to the researchers who discovered the fundamental basis of innate immune responses and their role in activating adaptive immunity. One half of the prize was awarded jointly to Bruce A. Beutler and Jules A. Hoffmann "for their discoveries concerning the activation of innate immunity", and the other half to Ralph M. Steinman "for his discovery of the dendritic cell and its role in adaptive immunity". In the work of Professor Hoffmann's group, the *Drosophila melanogaster* (*D. melanogaster*) Toll receptor was identified to be essential in the defense against fungal infections (1). This finding was soon followed by the discovery of the Toll-like receptors (TLRs) in mammals, opening new horizons for deeper understanding how mammalian immune responses are regulated (2, 3). In our Journal of Immunology Brief Reviews article from January 2011, we reviewed the literature leading to the understanding of the *Drosophila* Toll pathway function in both embryonic development and immunity (4). Here we revisit the topic of the *D. melanogaster* Toll signaling pathway and describe, in particular, the immune-related developments in Toll pathway research during the past decade, including findings concerning both humoral and cell-mediated arms of innate immunity.

Developments in microbe recognition, at the receptor level and in the core Toll pathway

The *Drosophila* Toll receptor differs from the mammalian TLRs in that the *Drosophila* Toll receptor functions as a cytokine receptor (reviewed in (5), whereas TLRs recognize foreign structures directly and thus are pattern recognition receptors (PRRs). In *Drosophila*, there are nine genes encoding Toll receptors (Toll-1 to Toll-9), out of which Toll-1 (Toll) has the main role as mediating innate immune signaling (4). Other Toll receptors may have tissue- and/or infection type specific roles (described below).

Events upstream of the *Drosophila* Toll receptor to activate the Toll pathway in different contexts have been thoroughly dissected already earlier, and are reviewed in Valanne *et al.* 2011 (Figure 1) (4). Recent developments include clarifying the structure of the Spätzle (Spz)/Toll receptor complex; in two independent studies it was shown that a single Spz dimer binds one Toll receptor ectodomain in 1:1 complex (6, 7). The stoichiometry of Spz binding to Toll is similar to some mammalian neurotrophins, where one cystine-knot dimer binds one receptor chain (7). Furthermore, Kellenberger *et al.* (8) have resolved the crystal structure of Grass, the clip serine protease involved in Toll pathway activation upstream of Sphinx1/2 / Spirit / Spheroide (8). In addition, the role of thioester-containing proteins (TEPs) in immune response has been studied, with the secreted TEPs (TEP1, 2, 3, and 4) shown to play a role in Toll pathway activation, likely by taking part in the recognition of certain gram-positive bacteria and fungi (9).

The activation of pathogen recognition receptors by microbial molecules has also been thoroughly studied (e.g. in (4) Figure 1, (10)). In the current model on Toll pathway activation, bacterial and fungal structures are recognized by specific PRRs, leading to the activation of downstream cascades and ultimately, the cleavage and activation of the Toll receptor ligand Spz. Recently, Gyc76C, a receptor guanylate cyclase, was shown to function as a parallel immune receptor to Toll, modulating NF-κB signaling downstream of MyD88 (11). Furthermore, it was shown that Gyc76C mediates both humoral responses (e.g. AMP induction) and cellular responses (hemocyte proliferation), but with distinct mechanisms: for the humoral response, Gyc76C-mediated AMP induction requires production of the secondary messenger cyclic guanosine monophosphate (cGMP), whereas hemocyte proliferation is cGMP-independent (12).

Another proteolytic cascade leading to Spz activation is initiated by proteases secreted by microbes, which can be considered as danger signals (i.e. damage-associated molecular patterns or danger-associated molecular patterns, DAMPs) (13–15). DAMPs can also be endogenous molecules generated upon injury or cellular damage, but here we discuss the danger signals coming from microbes upon infection. The mechanism behind the function of Persephone (Psh) in recognizing DAMPs upstream of Spätzle processing enzyme (SPE) was recently further studied (16). It was shown that certain fungal or bacterial proteases, which are important virulence factors for host colonization, prime Psh for the cleavage and activation by the endogenous cysteine cathepsin 26-29-p. Specifically, the microbial proteases act as danger signals to the host before tissue damage occurs, and the pro-domain of Psh functions as a bait for a broad range of these proteases. Subsequent action of the cysteine cathepsin 26-29-p on the primed Psh leads to the activation of the Toll pathway. This highlights the potential importance of cysteine cathepsins also in mammalian inflammatory diseases, a factor that has recently been discussed (e.g. (17)). Of note, it was recently discovered that *psh* is likely to be a relatively recent duplication of the serine protease gene *Hayan*, and that these two proteins redundantly activate the Toll pathway downstream of pattern recognition receptors (18). It is evident that this system of proteolytic activation by danger signals can sense a plethora of microbes, regardless of their origin, type, or specificity. Therefore, this finding leads to a conceptually novel immune system function in animals, although similar guard mechanisms have been known to play a role in plants (19). Recently, a parallel immune mechanism has been identified also in mammals; it was shown that the NLRP1 inflammasome is proteolytically activated by diverse microbial enzymes (20).

The core Toll pathway was extensively mapped already by 2011 (21, 22), but one important question remained – what is the kinase phosphorylating the *Drosophila* Inhibitor of κB (IκB) homolog Cactus (Cact)? Cact needs to be phosphorylated for its degradation and the subsequent activation of the pathway. After years of speculation, Daigneault and co-workers showed that Pelle phosphorylates Cact at the serines required for signal transduction and thus acts as the Cact kinase (23). Pelle can also phosphorylate the required sites of IκBα (23). Whereas the understanding of the core pathway has not changed much during the past ten years, much more insight has been gained relating to regulation and fine-tuning of the Toll pathway.

Regulation of the Toll pathway

As Toll signaling is central in inflammatory and immune responses, it needs to be tightly controlled. Many aspects of the regulation of the Toll pathway have been investigated in detail (Figure 1). At the level of modifying the structure of chromatin, Osa-containing Brahma complex (BAP) was shown to negatively regulate Toll pathway-mediated immune reactions both *in vitro* and *in vivo* in *Drosophila* (24). In the transcriptome study, Osa was also shown to regulate the expression of metabolic genes, highlighting the importance of the interplay between immunity and metabolism (24, 25). Another identified negative regulator of the Toll pathway is the retromer complex, shown to function upstream of the Toll receptor but downstream of SPE. Retromer is a protein complex originally identified in yeast (26). The complex is associated with the cytosolic side of the cell membrane and regulates the trafficking of protein cargo from endosomes to the trans-Golgi network (26, 27). Retromer is composed of five components: Sorting nexin 1/2 (SNX1/2), SNX5/6, Vacuolar protein sorting 29 (Vps29), Vps26, and Vps35. Zhou *et al.* (28) speculate that retromer is involved in an as yet unclear mechanism of Spz maturation. Besides general Toll pathway regulation, tissue-specific regulation mechanisms of the immune response have been studied in *Drosophila* respiratory

epithelium, i.e. trachea, where Tollo (Toll-8) was shown to negatively regulate the immune response signals coming via the Imd pathway. The ligand (or one of the ligands) to activate Tollo is a Spz homologue Spz2/DNT1, but the exact mechanism between Imd pathway and Tollo interplay in the tracheal tissue remains elusive (29).

Regulatory mechanisms studied in greater detail in recent years also include post-translational modifications such as ubiquitination and sumoylation. Both ubiquitination (reviewed in (30)) and sumoylation (reviewed in (31)) are mechanisms that can regulate immune pathway proteins, either by activating them, repressing them, or targeting them for degradation (e.g. (32, 33)). In the *Drosophila* Toll pathway, Pellino (Pli) has been identified as a Pelle-interacting factor (34–36). First, Pli was suggested to positively regulate Toll pathway activity, since ubiquitous overexpression of *Pli* resulted in enhanced Toll pathway target gene *Drosomycin* (*Drs*) expression (35). Somewhat controversially, it was later demonstrated that knockdown or overexpression of *Pli* in the fat body, or in *D. melanogaster* Schneider 2 (S2) cell line cells, has effects that suggest that Pli acts as a negative regulator of the Toll pathway in these contexts (36). The authors demonstrated that at the plasma membrane, Pli interacts with the adaptor protein MyD88, regulating its ubiquitination and targeting it for degradation (36). In mammals there are several Pli homologs that have opposing roles in different cells/tissues, indicating that the regulation mediated by Pli family members is complex, and appears to be context dependent (37). Looking further into MyD88-related regulatory mechanisms, a detailed study on MyD88 function showed that *Drosophila* MyD88 binds to the phosphatidylinositol 4,5-bisphosphate (PIP_2)-rich regions on the plasma membrane. PIP_2-guided localization of MyD88 on the membrane was shown to be essential for its function as a Toll pathway signaling adaptor and the subsequent activation of immune reactions. The authors concluded that *Drosophila* MyD88

144 serves as a sorting adaptor, and functionally is the equivalent of the mammalian sorting adaptor
145 TIRAP (38, 39).
146
147 Anjum and coworkers (40) showed that the concerted action of *Drosophila* β-Arrestin Kurtz
148 (Krz) and a sumo protease Ulp1 is needed to keep Toll signaling at bay in the fat body via
149 desumoylation of Dorsal (Dl). Silencing of *Krz* and *Ulp1* led to activation of Toll signaling and
150 was lethal to the larvae (40). Hegde and coworkers (41) show that in a sumoylation resistant Dl
151 mutant (Dl^{K382R}), Dl transcriptional activation is increased. This somewhat contradicts the
152 earlier finding (40), however Anjum and coworkers speculated that in their study there are
153 perturbations in the general sumoylation machinery, which may affect also other sumoylation
154 targets besides Dl (40).
155
156 At the level of translational regulation of Toll pathway proteins, Wang and colleagues (42)
157 provide evidence that Dicer-2, part of the RNAi machinery, is involved in translation of the
158 Toll protein by binding to the Toll mRNA 5' untranslated region. Through this mechanism,
159 Dicer-2 is involved in regulation of Toll pathway-mediated immune reactions.
160
161 Non-coding RNAs, including long non-coding RNAs (lncRNA), microRNAs (miRNA), and
162 small interfering RNAs (siRNAs), have emerged as an important regulatory mechanism across
163 a wide range of biological contexts. A limited number of recent studies have identified
164 examples of modulation of *Drosophila* Toll pathway activity by both miRNAs and lncRNAs.
165 The miRNA miR8, related to the miR200 family of microRNAs conserved in mammals,
166 appears to downregulate the Toll pathway by interacting with mRNAs of multiple Toll pathway
167 genes, including Toll and Dl (43, 44), with this occurring specifically in the fat body tissue of
168 the fly (43). Other miRNAs suggested to downregulate Toll signaling by targeting various

genes in the pathway are miR958 (45), miR964 (46), miR317 (47), as well as members of the miR959-962 cluster of RNAs (48). The lncRNA *CR11538* (49) has been shown to bind to Dif/Dl proteins to prevent transcription of immune effector genes, while *CR46018* (50) and *CR33942* (51) upregulate Toll signaling through a similar mechanism. Finally, Zhang *et al.* (52) identified the lncRNA *VINR* as being involved in the immune response against both *Drosophila* C virus (DCV), and bacterial infections, through a non-canonical activation of Toll signaling involving Cactin. However, the complete picture of how miRNAs, siRNAs and lncRNAs regulate the Toll pathway in different tissues and during immune challenge is yet to develop.

Toll pathway effector molecules

In *Drosophila*, the immune response against gram-negative bacteria is primarily orchestrated by another NF-κB signaling pathway called the Imd pathway, whereas the Toll pathway has a more important role in the defense against gram-positive bacteria and fungi (53). These responses are mediated through effector molecules. Marked progress in the past decade has been made in analyzing the Toll pathway effectors and their function. While many of the recently characterized effector molecules had already been identified nearly 25 years ago in a mass spectrometric analysis of immune-induced molecules (IMs) (54), the modern CRISPR/Cas9 gene editing technology has now facilitated the dissection of their roles in the *Drosophila* immune defense. Such molecules include the Daisho peptides Daisho1 and Daisho2 (previously called IM4 and IM14), which are related peptides with partially shared functions. Daishos are needed in the defense against a group of pathogenic, filamentous fungi (55). Another recently characterized gene based on the original IM findings is *Baramicin A* (*BaraA*) (56). The *BaraA* gene encodes a polypeptide precursor which is cleaved into multiple peptides that correspond to one third of the originally described IMs (IMs 5, 6, 8, 10, 12, 13,

22 and 24) (54, 56). The most abundant products of the *BaraA* gene are IM10 and IM10-like peptides, cleavage products from the other produced IMs. These have a synergistic antifungal effect with an antifungal agent, Pimaricin. Moreover, *BaraA* mutant flies are highly susceptible to *Beauveria bassiana* fungal infection, indicating that *BaraA* is required in the defense against fungi (56). Recently, it was shown that a Baramicin paralog, encoded from the IM24 Baramicin domain, also has non-immune functions in the nervous system (57).

Bomanins (Boms) make up another gene family that is induced upon the Toll pathway activation (58). Some of the Boms were found in the mass spectrometric analysis and previously designated as IMs (54), whereas others were found through bioinformatic analysis (58). Ten out of the twelve Boms are found in a cluster on chromosome 2 at cytogenetic position 55C, whereas the remaining two are located on chromosome 3. Deletion of the Bom55C cluster shows that it is specifically required for the Toll-mediated response against certain bacteria and fungi (59). In another study, it was shown that Boms are the main contributors to gram-positive bacterial and fungal resistance; the $Bom^{\Delta 55C}$ mutant flies are as susceptible to infections as Toll pathway mutants, whereas mutant flies lacking 14 antimicrobial peptide (AMP) genes that are induced upon systemic infection show a much milder phenotype (60). Bom peptides form three distinct groups: short, tailed, and two-headed (or bicipital), and were renamed accordingly a few years after initial characterization (S. A. Wasserman 2019, personal communication to FlyBase, Flybase ID: FBrf0243179). Furthermore, a key factor called Bombardier (Bbd), controlling expression of short-form Boms and therefore Toll pathway-mediated humoral immunity, was recently identified (61).

Furthermore, two novel peptide-encoding genes, namely *Induced By INfection* (*IBIN*) and *IBIN-like*, were recently identified as induced by gram-positive bacteria *Micrococcus luteus*

219 infection in *Drosophila* (62). It was previously thought that IBIN and IBIN-like are non-coding
220 RNA molecules (*CR44404* and *CR45045*, respectively), but they have been re-annotated as
221 peptide-encoding genes with strong homology to each other (63). The *M. luteus*-mediated
222 induction of *IBIN* expression is dependent on the Toll pathway, however, *IBIN* can be also
223 induced by gram-negative bacteria, in which case the Relish/Imd pathway is required. *IBIN*
224 overexpression has effects on the expression of metabolic genes, but the exact effector role of
225 IBIN molecules is not known (62). Other studies have recently shown that *IBIN* is induced
226 upon sight of parasitoid wasps (64) and social isolation (65), indicating an additional role for
227 IBIN peptides in other stress-related situations besides infection.
228
229 In addition to Osa (24) and IBIN, the connection between the Toll pathway and metabolism
230 was established in the gut: Peptidoglycan recognition protein SA (PGRP-SA) recognizes
231 intestinal bacteria on the surface of enterocytes, activates the intracellular Toll pathway and
232 thus increases the phosphorylation of 4E-BP/Thor transcription enabling fat catabolism and
233 maintenance of the gut microbiota (66).
234
235 **Sex differences in Toll pathway responses**
236 Female and male flies differ in their response to infection and this variation has been noted to
237 be pathogen-specific (reviewed in (67)). The Toll pathway has been shown to mediate sex-
238 specific differences in response to both bacterial and fungal infections. Besides involvement in
239 immunity, the Toll pathway also participates in the female specific process that occurs in the
240 eggs, the dorso-ventral embryonic patterning. As the transmembrane receptor *Toll* is shared
241 between the two processes, females have higher overall *Toll* expression levels due to
242 expression in the ovaries (68). However, in various infection models, males have better survival
243 rates and resistance compared to females (67). Duneau *et al.* (69) showed that in the absence

of Toll signaling males were less resistant than females when challenged with *Enterococcus faecalis*. They also showed that males exhibit higher expression of Toll pathway effectors at the basal level and when infected with *Providencia rettgeri,* and that the loss-of-function of the *psh* gene abolished the sex differences. Gene expression levels of *Drs* and *Metchnikowin* during the first 24h of infection (70), *Toll-5* upon infection, and *Toll-7* at the basal level (69) have been shown to be higher in males than in females. Loss of Toll has been additionally shown to affect the expression of *Attacins* and *Diptericins* in *Enterobacter cloacae* infected males more than in females (71). Males also seem to have better survival rates when exposed to certain fungal infections. Shahrestani *et al.* (72) showed that females were more susceptible to fungal entomopathogen *B. bassiana*, with loss-of-function mutations of Toll pathway genes removing the sex differences in survival. Resistance to *Candida albicans* was also altered more strongly in males in loss-of-function mutants of *Toll* and *Toll-7* (68). Belmonte and coworkers (67) speculated that the involvement of the Toll pathway in sex-specific differences in immunity may be due to the dual role of the Toll pathway in females, as the Toll pathway immune responses in females are somewhat restricted by potential consequences on egg development, a limitation that is absent in males. Although it is clear that the Toll pathway mediates sex-specific differences, the reasons for this are as yet unresolved.

Toll pathway in viral immunity

Immunity against viral infection in *Drosophila* appears to be largely dependent on RNAi, as well as the JAK/STAT and Imd pathways (73–76). Recent studies have presented evidence against a general major role for the Toll pathway in defense against viral infections in *Drosophila in vivo*, while suggesting that the pathway is involved in certain situations. In addition, several transcriptional profiling studies have shown no, or only very limited, upregulation of Toll pathway genes during viral infection of flies and/or S2 cells (77, 78). For

example, Liu *et al*. (79) showed upregulation of the Imd pathway target gene *Diptericin*, but not the Toll pathway effector gene *Drs*, in the brains of Zika-infected *Drosophila*.

Limited evidence for a role for the Toll pathway has been published. Kallithea virus has been shown to suppress Toll pathway activity in the fly (80), suggesting a potential role for Toll in response to viral infection. In a separate study, invertebrate Iridescent virus 6 (IIV-6) suppressed both Imd and Toll pathways (81). Separately, the Toll pathway in planthoppers was shown to be activated upon infection with a plant pathogen virus (82). In S2 cells, Flock House Virus (FHV) and vesicular stomatitis virus (VSV) have both been shown to trigger *Drs* expression (42), and while describing a transcriptional pausing mechanism for the control of virus response genes, Xu *et al.* (83) showed that expression of *Toll*, *Toll-2*, *Toll-7* and *Tollo* are all upregulated during infection with VSV and Sindbis virus (SINV). The gut has been suggested as a tissue in which Toll plays a role in responses to specific viruses, for example, DCV (84), despite apparent lack of upregulation in systemic infection with this virus.

Beyond Toll itself, other Toll family members may have roles in viral immunity. Toll-7 was suggested to be involved in antiviral autophagy in two articles with somewhat contradictory results as to the role of Toll-7 and the downstream signaling pathway. Nakamoto *et al.* (85) found higher VSV replication in Toll-2 and Toll-7 knockdown S2 cells, and in flies with Toll-7 knockdown. Toll-7 was suggested to act as a pattern recognition receptor, not dependent on canonical Toll signaling through MyD88. A second article (86) supports the role of Toll-7 in an autophagy reaction against specific viral infections, however, in this case, Toll-7 signaling was suggested to use the canonical Toll signaling pathway. Lamiable and colleagues (87) have since shown that in their experiments, Toll-7 was not needed for resistance to VSV infection, and that autophagy only plays a limited role in this reaction. Apart from the Toll family of

receptors, a long non-coding RNA, VINR, has been shown to act as a pattern recognition receptor, recognizing viral suppressors of the RNAi pathway, and triggering the expression of Toll and Imd target genes (52). VINR was shown to be relevant to limiting viral replication of DCV (but not of other viruses) in S2 cells, providing further evidence for the role of Toll and Imd effectors in response, in particular to DCV infection.

Toll pathway in blood cell homeostasis and cell-mediated immune response

The *Drosophila* blood cells, called hemocytes, can be classified into three main types: the macrophage-like plasmatocytes; crystal cells, central for melanization responses at wound sites and against microbes; and lamellocytes, an immune-inducible hemocyte type needed for the encapsulation and melanization response against parasitoids. Many thorough reviews on the *Drosophila* blood cell system and its similarities to its mammalian counterpart exist for an interested reader (for instance (88–90)). Despite the first findings on the role of Toll signaling in the formation of melanized masses via the action of lamellocytes having been made over 30 years ago (91–93), the intricacies of Toll signaling in the cellular innate immune response has been much less well studied than in the humoral response. Besides lamellocyte differentiation, the Toll-induced hemocyte phenotype includes the release of hemocytes from their sessile reservoirs, as well as hemocyte hyperproliferation. Multiple studies have further elaborated on the roles of Toll signaling in the control of immune cells, and on cell-mediated immune responses in the larval hematopoietic organ (the lymph gland) and in the mature hemocytes, or via signaling from other tissues, such as the fat body. Since lamellocyte differentiation occurs at the larval stages, the studies discussed below were conducted on larvae unless otherwise stated. Figure 2 gives a schematic summary of the findings discussed below, concentrating on the role of Toll signaling in differentiation of lamellocytes, which can be considered as a hallmark of hemocyte activation in *D. melanogaster*.

Qiu *et al.* (94) were the first to show that the Toll/Cact signaling axis is involved in the control of hematopoiesis in the lymph gland. Several papers have elaborated on the roles of Toll signaling in hematopoietic homeostasis in different compartments of the lymph gland. Gueguen *et al.* (95) showed that Dif and Dl are nuclear, and hence active, specifically in the posterior signaling center (PSC), which acts as a niche, maintaining hemocyte progenitor cells located in the medullary zone of the lymph gland. Not only PSC-specific overexpression of either *Dif* or *dl*, but also infection by parasitoid wasp *Leptopilina boulardi* eliciting the cell-mediated immune response including lamellocyte formation, increased the nuclear localization of the NF-κB factors in the niche, and resulted in lamellocyte differentiation in the lymph gland (95). Louradour *et al.* (96) showed that larvae mutant for various Toll pathway components exhibited delayed disruption of the lymph gland, and subsequently delayed release of lamellocytes as a response to *L. boulardi* parasitization, leading to reduced immune response against the parasitoids. They also showed that Toll signaling is activated in the PSC upon wasp infection via increased reactive oxygen species (ROS) production in a Psh-dependent manner, and that this activation requires Dif, but not Dl. In contrast, Dl, but not Dif, in the prohemocytes was shown to regulate the prohemocyte pool in the lymph gland medullary zone during steady-state conditions, and overexpression of *dl* or knockdown of *cact* in prohemocytes initiated their differentiation into lamellocytes (97).

Several studies have looked at the role of Toll signaling on hemocyte activation outside of the lymph gland. Schmid *et al.* (98) showed that although expressing the Toll gain-of function mutant ($Toll^{10b}$) in the fat body, midgut, or in mature hemocytes was sufficient to induce lamellocyte formation, Toll activation in the fat body was required for the full spectrum of the Toll-induced hemocyte phenotypes (98). They also showed that parasitization suppressed Toll

activation in the fat body, but that the response against *L. boulardi* does not seem to require Toll, neither in the fat body nor in the hemocytes. Similarly, Yang & Hultmark (99) reported that silencing of the Toll receptor in the fat body or in hemocytes does not affect the killing of *L. boulardi*. However, Toll signaling has been shown to be suppressed by parasitoid wasp infection also in other insects (for example (100)), suggesting a role for Toll signaling in the cell-mediated immune response against parasitoids. To that end, Yang *et al.* (101) observed that pupal ectoparasitoid *Pachycrepoideus vindemmiae* infection induces Toll signaling as measured by *Drs* induction.

Schmid and coauthors (102) focused on the molecular underpinnings of the Toll-induced hemocyte mobilization. In their deletion screen they identified the gene *immune response deficient* (*ird1*) mutant as a suppressor of this phenotype. Interestingly, other Toll-induced hemocyte traits, such as melanotic nodules and increased number of circulating hemocytes, were not suppressed, but rather enhanced in *ird1* mutants. The authors showed that Toll signaling was induced in *ird1* mutant larvae in the fat body, but not in hemocytes. *Ird1* encodes for a serine/threonine kinase important in several vesicle trafficking pathways, but it remained unclear how its loss may activate Toll signaling. The authors suggest that the observed re-localization of the Toll receptor in *ird1* mutant larvae might contribute to Toll activation. Also, when Yu and others (103) knocked down the Ras-like GTPases *Rab5* and *Rab11* with important roles in vesicle transport in hemocytes, Dif and Dl were localized into the nucleus and lamellocytes were formed, requiring Dif but not Dl.

The complex tissue-specific functions of Toll signaling are further highlighted in several papers discussing the link between a winged helix/forkhead transcription factor, Jumeau (Jumu) and Toll signaling. First, Zhang *et al.* (104) showed that simultaneous overexpression of *jumu* in

369 the fat body and in hemocytes, but not in either tissue individually, led to activation of Toll
370 signaling and formation of melanotic nodules and lamellocytes. Second, Hao & Jin (105)
371 showed that loss of *jumu* throughout the lymph gland induced lamellocyte differentiation in a
372 Dif-dependent manner. The authors note that Jumu might regulate Toll indirectly, via the
373 transcription factor Collier (105). In a third study, Hao *et al.* (106) showed Toll activation in
374 transheterozygous *jumu* mutants, both in the fat body and in hemocytes. Nuclear Dif and Dl
375 localization was accompanied by lamellocyte formation only in hemocytes, as shown by
376 silencing of *jumu* tissue-specifically.
377
378 As Toll signaling is responsive not only to pathogen-associated molecules, but also to various
379 DAMPs, it has been shown to alter the hemocyte response also via these signals. Ming *et al.*
380 (107) discovered that apoptosis-deficient *Drosophila* larvae systemically activate Toll
381 signaling as a response to DAMPs in the hemolymph. This activation led to classical Toll-
382 dependent effects on hemocytes: hyperproliferation and the formation of melanotic nodules,
383 and Spz secretion from the hemocytes into the hemolymph. The systemic Toll activation as a
384 response to DAMPs was dependent on the action of the serine protease Psh in the hemolymph
385 (107). Arefin *et al.* (108) showed that apoptosis induction in non-lamellocyte hemocytes
386 induced melanotic masses and lamellocyte differentiation, which was correlated with increased
387 activity of Toll signaling measured as increased expression of *Drs*. Incidentally, Shields et al.
388 (109) showed that in Apoptosis-induced Proliferation of epithelial cells, Toll-9 interacts with
389 Toll leading to the activation of the core Toll pathway. This results in nuclear translocation of
390 Dl and induced expression of pro-apoptotic genes *reaper* and *hid*, recruitment of hemocytes
391 and c-Jun N-terminal kinase (JNK) pathway activation (109). Evans *et al.* (110) looked at Toll
392 signaling in the lymph gland and in circulating hemocytes, in the context of sterile wounding.
393 They showed that injury alone was able to activate Spz in an SPE- and Grass-dependent manner

394 in the hemolymph. Spz, in turn, activated Toll signaling in hemocytes, initiating lamellocyte
395 differentiation via Toll-activated c-Jun N-terminal kinase (JNK) signaling. Rather than
396 microbe sensors, activation of Toll signaling in hemocytes required hydrogen peroxide (H_2O_2)
397 production at the wound site (110). Chakrabarti & Visweswariah (111) similarly showed that
398 in adult flies, a burst of reactive oxygen species (ROS) at the wound led to H_2O_2 production in
399 hemocytes, as well as activation of Toll signaling in those hemocytes. Toll activity was
400 required for the survival of the flies after wounding (111).
401
402 These studies emphasize the various roles of Toll signaling in the cell-mediated immune
403 response and especially in the control of hemocyte differentiation, in the lymph gland, in
404 hemocytes and via signals from the fat body. Recently, research on the *Drosophila* blood cell
405 system has moved into the single cell RNA sequencing era, enabling more detailed analysis of
406 hemocytes under various conditions. The data so far have already indicated enriched expression
407 of Toll pathway components in certain subtypes of plasmatocytes (112, 113). Further
408 experiments focusing on transcriptomics and proteomics at single cell level will aid in
409 dissecting the role of Toll signaling in detail in different hemocyte subtypes.
410
411 **Conclusions**
412 Toll pathway regulators and responses have been extensively studied with *Drosophila*,
413 especially upon systemic bacterial and fungal infection. However, the roles of Toll in viral and
414 parasitoid infections, as well as tissue-specific Toll pathway responses, and the effect of the
415 sex of the animal on Toll pathway activation and resulting outcomes, require further
416 investigation. Open questions for future research include, for example: what the signals from a
417 Toll-activated fat body to hemocytes are that result in hemocyte activation; what downstream

418 events are affected by Toll signaling in different tissues; and how do different effectors affect
419 immunity at the molecular level.

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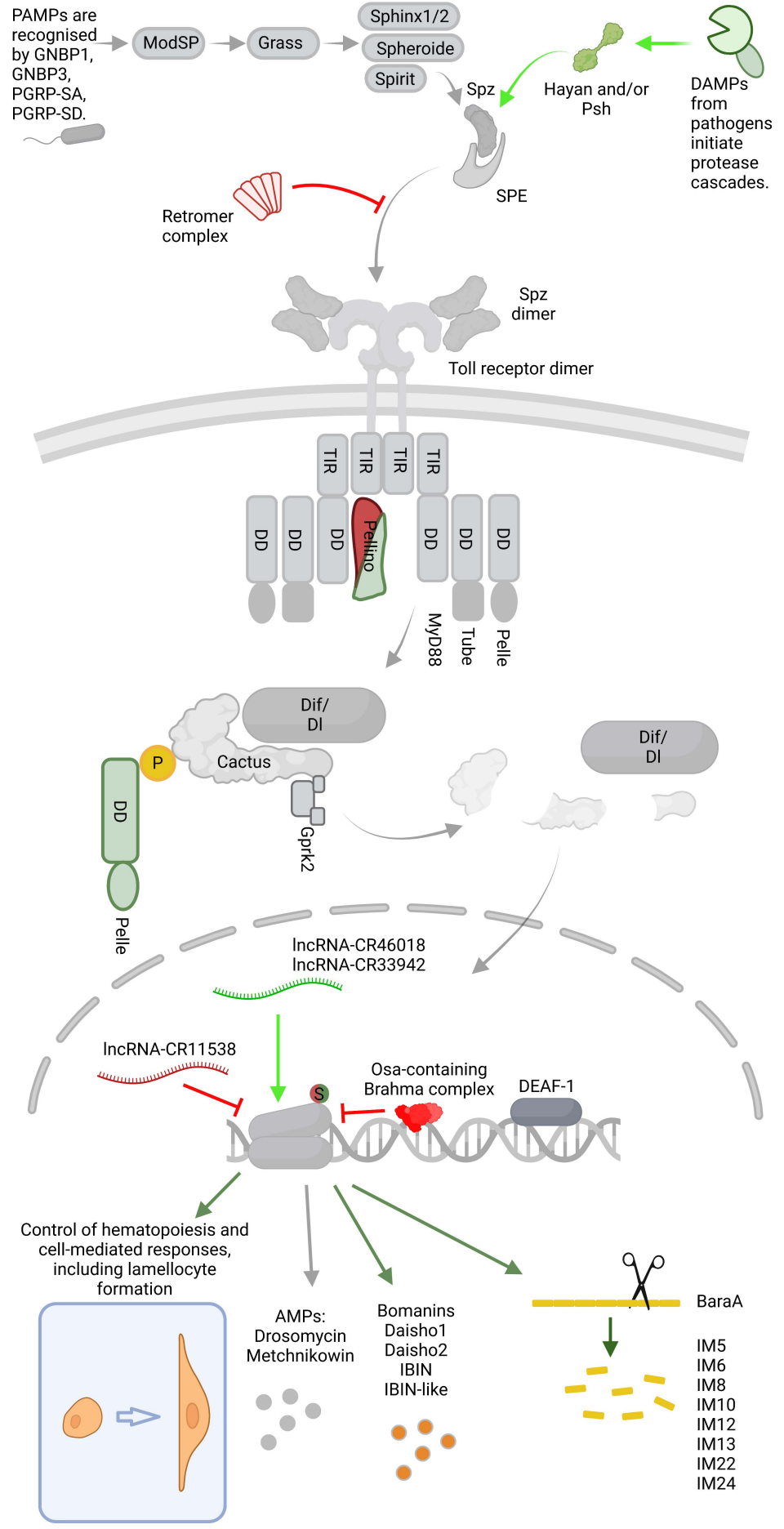
PAMPs are recognised by GNBP1, GNBP3, PGRP-SA, PGRP-SD.
ModSP
Grass
Sphinx1/2
Spheroide
Spirit
Spz
Hayan and/or Psh
DAMPs from pathogens initiate protease cascades.
SPE
Retromer complex
Spz dimer
Toll receptor dimer
TIR
DD
Pellino
MyD88
Tube
Pelle
Dif/ Dl
P
Cactus
Gprk2
lncRNA-CR46018
lncRNA-CR33942
lncRNA-CR11538
S
Osa-containing Brahma complex
DEAF-1
Control of hematopoiesis and cell-mediated responses, including lamellocyte formation
AMPs: Drosomycin Metchnikowin
Bomanins Daisho1 Daisho2 IBIN IBIN-like
BaraA
IM5
IM6
IM8
IM10
IM12
IM13
IM22
IM24

Figure legends

Figure 1. Overview of the Toll pathway signaling mechanism in *Drosophila*. Pathway components known previously (prior to 2011, described in (4)) shown in grey tones. Newly discovered positive regulators of the pathway are shown in green, negative regulators in red, and novel effectors in orange and yellow colors. Recognition of pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) leads to maturation of Spätzle processing enzyme (SPE) via either Grass or Persephone (Psh)/Hayan pathways. Spätzle (Spz) is processed by SPE. Processed Spz forms dimers and binds to Toll, which itself dimerizes, allowing transduction of the signal into the cell. The intracellular signaling cascade, involving MyD88, Tube, and Pelle (DD = Death Domain, TIR = Toll/IL-1R), leads to phosphorylation (P) by Pelle of Cactus, resulting in the degradation of Cactus, which releases Dorsal-related immunity factor (Dif) and Dorsal (Dl) NF-κB transcription factors. These transcription factors move into the nucleus where they form a dimer and activate the transcription of immune response genes, resulting in the production of AMPs, Bomanins, Daisho1 and Daisho2, IBIN and IBIN-like, and other peptides, including those resulting from the cleavage of the *BaraA* gene product. Dif/Dl-mediated transcription can also result in cell-mediated immune processes, including the formation of lamellocytes. Dif and Dl transcription factor activity can be regulated positively or negatively. For example, evidence for both positive and negative regulatory effects of sumoylation (S) of Dif/Dl have been shown. Created with BioRender.com.

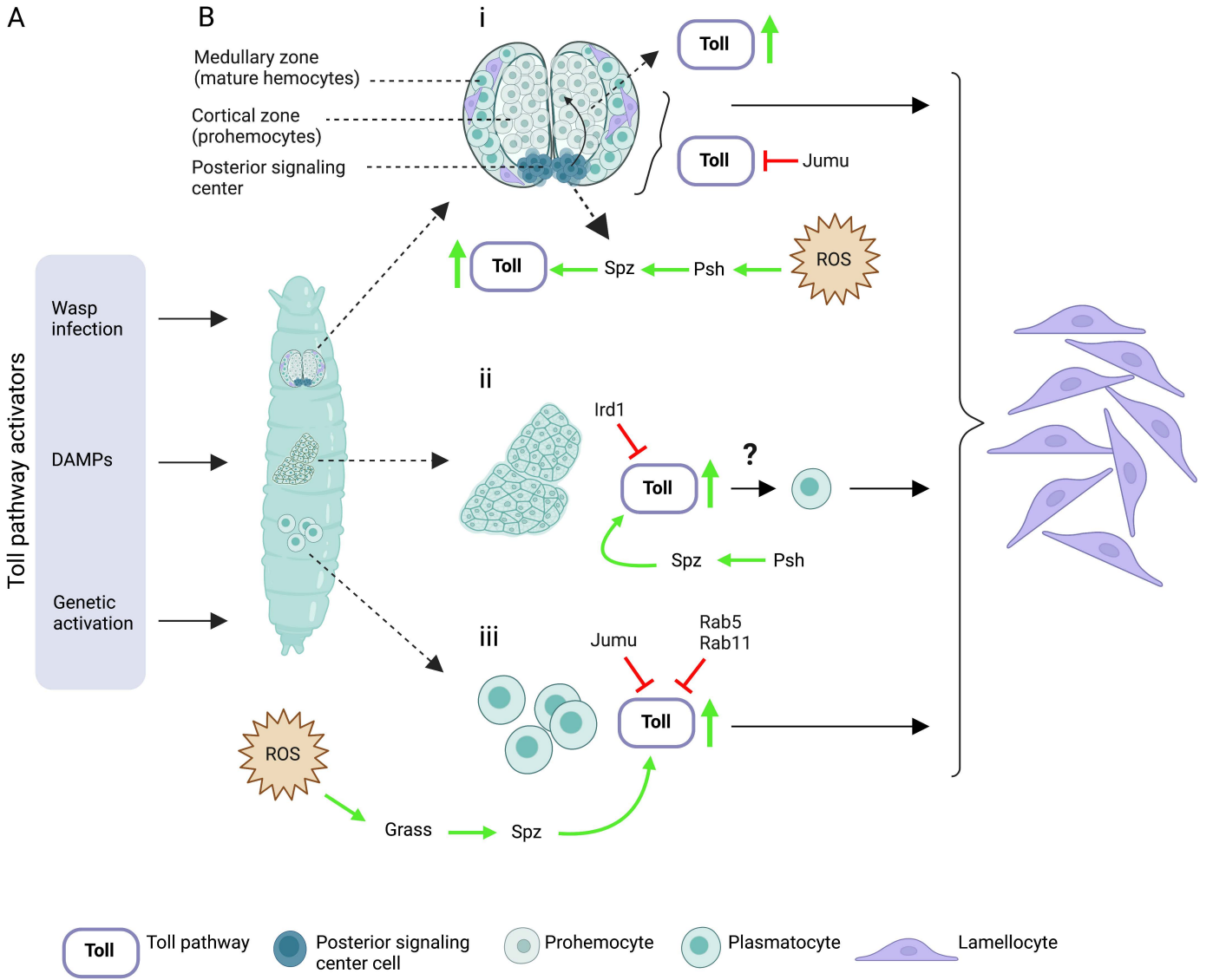
A
B
i
Medullary zone
(mature hemocytes)
Cortical zone
(prohemocytes)
Posterior signaling
center
Toll
Toll
Jumu
Toll
Spz
Psh
ROS
Toll pathway activators
Wasp
infection
DAMPs
Genetic
activation
ii
Ird1
Toll
?
Spz
Psh
iii
Jumu
Rab5
Rab11
Toll
ROS
Grass
Spz
Toll
Toll pathway
Posterior signaling
center cell
Prohemocyte
Plasmatocyte
Lamellocyte

Figure 2. Activation of the Toll signaling pathway in several tissues in the *Drosophila* larvae is involved in lamellocyte differentiation. Activation of Toll signaling in several tissues can lead to differentiation to an immune-induced hemocyte type, the lamellocyte. This schematic gives a simplified overview of the roles of Toll in lamellocyte differentiation. **A)** Several upstream factors can lead to Toll activation and subsequent lamellocyte formation, including a parasitoid wasp laying its eggs into the larval hemocoel; through recognition of danger-associated molecular patterns (DAMPs) such as reactive oxygen species (ROS); or via genetic activation of the signaling pathway. **B)** Main sites where Toll activation is known to induce the differentiation of lamellocytes are **i)** lymph gland, **ii)** fat body, and **iii)** plasmatocytes (the main circulating hemocyte type in a healthy larva). Increased nuclear localization of the NF-κB transcription factors Dorsal-related immunity factor (Dif) and Dorsal (Dl) in these tissues leads to Toll-induced gene expression requiring Dif and/or Dl context-dependently, which in turn leads to lamellocyte formation via largely unresolved downstream effectors and signaling events. **i)** In the lymph gland, Toll activation, either in prohemocytes residing in the medullary zone, or in the posterior signaling center, a group of cells controlling the prohemocyte pool, can lead to prohemocyte differentiation into lamellocytes. Mature lamellocytes are found in the cortical zone of the lymph gland from where they are released into the circulation. Evidence does point towards the importance of lymph gland Toll activation in the cell-mediated immune response against parasitoids. **ii)** Toll activation in the fat body triggers lamellocyte differentiation, possibly via Spätzle processing enzyme (SPE) secretion from the fat body and/or via some other, yet unidentified diffusible signal. Since wasp parasitization seems to interfere with Toll signaling in the fat body, the role of Toll in the fat body in the fight against parasitoids remains somewhat unclear. **iii)** Toll activation in circulating hemocytes induces their transdifferentiation into lamellocytes, possibly involving

additional signaling events downstream of Toll signaling pathway. Created with BioRender.com.