

Abstracts: Reviews

Schmidt *et al.*, 2008, Insect and veterbrate immunity: key similarities versus differences.

Abbreviations:

PAMP: pathogen-associated molecular pattern.

PRR: pattern-recognition receptors.

LPS: lipopolysaccharide.

PGN: peptidoglycan.

LTA: lipoteichoic acid.

Distinction between self and non-self relies on pattern-recognition receptors (PRRs) that bind to diagnostic sites for potential pathogens, or pathogen-associated molecular patterns (PAMPs). One precondition for sensing non-self by PAMP recognition is that these molecular patterns are conserved enough to allow the host to evolve binding proteins before the pathogen is able to eliminate or modify the target site. Common PAMPs include bacterial lipopolysaccharide (LPS), peptidoglycan (PGN), lipoteichoic acid (LTA) and fungal beta-1, 3-glucans.

Extracellular lipid particles are involved in systemic immune response to pathogens. Apolipoprotein III is sensitive to both particle lipid composition and immune elicitors. Moreover, lipid particles are associated with typical immune proteins including prophenoloxidase and its upstream proteins, such as LPS- and PGN-binding proteins.

Recognition of self (histocompatibility and self-incompatibility) and altered-self (apoptotic and tumor cells)

Antimicrobial peptides are defense molecules against microbes by permeation and disruption of target membranes. They often kill microorganism via non-receptor-mediated mechanisms, although some bind to bacterial cell wall components (*e.g.* nisin Z).

Phagocytosis is the cellular uptake of particular substrate. It is a fundamental cellular process in eukaryotes and essential for the clearance of damaging objects in multicellular organisms. In many animals, specialized cells engage in phagocytosis, such as phagocytes in vertebrates and macrophage-like hemocytes in insects.

Endocytosis

28 Adaptive immunity of vertebrates is fundamentally different from innate immunity. In adaptive immu-
29 nity, the anticipatory nature of antibody repertoires is capable of binding epitopes never encountered by
30 the organism or its predecessors using direct antibody-epitope specific binding. Self-recognizing antibody-
31 producing cells are removed by clonal selection during ontogeny. The specific propagation of antibody-
32 producing immune cells provides the basis for an immunological memory. Instead, in innate immunity,
33 PRRs are acquired through evolutionary processes resulting from exposure to pathogens over generations.
34 Retaining pathogen-binding proteins and removing self-recognizing proteins are facilitated at population
35 level.

36 Although lack of adaptive immunity, insects are able to induce immune activity after sub-lethal en-
37 counters with pathogens. Exposure to sub-lethal concentration of damaging objects enables latter survival
38 under lethal level. This immune induction and protection comes with fitness cost, which is often expressed
39 as a delay in development. Moreover, the induction of immune defense can be maternally transmitted
40 to subsequent generations, occurring by potential epigenetic mechanisms or the incorporation of female-
41 derived immune-inducible material into oocytes.

42 **Strand, 2008, Insect hemocytes and their role in immunity.**

43 1 Abbreviations

44 AMP: antimicrobial peptide.

45 PO: phenoloxidase.

46 PPO1: proPO 1.

47 JNK: Jun kinase.

48 PSC: posterior signaling center.

49 Srp: Serpent.

50 JAK: Janus kinase.

51 STAT: signal transducers and activators of transcription.

52 gcm: glial cell missing.

53 PRR: pattern recognition receptor.

54 LPS: lipopolysaccharide.

55 PGN: peptidoglycan.

56 LPSBP: LPS-binding protein.

57 GNBP: Gram-negative binding protein.

58 PGRP: PGN recognition protein.

59 GRP: glucan recognition protein.

60 Dscam: Down's syndrome cell adhesion molecule.

61 SR: scavenger receptor.

62 SPZ: Spaetzle.

63 NF- κ B: nuclear factor κ B.

64 The innate immune system of insects consists of humoral and cellular defense response. Humoral
65 defenses refer to soluble molecules including antimicrobial peptides (AMPs), complement-like proteins
66 and products from proteolytic cascades such as phenoloxidase (PO) pathway. Cellular defenses refer to
67 responses like phagocytosis, encapsulation and clotting that are directly mediated by hemocytes.

68 Hemocytes have similar function in immunity across insects, but naming of hemocyte types varies
69 among taxa. *Drosophila* larvae contain three terminally differentiated hemocyte types: plasmatocytes,
70 crystal cells and lamellocytes. Plasmatocytes represent 90-95% of mature hemocytes, are strongly adhe-
71 sive *in vitro*. and function as professional phagocytes that engulf pathogens and dead cells. Molecular
72 markers for plasmatocytes include extracellular matrix protein peroxidase and a surface factor P1 anti-
73 gen. Crystal cells represent about 5% of mature hemocytes. They are non-adhesive rounded cells that
74 express PO cascade components such as proPO 1 (PPO1). Lamellocytes are absent in healthy *Drosophila*
75 larvae, but rapidly differentiated from prohemocytes after being attacked by parasitoid wasps and during
76 metamorphosis. They are large, flat, adhesive cells that express reporters related to Jun kinase (JNK)
77 signaling and L1 antigen. The main function of lamellocytes is encapsulation of parasitoids and other large
78 foreign targets. Each of these hemocyte types differentiate from precursor prohemocytes that originate
79 from pre-prohemocytes, which mainly reside in hematopoietic organs, and a small number in circulation.

80 In Lepidoptera, main differentiated hemocytes in circulation are granulocytes, plasmatocyte, spherule
81 cells and oenocytoids. Granulocytes are the most abundant and characterized by the granules in their
82 cytoplasm, the ability to adhere and spread on foreign surface in primary culture, and the tendency to
83 spread systemically. They function as professional phagocytes. Plasmatocytes are usually larger than
84 granulocytes, spread asymmetrically on foreign surfaces, and are the main capsule-forming hemocytes.
85 Non-adhesive hemocytes in larval stage Lepidoptera include oenocytoids that contain PO cascade com-
86 ponents, and spherule cells that are potential sources of cuticular components.

87 In mosquitoes, hemocyte types include granulocytes, oenocytoids and prohemocytes. Granulocytes
88 are strongly adhesive, phagocytic, and the most abundant cell types. They express PO activity induced by
89 immune challenge. Oenocytoids are non-adhesive and constitutively express PO activity. Prohemocytes
90 are characterized by uniform size, rounded morphology and large nuclear. It is unknown whether they
91 differentiate into granulocytes/oenocytoids.

92 Hemocytes arise during two stages of development. The first population of hemocytes arises during

embryogenesis from head or dorsal mesoderm, and the second is produced during the larval or nymphal stages in mesodermally derived hematopoietic organs. The hematopoietic organs of *Drosophila* are lymph glands that form bilaterally along the anterior part of the dorsal vessel during embryogenesis. By the third instar, each lymph gland consists of an anterior primary lobe and several posterior secondary lobes separated by pericardial cells. The primary lobe has three zones: (1) a posterior signaling center (PSC) that contains cells marked by the expression of transcription factor Collier and Notch ligand Serrate; (2) a medullary zone that contains quiescent prohemocytes; (3) a cortical zone that contains plasmatocytes, crystal cells and following parasitoid attack, lamellocytes. Secondary lobes contain pre-prohemocytes, prohemocytes and some plasmatocytes.

Earliest lymph gland cells, hemocyte precursor cells, are identified by expression of GATA transcription factor homolog Serpent (Srp). As transition to pre-prohemocytes, they initiate expression of receptor tyrosine kinase Pvr followed by expressing JAK/STAT (Janus kinase/signal transducers and activators of transcription) signaling pathway receptor Dome, which characterizes maturation of prohemocytes. In differentiation of prohemocytes into hemocyte types, Dome is down-regulated. Specification of plasmatocytes requires expression of transcription factor glial cell missing (gcm) and gcm2, while crystal cell specification requires Runt-domain protein Lozenge (Lz) and Serrate signaling through Notch. The PSC along with JAK/STAT and JNK signaling have been implicated in differentiation of lamellocytes.

The maintenance of hemocytes in circulation involves two aspects: production and release of cells from lymph glands, and proliferation of hemocytes already in circulation. Furthermore, the number of circulating hemocytes increases rapidly in response to stress, wounding or infection.

Immune responses mediated by hemocytes are phagocytosis, encapsulation and clotting. Phagocytosis is a conserved defense response in which individual cells internalize and destroy targets. It depends on receptor-mediated recognition and binding of the target to a hemocyte followed by formation of a phagosome and engulfment of the target via actin polymerization-dependent mechanisms. The phagosome then matures to a phagolysosome by a series of fission and fusion events with endosomes and lysosomes. Insect hemocytes phagocytize bacteria, yeast, fungi, protozoans, apoptotic bodies and inanimate materials like synthetic beads and ink particles.

Encapsulation refers to the envelopment of large targets by multiple hemocytes. In *Drosophila*, the capsules formed around invaders are mainly comprised of lamellocytes. In Lepidoptera, formation of capsules is mainly conducted by plasmatocytes, while cooperation of granulocytes are sometimes required for recognition and encapsulation of targets. Besides, melanin is often deposited within and around the capsules.

Coagulation of insect hemocytes occurs at sites of external wounding. Soft clots initially con-

126 sist of fibrous matrix embedded with hemocytes, mainly granulocytes (Lepidoptera) or plasmatocytes
127 (*Drosophila*). This is followed by clot hardening due to cross-linking of proteins and melanization.

128 Defense responses including phagocytosis and encapsulation are dependent on recognition of targets as
129 foreign, followed by activation of downstream signaling and effector responses. Some foreign invaders are
130 recognized by humoral pattern recognition receptors (PRRs), which bind to targets to enhance recognition
131 by other receptors on hemocyte surface. This process is opsonization. Other targets are recognized directly
132 by hemocyte surface receptors.

133 Humoral PRRs can opsonize microorganisms by binding to lipopolysaccharides (LPSs), peptidogly-
134 cans (PGNs) and glucans. These include hemolin, LPS-binding proteins (LPSBPs), Gram-negative bind-
135 ing protein (GNBPs), soluble PGN recognition proteins (PGRP-SA and PGRP-SD), glucan recognition
136 proteins (GRPs), soluble Down's syndrome cell adhesion molecule (Dscam) and complement-like TEP
137 proteins. Another group of PGRPs (PGRP-SB1, -SC1a, -SC1b, -SC2) enzymatically degrade PGN. This
138 activity kill some bacteria and releases PGN fragments triggering hemocyte effector responses. Other hu-
139 moral molecules implicated in pathogen recognition and opsonization include leucine-rich repeat proteins,
140 glutamine-rich protein and immunoelectins. The sources of humoral PRRs include hemocytes and other
141 immune tissues, *e.g.* the fat body.

142 Cell surface receptors involved in opsonin-independent immunity include Peste, a class B scavenger
143 receptor (SR) (or CD36 family member); dSR-CI, a class C SR; transmembrane protein Eater; membrane
144 bound PGRPs (PGRP-LC and its co-receptor PGRP-LE); transmembrane form of Dscam; class B SR
145 Croquemort; low-density lipoprotein (LDL) receptor-related protein LRP1. A long version of PGRP-LE
146 can act as intracellular receptor recognizing bacteria. Other proteins implicated to be cellular receptors
147 include integrins, tetraspanin proteins, neuroglian (an immunoglobulin superfamily member).

148 Cytokines are extracellular molecules that regulate hemocyte function. These include cysteine-knot-
149 like growth factor Spaetzle (SPZ) that is activated by a proteolytic cascade and interacts with Toll
150 receptors located on cell membrane. This leads to activation of nuclear factor κ B (NF- κ B) transcription
151 factors, which initiate a number of immune genes including several AMPs. Upstream PRRs involved in
152 initiating proteolytic cascade that lead to SPZ activation include PGRP-SA and soluble GNRP. Cytokine
153 PSP is also processed from a precursor protein by a proteolytic cascade. After binding to its membrane
154 receptor, PSP simulates plasmotocytes to adhere and spread on foreign surfaces.

155 In addition to Toll signaling, other pathways also are also activated in hemocytes by cytokine and/or
156 binding of foreign to surface receptors. These include Imd pathway activated by PGRP-LC binding with
157 Gram-negative bacteria. Imd signaling induces expression of immune effector genes. TEP proteins are
158 involved in activation of JAK/STAT signaling, while JNK signaling is associated with phagocytosis and

159 adhesion.

160 **Waterhouse *et al.*, 2020, Characterization of insect immune systems from genomic data.**

161 Identification of genes involved in physiological processes can be conducted by homology search or tran-
162 scriptomic analysis. Homology search works well for evolutionarily conserved and well-studied canonical
163 gene repertoires, but lose evolutionarily novel or not well-studied genes, which can be complemented by
164 transcriptomic analysis.

165 The first step of characterizing canonical gene repertoires in a newly sequenced genome is to compile
166 reference sequences, *i.e.* protein sequences of gene repertoires from reference species that have been
167 characterized. It requires define a scope of gene families to be included and select appropriate species
168 from which reference sequences are drawn. For immune gene identification, principle components of
169 immune responses should be included, *i.e.* recognition of antigens, signaling transduction and effectors
170 (??). As for reference species, characterized species of the same order are the most useful, as the lower
171 sequence divergence between more closely related species improves the success of sequence homology
172 searches. Besides, closely related species share similar gene family components with less gene gain/loss
173 events.

174 **Gram-negative binding proteins:** Gram-negative binding proteins (GNBPs) or beta-1,3-glucan-
175 binding proteins (BGBPs) are a family of carbohydrate-binding pattern recognition receptors.

176 **Peptidoglycan binding proteins:** PGRPs are pattern recognition receptors capable of recognizing
177 the peptidoglycan from bacterial cell walls.

178 **Fibrinogen-related proteins:** FREPs (also known as FBNs) are a family of pattern recognition
179 receptors with homology to the C terminus of the fibrinogen beta- and gamma-chains.

180 **Galectins:** GALEs bind specifically to beta-galactoside sugars and can function as pattern recognition
181 receptors in innate immunity.

182 **MD-2-like proteins:** MLs, also known as Niemann-pick type C-2 proteins, possess myeloid-
183 differentiation-2-related lipid-recognition domains involved in recognizing lipopolysaccharide.

184 **Nimrods:** NIMs have been shown to bind bacteria leading to their phagocytosis by hemocytes.

185 **Scavenger receptors:** SCRs are made up of different classes that function as pattern recognition
186 receptors for a broad range of ligands including from pathogens.

187 **Spaetzle-like proteins:** The cleavage of Spaetzle results in binding of the product to the toll receptor
188 and subsequent activation of the toll pathway; SPZs contain a cystine knot domain.

189

190 **IMD pathway:** Immune deficiency pathway is characterized by peptidoglycan recognition protein
191 receptors, intracellular signal transducers and modulators, and the NF- κ B transcription factor relish.

192 **Toll pathway:** The intracellular components of Toll pathway signaling are homologous to the Toll-like
193 receptor innate immune pathway in mammals, culminating in activation of the NF- κ B transcription
194 factors dorsal and DIF in *Drosophila*.

195 **JAK/STAT pathway:** The Janus kinase protein (JAK) and the signal transducer and activator of
196 transcription (STAT) are two core components of the JAK/STAT pathway, which is involved in cellular
197 responses to stress or injury.

198 **RNAi pathway:** RNA interference protects against viral infections employing dicer and Argonaute
199 proteins as well as helicases to identify and destroy exogenous double-stranded RNAs.

200 **Caspase:** Cysteine-aspartic proteases are involved in immune signaling cascades and apoptosis.

201 **CLIP-domain serine protease:** Several CLIP proteases have roles as activators or modulators of
202 immune signaling cascades.

203 **Inhibitor of apoptosis:** IAPs are important in antiviral responses and are involved in regulating
204 immune signaling and suppressing apoptotic cell death.

205 **Serine protease inhibitors:** Protease inhibition by serpins, or SRPNs, modulates many signaling
206 cascades; they act as suicide substrates to inhibit their target proteases.

207 **Thioester-containing proteins:** TEPs are related to vertebrate complement factors and alpha2-
208 macroglobulin protease inhibitors; their activation through proteolytic cleavage leads to phagocytosis or
209 killing of pathogens.

210

211 **Antimicrobial peptide:** Antimicrobial peptides (AMPs) are the classical effector molecules of in-
212 nate immunity; they include defensins, cecropins, and attacins that are involved in bacterial killing by
213 disrupting their membranes.

214 **Lysozymes:** LYSs are key effector enzymes that hydrolyze peptidoglycans present in the cell walls of
215 many bacteria, causing cell lysis.

216 **C-type lectins:** C-type lectins (CTL) are carbohydrate-binding proteins with roles in pathogen op-
217 sonization, encapsulation, and melanization, as well as immune signaling cascades.

218 **Prophenoloxidases:** PPOs are key enzymes in the melanization cascade that helps to kill invading
219 pathogens and is important for wound healing.

220 **Peroxidases:** PRDXs are enzymes involved in the metabolism of reactive oxygen species (ROS) that are
221 toxic to pathogens.

222 **Superoxide dismutases:** SODs are antioxidant enzymes involved in the metabolism of toxic superoxide
223 into oxygen or hydrogen peroxide.

224 **Nazario-Toole *et al.*, 2017, Phagocytosis in insect immunity.** Abbreviation:

225 SC: scavenger receptor.
 226 EGF: epidermal growth factor.
 227 PGRP: peptidoglycan recognition receptor.
 228 PGN: peptidoglycan.
 229 AMP: antimicrobial peptide.
 230 Dscam: Down syndrome adhesion molecular.
 231 IgSF: immunoglobulin superfamily.
 232 TEP: thioester-containing protein.
 233 Mcr: macroglobulin complement related.
 234 GEF: guanine nucleotide exchange factor.
 235 ESCRT: endosomal sorting complex required for transport.
 236 VPS-C: vacuolar protein sorting-C.

237 Phagocytosis is initiated when phagocytic cell surface receptors recognize their ligands and trigger the
 238 engulfment of targets into phagosome. Phagocytic receptors can recognize targets directly, or recognize
 239 opsonins coating targets. Additionally, due to the diversity of targets for phagocytosis, there is overlap
 240 and redundancy in receptor-ligand specificities to facilitate recognition. It also provides evolutionary
 241 advantage as it allows recognition of pathogens that have developed mechanisms to evade detection by a
 242 particular receptor.

243 Scavenger receptors (SRs) are a family of structurally diversified transmembrane proteins, subdivided
 244 into nine classes (Class A-I). They exhibit broad ligand specificity, including both altered self and molec-
 245 ular patterns from invaders. Croquemort in *Drosophila* is homolog of mammalian CD36 (France *et al.*,
 246 1996). It mediates phagocytosis of apoptotic cells and participates immunity against bacteria (France
 247 *et al.*, 1999; Stuart *et al.*, 2005). Class C SRs are unique to insects, with four members in *Drosophila*:
 248 SR-CI, -CII, -CIII and -CIV. SR-CI recognizes bacteria, mediating their phagocytosis (Ramet *et al.*, 2001;
 249 Ulvila *et al.*, 2006). *Drosophila* Peste is a class B SRs and homolog of mammalian CD36. It is involved
 250 in phagocytosis of bacteria (Philips *et al.*, 2005; Agaisse *et al.*, 2005).

251 Nimrod family is characterized by epidermal growth factor (EGF)-like repeats called NIM repeats
 252 (Kurucz *et al.*, 2007). It is divided into three groups: (1) draper-type, including *Drosophila* nimrod
 253 A and draper; (2) nimrod-B type, including *Drosophila* nimrod B 1-5; (3) nimrod-C type, including
 254 *Drosophila* nimrod C 1-4 and eater. *Drosophila* eater protein mediates phagocytosis of bacteria as a
 255 pattern recognition receptor (Ramet *et al.*, 2002; Kocks *et al.*, 2005; Chung and Kocks, 2011). Nimrod
 256 C1 (NimC1) is located on hemocyte plasm membrane and bound to bacteria for phagocytosis (Kurucz *et*
 257 *al.*, 2007). *Drosophila* draper is identified as phagocytosis receptor (Freeman *et al.*, 2003). It is involved

258 in phagocytosis of apoptotic cells (Manaka *et al.*, 2004; Kuraishi *et al.*, 2009; Tung *et al.*, 2013) and
259 bacteria (Cuttell *et al.*, 2008; Hashimoto *et al.*, 2009).

260 Peptidoglycan-recognition receptors (PGRPs) bind to peptidoglycan (PGN), a polymer restricted to
261 bacterial cell wall. There are 13 PGRP genes in *Drosophila*. They are upstream of Toll and IMD signaling
262 pathways that regulate the expression of antimicrobial peptides (AMPs) and other effectors. In *Drosophila*
263 PGRPs, there are six long (L) form proteins, four of which are located at plasm membrane (Werner *et*
264 *al.*, 2000). The remaining seven short (S) from proteins predicted to be secreted (Werner *et al.*, 2000).
265 Members of non-catalytic group (PGRP-SA, -SD, -LA, -LC, -LD, -LE, -LF) serve as pattern recognition
266 receptors. They lack the critical cysteine residue in the enzymatic pocket of PGRP domian and are unable
267 to degrade PGN (Mellroth *et al.*, 2003). Catalytic PGRPs (PGRP-SC1, -SC2, -LB, -SB1, -SB2) posses
268 amidase activity and degrade PGN (Zaidman-Remy *et al.*, 2011). PGRP-SC1a is a receptor for bacteria
269 (Garver *et al.*, 2006). Its catalytic activity is required for mediating phagocytosis (Koundakjian *et al.*,
270 2004). PGRP-SA is a pattern recognition receptor with dual roles in *Drosophila* humoral and cellular
271 immunity. It activates Toll pathway and thus up-regulates drosomycin, an AMP (Michel *et al.*, 2001).
272 PGRP-SA is also important for phagocytosis of Gram-negative bacteria (Garver *et al.*, 2006). PGRP-LC
273 is membrane-bound and mediates phagocytosis of Gram-negative but not Gram-positive bacteria (Ramet
274 *et al.*, 2002). It is also the major upstream receptor of IMD pathway (Ramet *et al.*, 2002; Choe *et al.*,
275 2002; Gottar *et al.*, 2002).

276 Integrin functions as a heterodimer of two transmembrane subunits, α and β integrin. In *Drosophila*,
277 there are 5 genes coding α integrin, and 2 coding β integrin (Brown *et al.*, 2000). Integrin heterodimer
278 α PS3 and β ν is a receptor for bacteria and apoptotic cells (Nagaosa *et al.*, 2011; Nonaka *et al.*, 2013;
279 Shiratsuchi *et al.*, 2012).

280 Down syndrome adhesion molecular (Dscam) is a immunoglobulin superfamily (IgSF) in *Drosophila*.
281 There are four Dscam-like genes and *Dscam1* is the most extensively characterized (Armitage *et al.*,
282 2012). *Dscam1* is arranged into clusters of variable exons (exon 4, 6, 9, 17) that are flanked by constant
283 exons. Via alternative splicing, large isoform repertoires are generated for recognition of diverse ligands
284 (Schmucker *et al.*, 2000). *Dscam1* expresses in immune competent tissues of *Drosophila* and acts as
285 phagocytosis receptor (Watson *et al.*, 2005).

286 Opsonization is the process by which humoral molecules bind to pathogens and promotes phagocytosis.
287 In mammals, antibodies and complement factors act as opsonins. Activated complement factors form
288 covalent binding pathogens or altered self, and mark them for phagocytosis.

289 Insect thioester-containing proteins (TEPs) share sequence similarity with vertebrate complement
290 factor. In *Drosophila*, there are six TEPs (TEPI-VI). The present of signal peptide indicates they are

secreted proteins. TEPV does not seem to be expressed (Lagueux *et al.*, 2000). TEPI-IV are closely related to mammalian complement factors as they share a CGEQ motif critical for the formation of thioester bonds with targets. TEPVI, also called *macroglobulin complement related (Mcr)*, lacks the critical cysteine residue in the thioester-binding site (Stroschein-Stevenson *et al.*, 2006).

Signaling from bound phagocytic receptors triggers coordinated rearrangement of the actin cytoskeleton. GTPase of Ras superfamily, including Rho-GTPase Cdc42, Rac1 and Rac2, are recruited to the plasma membrane. They are activated by binding with GTP, which is facilitated by guanine nucleotide exchange factors (GEFs); and inhibited by hydrolysis of GTP by guanine nucleotide disassociation inhibitors.

Drosophila Zir is a Rho-GEF that interacts with Cdc42 and Rac2 to mediate larval phagocytosis (Sampson *et al.*, 2012). Rac2 activates WAVE. WAVE then activates Arp 2/3 complex, which stimulates actin nucleation, the initial step for the formation of new filament structure. Cdc42 activates WAS(p), which activates Arp 2/3 complex. Cdc 42, Rac1, Rac2 and Arp 2/3 complex are all involved in phagocytosis (Agaisse *et al.*, 2005; Philips *et al.*, 2005; Stroschein-Stevenson *et al.*, 2006; Stuart *et al.*, 2005).

The process of internalization of targets forms a membrane-bound vesicle, the phagosome, which contains targets for degradation. Phagosome formation is followed by a series of ordered fission/fusion events with components of endosomal pathway. This process, termed as phagocytosome maturation, produces a highly acidic and hydrolytic phagolysosome designed to destroy the targets. Phagocytosome maturation involves interactions with early endosomes, recycling endosomes, late endosomes and lysosomes. Involved proteins include Rab GTPase, phosphatidylinositol 3-kinase, vacuolar hydron-ATPase, endosomal sorting complex required for transport (ESCRT) and vacuolar protein sorting-C (VPS-C) complex.

Phagosome fuse with early endosome quickly (Mayorga *et al.*, 1991). GTPase Dynamin recruits Rab5 to newly formed phagosome (Bucci *et al.*, 1992; Kinchen *et al.*, 2008). Rab5 recruits effectors to early endosomal/phagosomal membrane, including early endosome antigen 1 (EEA1), SNARE proteins required for membrane fusion, Vps34 and Vps15 (also called p150, regulatory subunit of Vps34).

Vps15 is a serine-threonine kinase recruiting Vps34 to early phagosome. Vps34 is a class III phosphatidylinositol 3-kinase (PI3-kinase) generating phosphatidylinositol-3-phosphate (PI3P) on early phagosome membrane (Vieira *et al.*, 2001). PI3P interacts with Fab1, YOTB, Vac1 and EEA1 via their conserved FYVE domain. In *Drosophila*, PI3-kinase 59F (Pi3K59F) is homolog of mammalian Vps34 and functions in cellular immune responses (Qin *et al.*, 2008; Qin *et al.*, 2011). Rebenosyn-5, *Drosophila* homolog of EEA1, contains a FYVE domain that binds to PI3P and Rab5 on the phagosome surface, and is required for fusion of early endosomes and phagosomes (Morrison *et al.*, 2008; Simonsen *et al.*, 1998).

Vacuolar hydron-ATPase (V-ATPase) complex presents on phagosome membrane and is required for

324 acidification of phagosomal lumen (Beyenbach and Wieczorek, 2006). In *Drosophila*, 8 subunits of V-
325 ATPase are important for phagocytosis (Cheng *et al.*, 2005).

326 During phagosome maturation, multivesicular bodies (MVBs) appear within the phagosome by inward
327 budding and scission of phagosome membrane. Transmembrane proteins that are destined for degradation
328 are ubiquitinated and sorted into MVBs (Lee *et al.*, 2000).

329 After MVB formation, phagosome transitions to late stage, characterized by acidic lumen and sev-
330 eral molecules including lysosomal-associated membrane proteins (LAMPs) and hydrolase. LAMPs, *e.g.*
331 *Drosophila* Lamp1 (also called CG3305), are required for the last step of phagosome maturation, the
332 fusion of phagosome with lysosome (Huynt *et al.*, 2007; Peltan *et al.*, 2012).

333 Additional V-ATPase are acquired by late phagosomes, and the vesicles also acquire Rab GTPase
334 Rab7, a marker of late phagosome (Desjardins *et al.*, 1994). Rab7 recruits effectors such as Rab-interacting
335 lysosomal protein, facilitating the movement of phagosome (Harrison *et al.*, 2003; Jordens *et al.*, 2001).

336 VPS-C complexes interact with SNAREs and Rabs during phagosome maturation. There are two
337 VPS-C complexes: CORVET and HOPS. CORVET interacts with Rab5-GTP and promotes early en-
338 dosome/phagosome fusion. HOPS interacts with Rab7-GTP on late endosomes/MVBs and promotes
339 fusion with lysosomes. CORVET and HOPS are composed of four shared class C subunits (Vps11,
340 Vps16, Vps18 and Vps33) and two Rab-specific subunits. In *Drosophila*, Vps33 and Vps16 have two
341 homologs: car and Vps33B, Vps16A and Vps16B (Li and Blissard *et al.*, 2015; Pulipparacharuvil *et al.*,
342 2005). Vps16A and Vps16B are predicted to associate with HOPS complexes (Pulipparacharuvil *et al.*,
343 2005). Vps16A is required for fusion of autophagosomes with lysosomes (Takats *et al.*, 2015). Vps16B
344 mediates phagosome to lysosome fusion (Akbar *et al.*, 2011).

345 The final step of phagosome maturation is the formation of phagolysosome (pH about 4.5). Phagolyso-
346 somes are equipped with host factors that impede microbial growth while attacking and degrading
347 pathogens. Cofactors of bacterial housekeeping enzymes, such as Fe^{2+} , Zn^{2+} and Mn^{2+} , are removed
348 from phagolysosome lumen by sequestration by lactoferrin and removing by membrane-bound protein
349 NRAMP. Reactive oxygen (ROS) and nitrogen (RNS) attack bacteria. ROS is generated by membrane-
350 bound NOX2 NADPH oxidase, which transfers electrons from cytosolic NADPH to molecular oxygen,
351 and releases O_2^- to phagolysosome lumen. Superoxide dismutase converts O_2^- into H_2O_2 , which can be
352 converted into ROS like hypochlorous acid and chloramines. RNS is generated by iNOS, the enzyme
353 catalyses the formation of nitric oxide on cytoplasmic side of phagolysosome. Nitric oxide diffuses into
354 phagolysosome lumen, where it encounters ROS and is converted into various RNS that are highly toxic
355 to bacteria. Phagosomes are also equipped with other bactericidal elements: AMPs, peptidase, lipase and
356 hydrolyase.

Nakhleh *et al.*, 2017, **The melanization response in insect immunity.**

Melanization is an immune response triggered locally in response to cuticle injury or systemically following microbial invasion. It is characterized by synthesis of melanin and cross-linking with molecules on microbial surfaces, resulting in killing of invaders. Melanization is also linked with coagulation system: coagulation initiates clotting process and melanization contributes to hardening clots (Eleftherianos and Revenis, 2011). Besides, it is essential for cuticle sclerotization or tanning that leads to hardening of exoskeleton by cross-linking cuticular proteins by quinones (Andersen, 2010).

Phenoloxidase (PO) is a key enzyme in melanin synthesis. It mediates the oxidation of tyrosine to dihydroxyphenylalanine, and the oxidation of dihydroxyphenylalanine and dopamine to respective quinones, precursors of melanin (Vavricka *et al.*, 2020). PO is produced as prophenoloxidase (PPO), which is converted to active PO by a clip domain serine proteinase (CLIP). CLIPs are specific to invertebrates and act in cascades to modulate coagulation, melanization and activation of Toll pathway that activates antimicrobial peptides (AMPs) synthesis. CLIPs lack one or more of the three residues (His, Asp, Ser) that form catalytic triad are non-catalytic, or called clip-domain containing serine proteinase homologs (cSPHs). The rest catalytic CLIPs are known as clip-domain containing serine proteinase (cSP).

The most upstream proteinase that has been characterized in PPO activation cascades is a modular serine proteinase (ModSp) that lacks clip domain but contains other domain for interactions (Buchon *et al.*, 2009; Ji *et al.*, 2004; Roh *et al.*, 2009; Takahashi *et al.*, 2015). ModSps are often autoactivated and lead to proteolytic cleavage and activation of a CLIPC, which activates a CLIPB that functions as PPO-activating proteinase (Kanost and Jiang, 2015). CLIP cascades controlling PPO activation is regulated by serpins, a family of serine proteinase inhibitors.

Insect melanogenesis is initiated by hydroxylation of phenylalanine by phenylalanine 4-monooxygenase (PAH), which forms rate-limiting substrate tyrosine (Futahashi and Fujiwara, 2005; Gorman *et al.*, 2007). The tyrosinase-like POs catalyses oxidation of tyrosine into dihydroxyphenylalanine (Dopa), and oxidation of Dopa into dopaquinone. With thiol compounds, dopaquinone is converted to cysteinyl and glutathionyl conjugates that mediate synthesis of cutaneous redish pigment phoemelanin. Without thiol compounds, dopaquinone undergoes spontaneous cyclization into dopachrome, which in turn is decarboxylated by dopachrome conversion enzyme to generate 5,6-dihydroxyindole (DHI). Following PO-mediated DHI oxidation, indole quinones polymerize and give rise to heteropolymer eumelanin. DHI-eumelanin can also be derived from dopamine produced early on decarboxylation of dopa by dopa decarboxylase (DDC).

The infection-induced melanization in *Drosophila melanogaster* requires two CLIPs: MP1 and MP2. The proteinase cascade for PPO activation includes MP1 and MP2, while its upstream pattern recognition receptors (PRRs) remain unclear (Tang *et al.*, 2008; An *et al.*, 2013). However, PRRs including PGRP-LE

(Takehana *et al.*, 2002) and GGBP3 (Matskevich *et al.*, 2010) are involved in melanization without linking to MP1-MP2 module. Additionally, another CLIP called Hyan is a key activator of PPO in systemic wound responses (Nam *et al.*, 2012).

In *Manduca sexta*, beta-glucan recognition proteins betaGRP1 and betaGRP2 trigger PPO activation (Jiang *et al.*, 2004; Ma and Kanost, 2000). Binding of betaGRP2 recruits ModSp HP14, which is autoactivated (Wang and Jiang, 2006) and cleaves cSP proHP21 into active HP21. HP21 cleaves PPO-activating proteinase-2 zymogen (PAP-2) into active PAP-2, the terminal cSP in the cascade that processes PPO into PO (Wang and Jiang, 2007). Additionally, HP21 also cleaves PAP-3 (Gorman *et al.*, 2007), which activates PPO directly (Jiang *et al.*, 1998; Jiang *et al.*, 2003; Jiang *et al.*, 2003). PAP-1 is also a direct activator of PPO, but is regulated by a pathway different from HP14-HP21, but requires HP6 (Ann *et al.*, 2009). Two cSPs, SPH1 and SPH2, seem to be required as cofactors for PPO cleavage (Gupta *et al.*, 2005; Yu *et al.*, 2003). HP6 also controls Toll pathway by cleaving HP8 (An *et al.*, 2009). PPO cascade is subject to a positive feedback. PAP-1 activates HP6, hence increases PAP-1 activation (Wang and Jiang, 2008). PAP-3 cleaves PPO as well as SPH1, SPH2, PAP-3, and thus leading to a positive feedback loop (Wang *et al.*, 2014). Besides, PAP-3 is targeted by several serpins including serpin 1J (Jiang *et al.*, 2003), serpin-3 (Christen *et al.*, 2012), serpin-6 (Wang and Jiang, 2004) and serpin-7 (Suwanchaichinda *et al.*, 2013). Serpin-4 and -5 are also involved in regulation of PPO cascade upstream of PAPs (Tong and Kanost, 2005). Serpin-4 inhibits HP21, HP6 and HP1 (Tong *et al.*, 2005). Serpin-5 inhibits HP6 and HP1 (An and Kanost, 2010).

In *Tenebrio molitor*, PGRP-SA and GGBP1 act as upstream PRRs of PPO cascade (Park *et al.*, 2006). They recruit an autoactivated ModSp, which cleaves downstream cSP called SAE (Kim *et al.*, 2008). SPE activates Toll pathway and PPO, and process a precursor of cSPH1 (Kan *et al.*, 2008). cSPH1 ligand PO to microbial surface (Zhang *et al.*, 2003). PPO cascade is inhibited by serpin 40, serpin 55, serpin 48 (Jiang *et al.*, 2009), and a melanization-inhibiting protein (MIP) inhibits melanization (Zhao *et al.*, 2005).

In *Anopheles gambiae*, complement-like thioester-containing protein 1 (TEP1) promotes melanization (Povelones *et al.*, 2013) and its downstream includes CLIPA8, a cSPH cleaved during melanization response (Volz *et al.*, 2006; Schnitger *et al.*, 2007). CLIPA2 is another cSPH that inhibits melanization by controlling TEP1 (Volz *et al.*, 2006; Kamareddine *et al.*, 2016; Yassine *et al.*, 2014). SPCLIP1 activates TEP1 as cSPH (Povelones *et al.*, 2013). Other cSPs required for melanization include CLIPB17, CLIPB8, CLIPB3 and CLIPB4 (Volz *et al.*, 2006). Serpin 2 inhibits PPO cascade by targeting several cSPs (Michel *et al.*, 2005). One of its targets is CLIPB9, which is predicted as a PAP (An *et al.*, 2011).

In *Aedes aegypti*, tissue melanization requires two cSPs, IMP1 and CLIPB8, and is inhibited by serpin-

2 (Zou *et al.*, 2010). Hemolymph melanization requires two cSPs, IMD1 and IMD2, and is inhibited by serpin-1 (Zou *et al.*, 2010). Additionally, modular serine protease CLSP2 also inhibits hemolymph PPO (Wang *et al.*, 2015).

There is extensive crosstalk between PO cascade and other humoral immune pathways, especially the Toll pathway. In *Drosophila melanogaster*, this link is through Spn27A (serpin 27A). Toll activation requires depletion of Spn27A from Hemolymph, which activates PO cascade (De Gregorio *et al.*, 2002; Ligoxygakis *et al.*, 2002). Spn27A inhibits PO cascade by binding to MP2 (An *et al.*, 2013). Additionally, PO cascade can be triggered by fungal receptor GGBP3 in a Toll-independent manner (Matskevich *et al.*, 2010). In *Anopheles gambiae*, upregulation of Toll leads to increased melanization, which is partially due to increased expression of TEP1 (Frolet *et al.*, 2006). Besides, the Imd/Rel2 pathway triggered by PGRP-LC inhibits melanization, which is partially due to activation of CLIPA2 that inhibits TEP1 (Frolet *et al.*, 2006; Meister *et al.*, 2005). In *Aedes aegypti*, Toll pathway activates melanization by controlling expression of two cSPs (IMP1 and IMP2) and several PPO genes (Zou *et al.*, 2010).

PO cascade and Toll can be controlled by common upstream signals. In *Tenebrio molitor*, SPE cleaves PPO and cSPH1 (Kan *et al.*, 2008), as well as Spz that activates Toll (Kim *et al.*, 2008). In *Manduca sexta*, HP6 activates cleaves Spz, resulting in Toll activation; and activates PPO by cleaving proPAP1 (Ann *et al.*, 2009). In *Bombyx mori*, serpin-5 regulates both Toll and PPO (Li *et al.*, 2016). In *Drosophila*, PGRP-LE activates Imd pathway and PPO cascade (Takehana *et al.*, 2002; Takehana *et al.*, 2004).

Hillyer, 2016, Insect immunology and hematopoiesis. The most encompassing physical barrier of insects is the cuticle. This chitinous, hydrophobic material forms the exoskeleton, and also lines foregut, hindgut and tracheal system. Pathogens enter body through cuticle via wound or enzymatic digestion. Ingestion is another routine for pathogen entrance.

Multiple insect cells and tissues are involved in immunity. Hemocytes are the primary immune cells. They circulate with hemolymph (circulating hemocytes) or attach to tissues (sessile hemocytes). These cells drive cellular and humoral immunity. Fat body is composed of loosely associated cells that are rich in lipids and glycogen, lines the integument of hemocoel. It functions in energy storage and synthesis of vitellogenin precursors that are required for egg production. Fat body also produces antimicrobial peptide. Midgut mainly functions in digestion and nutrition absorption. It produces nitric oxide synthesis and other lytic effectors killing pathogens. Salivary glands are primarily involved in feeding and usually located in the anterior of thorax. It is involved in immunity.

Immune responses are initiated by recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs). Among PRR families are

(1) PGRP: peptidoglycan recognition protein, characterized by peptidoglycan-binding domain;

- 456 (2) Ig: immunoglobulin domain proteins;
- 457 (3) FREP: fibrinogen-related protein, or fibrinogen domain immunorecognition (FBN), contain fibrinogen-like
- 458 domain;
- 459 (4) TEP: thioester-containing protein;
- 460 (5) betaGRP: beta-1,3-glucan recognition proteins, or Gram-negative bacterial-binding protein (GNBP);
- 461 (6) Galectin: bind specifically to beta-galactoside sugars;
- 462 (7) CTL: C-type lectin;
- 463 (8) LRR: leucine-rich repeat containing protein;
- 464 (9) DSCAM: down syndrome cell adhesion molecule;
- 465 (10) Nimrod: include eater and draper in *Drosophila melanogaster*;
- 466 (11) ML: MD-2-like protein, or Niemann-Pick type C-2 protein, involved in recognizing lipopolysaccharide;
- 467 (12) SR: scavenger receptor, include croquemort and peste in *Drosophila melanogaster*;
- 468 (13) Integrin.

469 Toll pathway functions in both development and immunity. In immunity, Toll signaling is effective in
 470 combating Gram-positive bacteria, fungi and viruses. Toll pathway includes

- 471 (1) SPZ: Spatzle/spaetzle, extracellular cytokine;
- 472 (2) Toll: or toll-like receptor (TLR).
- 473 (3) MyD88: myeloid differentiation primary response 88;
- 474 (4) Tube: or interleukin-1 receptor-associated kinase 4 (IRAK4);
- 475 (5) Pelle: orthologous to human interleukin 1 receptor associated kinase 1 (IRAK1);
- 476 (6) Dorsal;
- 477 (7) Dif: Dorsal-related immune factor;
- 478 (8) Cactus: orthologous to human NF-kappaB inhibitor alpha (NFKBIA).

479 In Toll signaling, SPZ is activated by cleavage. SPZ binds to cellular receptor Toll. Toll recruits
 480 MyD88, Tube and Pelle. Pelle acts as serine/threonine-protein kinase, phosphorylating Cactus. Thus,
 481 NF-kappaB transcription factor Dorsal and Dif are released from Cactus and relocated to nuclear.

482 Imd signaling is effective in combating Gram-negative bacteria and viruses. Players in Imd signaling
 483 include:

- 484 (1) Imd: immune deficiency, or AGAP004959-PA/-PB in *Anopheles gambiae* str. PEST, or receptor-
 485 interacting serine/threonine-protein kinase 1-like, or LOC5572865 in *Aedes aegypti*;
- 486 (2) TAK1: transforming growth factor (TGF)-beta activated kinase 1, orthologous to human mitogen-
 487 activated protein kinase kinase kinase 7 (MAP3K7);
- 488 (3) Tab: TAK1-associated binding protein, or MAP3K7 binding protein;

- 489 (4) IKKgamma: inhibitor of NF-kappaB (IkappaB) kinase subunit gamma, or Kenny in *Drosophila*
490 *melanogaster*, or NF-kappaB essential modulator (NEMO), or optineurin;
- 491 (5) IKKbeta: IkappaB kinase subunit beta;
- 492 (6) Fadd: fas-associated death domain, or Fas associated via death domain;
- 493 (7) Dredd: death-related ced3/Nedd2-like caspase, or caspase 8, orthologous to human caspase 10;
- 494 (8) Relish: or nuclear factor NF-kappa-B p110 subunit;
- 495 (9) Diap: death-associated inhibitor of apoptosis, or inhibitor of apoptosis (Iap);
- 496 (10) Effete: or ubiquitin-conjugating enzyme E2-17 kDa;
- 497 (11) UEV1A: ubiquitin-conjugating enzyme variant 1A, or ubiquitin conjugating enzyme E2 variant 2;
- 498 (12) Bendless: or ubiquitin-conjugating enzyme E2 N, or ubiquitin-conjugating enzyme 13 (UBC13);
- 499 (13) Caspar: or fas-associated factor 1 (FAF1);
- 500 (14) Hemipterous: or dual specific mitogen activated protein kinase kinase 7 (MAP2K7), or MAP2K7;
- 501 (15) Basket: or stress-activated protein kinase JNK;
- 502 (16) Jra: Jun-related antigen, or transcription factor AP-1, or transcription factor jun-D-like;
- 503 (17) Kayak.

504 In Imd signaling, PRRs activate cascade composed of Imd, Fadd and Dredd. Dredd activates tran-
505 scription factor Relish. Another line for Relish activation includes Imd, Tab, TAK1, IKKgamma and
506 IKKbeta. Both signals require ubiquitination mediated by a protein complex including Diap, Effete,
507 UEV1A, Bendless. Caspar inhibits Dredd-mediated activation of relish. TAK1 also activates JNK (Jun
508 amino-terminal kinase) signaling, including Hemipterous, Basket, Jra and transcription factor Kayak.

509 JAK/STAT signaling functions in development and immunity. In immunity, it activates antimicrobial
510 genes like nitric oxide synthase and functions in antibacterial and antiviral responses. JAK/STAT signal-
511 ing includes

- 512 (1) Unpaired
- 513 (2) Domeless.
- 514 (3) Hopscotch: orthologous to several human genes including JAK1 (Janus kinase 1) and JAK3 (Janus
515 kinase 3).
- 516 (4) Stat: signal-transducer and activator of transcription protein.
- 517 (5) Socs: suppressor of cytokine signaling;
- 518 (6) Pias: protein inhibitor of activated Stat as E3 SUMO-protein ligase, or suppressor of variegation 2-10
519 (Su(var)2-10) in *Drosophila melanogaster*.

520 In JAK/STAT signaling, extracellular protein Unpaired activates membrane protein Domeless. Dome-
521 less activates Hopscotch and Stat. Stat relocates to nuclear, acting as transcription factor. Socs and Pias

522 inhibit JAK/STAT signaling.

523 Phagocytosis is a rapid process conducted by hemocytes. PRRs that have been shown to be involved
524 in phagocytosis include TEPs, Nimrods, DSCAMs, beta-integrins and PGRPs. The intracellular signaling
525 in phagocytosis remains poorly understood. In mosquitoes,

526 (1) CED2: cell death abnormality 2;

527 (2) CED5;

528 (3) CED6

529 are involved in signaling regulate internalization of bacteria (Moita *et al.*, 2005).

530 Melanization is an enzymatic process involved in cuticle hardening, egg chorion tanning, wound heal-
531 ing and immunity and is mainly conducted by hemocytes. In immunity, melanization functions in killing
532 bacteria, fungi, protozoa parasites, nematode worms and parasitoid wasps. It is manifested as a darkened
533 proteinaceous capsule that surrounds pathogens, and kills pathogens via oxidative damage or starvation.
534 Players in melanization include:

535 (1) PAH: phenylalanine hydroxylase, or phenylalanine 4-monooxygenase, or Henna in *Drosophila*
536 *melanogaster*;

537 (2) PO: phenoloxidase, or phenol oxidase, formed via cleavage of prophenoloxidase (PPO);

538 (3) DCE: dopachrome conversion enzyme or dopachrome decarboxylase/tautomerase, known as yellow in
539 *Drosophila melanogaster*;

540 (4) DDC: dopa decarboxylase, aromatic-L-amino-acid decarboxylase (AADC or AAAD), tryptophan de-
541 carboxylase or 5-hydroxytryptophan decarboxylase, decarboxylates dopa into dopamine, which is oxidized
542 into dopaminequinone by PO, and further converts into dopaminechrome non-enzymatically, and further
543 into DHI non-enzymatically.

544 (5) ModSp: modular serine protease that lacks clip domain but contains other domain for interactions;

545 (6) cSP: clip domain-containing serine protease, includes *Drosophila melanogaster* snake, easter, serine
546 protease 7 (SP7), serine protease immune response integrator (spirit), persephone, spatzle-processing en-
547 zyme (SPE), Gram-positive specific serine protease (grass), melanization protease 1 (MP1), hayan, Ser7,
548 lethal (2) k05911, activated by ModSp cleavage and activates PO by cleavage.

549 (7) serpin: serine protease inhibitors.

550 In synthesis of melanine, PAH hydroxylates phenylalanine to tyrosine. PO oxidizes tyrosine into
551 dihydroxyphenylalanine (Dopa), and further into dopaquinone. Dopaquinone is oxidized into dopachrome
552 non-enzymatically. DCE decarboxylates dopachrome into 5,6-dihydroxyindole (DHI). Another way from
553 Dopa to DHI is: DDC decarboxylates dopa into dopamine, which is oxidized into dopaminequinone
554 by PO. Dopaminequinone is further converted into dopaminechrome non-enzymatically, and further into

554 DHI non-enzymatically. Finally, following PO-mediated DHI oxidation, indole-5,6-quinones polymerize
556 and give rise to heteropolymer eumelanin. PO activity is controlled by ModSP, cSP and Serpin.

557 Encapsulation is a cellular immune response against pathogens that are too large to be phagocy-
558 tosed. In encapsulation, hemocytes attach to form a capsule surrounding pathogens. The capsule may
559 be melanized. In Lepidoptera, hemocyte adhesion is dependent on binding of integrin to specific sites
560 defined by Arg-Gly-Asp (RGD) sequence.

561 Nodulation is an immune response in which hemocyte adhere to large aggregates of bacteria and form
562 layers, usually followed by melanization. Underlying molecular mechanism of nodulation remains poorly
563 understood, but it relies on eicosanoid-based signaling and extracellular matrix-like protein Noduler.

564 Lysis of pathogens is resulted from disruption of cellular membrane by immune effectors including
565 (1) AMP: antimicrobial peptide, small secreted peptide including apisimin, attacin, cecropin, defensin,
566 dipteracin, drosocin, drosomycin, gambicin, gloverin, holitricin, jelleine, lebocin, melittin, metchnikowin,
567 moricin, persulcatusin, ponericin, pyrrhocoricin, sapecin;
568 (2) Lysozymes: or muramidase, or N-acetylmuramide glycanhydrolase, hydrolyze beta-1,4-glycosidic link-
569 age between N-acetylmuramic and N-acetylglucosamine of peptidoglycan;
570 (3) Transferrin: binds to Fe;
571 (4) Chitinase: degrades chitin and is involved in antifungal responses.

572 Reactive species are effect in lysis. Synthesis of reactive species include

573 (1) DUOX: dual oxidase, generates hydrogen peroxide;
574 (2) NOX: NADPH oxidase, generates hydrogen peroxide;
575 (2) NOS: nitric oxide synthase, generates nitric oxide;
576 (3) SOD: superoxide dismutase, catalyzes the dismutation (or partitioning) of the superoxide radical into
577 ordinary molecular oxygen and hydrogen peroxide;
578 (4) peroxidase: also peroxide reductase, peroxiredoxin, break up peroxides.

579 In RNA interference (RNAi) pathways, small RNA (sRNA) associates with Argonaute protein, forming
580 RNA induced silencing complex (RISC). RISC recognizes targets by complementary bases, and silences
581 targets in an Argonaute-mediated manner. RNAi functions in antiviral responses, gene expression regu-
582 lation and anti-transposon responses. In insects, there are three RNAi pathways: micro-RNA (miRNA),
583 small-interfering-RNA (siRNA) and piwi-interacting-RNA (piRNA).

584 miRNA pathway is mainly involved in gene expression regulation. Players in miRNA pathway include:

585 (1) Drosha;
586 (2) Pasha: partner of Drosha, or microprocessor complex subunit DGCR8.
587 (3) Dicer 1: endoribonuclease;

588 (4) Loquacious: or interferon-inducible double-stranded RNA-dependent protein kinase activator A ho-
589 molog, or TARBP2.

590 (5) Argonaute 1.

591 miRNA originates from nuclear genome, and is processed by nuclear protein Dicer and Pasha. Matured
592 miRNA relocates to cytoplasm, and is further processed by Dicer 1 and Loquacious. Then fully-matured
593 miRNA is loaded to Argonaute 1.

594 siRNA pathway is involved in defenses against viral dsRNA and transposonal elements.

595 (6) Dicer 2;

596 (7) R2D2: or double-stranded RNA-binding protein Staufen homolog;

597 (8) Argonaute 2.

598 Viral dsRNA is processed by Dicer 2 and R2D2, forming siRNA. siRNA is loaded into Argonaute 2. In
599 anti-transposonal elements, dsRNA is processed by Dicer 2 and Loquacious.

600 piRNA pathway is involved in defenses against transposonal element in germline.

601 (9) Zucchini: or mitochondrial cardiolipin hydrolase;

602 (10) Piwi: P-element induced wimpy testis, or Argonaute 3, or Aubergine, or Piwi-like protein Siwi;

603 Transposon transcripts is processed by Zucchini, forming piRNA. piRNA is loaded into Piwi.

604 Autophagy is a process of degradation of intracellular materials, and is involved in elimination of in-
605 tracellular bacteria and viruses. In *Drosophila*, autophagy defends against vesicular stomatitis virus and
606 Rift Valley fever virus, but enhances infection of Sindbis virus. Major players in autophagy include:

607 (1) PI3K: phosphatidylinositol 3-kinase, or phosphoinositide 3-kinase;

608 (2) AKT: or RAC serine/threonine-protein kinase;

609 (3) TOR: target of rapamycin, protein kinase.

610 (4) Atg1: autophagy-related (Atg) 1, or unc-51 like autophagy activating kinase (ULK), or unc-51, a
611 serine/threonine protein kinase;

612 (5) Atg13: serine/threonine protein kinase regulatory subunit;

613 (6) Atg14: or Beclin 1-associated autophagy-related key regulator;

614 (7) Vps15: vacuolar protein sorting (Vps) 15, or phosphoinositide 3-kinase regulatory subunit 4;

615 (8) Vps34: phosphatidylinositol 3-kinase 59F, or phosphatidylinositol 3-kinase catalytic subunit type 3;

616 (9) Atg5;

617 (10) Atg12;

618 (11) Atg8: or gamma-aminobutyric acid receptor-associated protein (GABARAP).

619 In immunity, autophagy initiates with PI3K-AKT signaling, inactivating TOR. TOR inactivation
620 activates protein complex containing Atg1 and Atg13, which leads to nucleation of autophagosomal mem-

brane via a complex containing Atg14, Vps15 and Vps34. Then autophagosome is elongated, dependent on Atg5, Atg12 and Atg8.

Apoptosis is a form of programmed cell death that often functions in antiviral responses. Key players include:

- (1) Dronc: death regulator Nedd2-like caspase, or Nedd2-like caspase (Nc);
- (2) Dark: death-associated APAF1-related killer, or apoptotic protease-activating factor 1 (APAF1);
- (3) Drice: death related ICE-like caspase;
- (4) DCP1: death caspase-1.

Dronc and Dark form a protein complex, and Dronc activates downstream caspase including Drice and DCP1.

Gerardoet al., 2020, Evolution of animal immunity in the light of beneficial symbioses.

There are three key ways for host-symbiont interactions. First, host immunity can play a role in regulation of symbionts. Second, symbionts can protect host against pathogens. Third, symbionts can influence the maturation of host immune system.

Host can regulate symbiont populations. For example, cereal weevil requires endosymbionts for exoskeleton development, after which symbionts are eliminated by apoptosis of bacteriocytes. Bean bug *Riptortus pedestris* up-regulates immunity and digests bacteriocytes before moulting, reducing symbiont populations as moulting is energy-costing and leaves bean bug vulnerable to infections and injuries.

The influence of host immunity on symbiosis likely dependent on symbiont transmission mode. In horizontal transmission, host select proper symbionts from pools of microorganisms. In vertical transmission, symbionts are transmitted vertically from parents (often mothers) to offsprings.

For vertebrate hosts, horizontal transmission is the major mode. Vertebrates often harbour a large and diversified community of microbes. By leveraging innate and adaptive immunity, vertebrates can mount rapid and robust responses to large numbers of microorganisms. In mice, immune system discriminates between pathogens and symbionts, and segregates symbionts to proper host tissues. In mice gut, epithelium tissue is protected from lumen by a mucus layer. Both pathogens and symbionts can enter gut lumen. Pathogens are segregated from mucus layer by immune responses. Symbionts enter mucus layer and are segregated from epithelium by antimicrobial peptides and immunoglobulins.

Invertebrates likely to regulate horizontally-transmitted symbiosis by compartmentalized innate immune responses. By compartmentalization, hosts can invest the most energy into screening microorganisms and mounting immunity in regions exposed to a wide array of microorganisms, while reduce immune investment elsewhere. For example, Hawaiian bobtail squid *Euprymna scolopes* screens large quantities of microbes by defenses including physical barrier, morphological changes and innate immunity. Thus, squid

limits colonization in light organ to bacteria with specific characteristics, including symbiotic molecular patterns, biofilm formation, bioluminescence and nitric oxide resistance. In this way, squid limits colonization of specific strains of *Vibrio fischeri* in light organ. Fruit fly *Drosophila melanogaster* uses physical barrier, morphological changes and compartmentalized immune expressions to eliminate pathogens and to limit few symbionts in gut microbiome.

In vertical transmission, hosts pass few symbionts directly to offsprings in ways including providing symbiont-enclosed capsules, smearing eggs with symbionts, and symbiotic infection of embryo. Passaged symbionts undergo population bottlenecks and have little chance for getting virulence factors via horizontal gene transfer with environmental microbes. In many cases, vertical transmission is coupled with sequestration of symbionts into specialized cells. Sequestration allows host to limit symbiont populations and reduce horizontal gene transfer with fewer investment. For example, in cereal weevil, antimicrobial peptides are not expressed in bacteriocytes except ColA, whose knock-out leads to symbiont overproliferation and escape to other tissues.

The evolution of immunity in symbiont regulation is likely dependent on transmission mode. Vertical transmission has evolved multiple times among invertebrates. Compared with horizontal transmission, it may allow reduced investment in symbiont regulation because (1) horizontal transmission requires screening for symbionts and discarding pathogens from environmental pools; (1) fitness of vertically-transmitted symbionts is dependent on host fitness, and therefore, they are less likely to exploit hosts; (2) vertical transmission limits chance for horizontal gene transfer from environments, reducing possibility that symbionts acquire virulence factors; (3) vertically-transmitted symbionts are often sequestered into host cells, allowing tightly control via nutrition availability. Therefore, it is possible that selection pressure on immunity is weaker in hosts with vertically-transmitted symbionts than hosts with horizontally-transmitted symbionts. However, vertical transmission provides less flexibility in the face of changing environment conditions, which can be especially important for long-living hosts. Adaptive immunity, in turn, is assumed to have evolved to affording regulation of a diversified symbiont communities, as complex symbiont communities are often found in vertebrates. However, adaptive immunity only evolved independently twice in jawed vertebrates and jawless vertebrates, indicating the co-occurrence of complex microbiomes and adaptive immunity is resulted from common ancestors instead of convergent evolution under selection.

Garcia *et al.*, 2014, The symbiont side of symbiosis: do microbes really benefit?

It has been presumed that microbial symbionts receive host-derived nutrients or a competition-free environment with reduced predation, but there have been few empirical tests, or even critical assessments, of these assumptions. Evaluation of these hypotheses based on available evidence indicates reduced competition and predation are not universal benefits for symbionts. Some symbionts do receive nutrients from

687 their host, but this has not always been linked to a corresponding increase in symbiont fitness.

688 **Palmer *et al.*, 2015, Comparative genomics reveals the origin and diversity of Arthropod**
689 **immune system.**

690 Immune gene families are searched in predicted peptide sets of arthropod species, including insect
691 *Drosophila melanogaster*, crustacean *Daphnia pulex* (water flea), myriapod *Strigamia maritima* (coastal
692 centipede) and five chelicerates: *Mesobuthus martensii* (Chinese scorpion), *Parasteatoda tepidariorum*
693 (house spider), *Ixodes scapularis* (deer tick), *Metaseiulus occidentalis* (western orchard predatory mite),
694 *Tetranychus urticae* (red spider mite). Arthropod Toll-like receptors (TLRs) are a dynamically evolving
695 gene family that includes relatives of vertebrate TLRs. The Toll signaling pathway is conserved across
696 arthropods. The IMD signaling pathway is highly reduced in chelicerates. *IMD* is frequently absent.
697 *Relish* is often present. ANK domain of *Relish* is occasionally absent, indicating Dredd-independent
698 activation. The JAK/STAT signaling pathway is highly conserved. β -1,3 glucan recognition proteins
699 (β GRPs) have been lost in chelicerates. Arthropod TEPs include relatives of vertebrate C3 complement
700 factors and proteins lacking the thioester motif. Gene duplication generates diversity in the immune recep-
701 tor Down syndrome cell adhesion molecule (Dscam). Argonaute 2 evolves rapidly and is often duplicated
702 with variable copy numbers.

703 **Viljakainen, 2015, Evolutionary genetics of insect innate immunity.**

704 Toll and Imd signaling pathways are well conserved across insects. Antimicrobial peptides (AMPs) are the
705 most labile component of insect immunity showing rapid gene birth-death dynamics and lineage-specific
706 gene families. Immune genes and especially recognition genes are frequently targets of positive selection
707 driven by host-pathogen arms races. Homology-based annotation is useful but to some extent restricted
708 approach to find immune-related genes in a newly sequenced genome. Novel immune genes have been
709 found in many insects and should be looked for in future research.

710 **Boehm, 2012 Evolution of vertebrate immunity.**

711 Could it be possible then that an immune system employing structurally diversified antigen receptors facil-
712 itated increased species-richness in autochthonous microbial communities, for example, in the intestine?
713 The selective advantage of increasing antigen receptor diversity with respect to the species-richness of
714 microbiomes is illustrated by the role of secreted antibodies, such as IgA in mammals, in the maintenance
715 of microbial homeostasis on mucosal surfaces; defective structural diversification of secreted antibodies is
716 associated with dysbiosis, which is characterized by generally lower species diversity and an ‘unhealthy’
717 composition of the microbiome. Autoimmunity can be a price for the evolution of adaptive immunity.

718 **McFall-Ngai, 2007, Care for the community.**

719 A memory-based immune system may have evolved in vertebrates because of the need to recognize and

720 manage complex communities of beneficial microbes. Invertebrates are no less challenged by the microbial
721 world than vertebrates, nor are they less able to remain healthy by entirely relying on innate immunity.
722 Invertebrates often harbor much less diversified symbiont communities compared with vertebrates. There
723 are three possible strategies for management of symbionts in invertebrates: maintain symbionts intra-
724 cellularly; build physical barrier between host tissue and symbionts; express a high number of specific
725 recognition components of innate immunity.

726 **Hoang King, 2022, Symbiont-mediated immune priming in animals through an evolu-**
727 **tionary lens.**

728 While research on symbiont-mediated immune priming (SMIP) has focused on ecological impacts and
729 agriculturally important organisms, the evolutionary implications of SMIP are less clear. Here, we review
730 recent advances made in elucidating the ecological and molecular mechanisms by which SMIP occurs.
731 We draw on current works to discuss the potential for this phenomenon to drive host, parasite, and sym-
732 biont evolution. We also suggest approaches that can be used to address questions regarding the impact
733 of immune priming on host-microbe dynamics and population structures. Finally, due to the transient
734 nature of some symbionts involved in SMIP, we discuss what it means to be a protective symbiont from
735 ecological and evolutionary perspectives and how such interactions can affect long-term persistence of the
736 symbiosis.

737 **Sharp Hoster, 2022, Host control and the evolution of cooperation in host microbiomes.**

738 It is often suggested that the mutual benefits of host-microbe relationships can alone explain cooperative
739 evolution. Here, we evaluate this hypothesis with evolutionary modelling. Our model predicts that
740 mutual benefits are insufficient to drive cooperation in systems like the human microbiome, because
741 of competition between symbionts. However, cooperation can emerge if hosts can exert control over
742 symbionts, so long as there are constraints that limit symbiont counter evolution. We test our model
743 with genomic data of two bacterial traits monitored by animal immune systems. In both cases, bacteria
744 have evolved as predicted under host control, tending to lose flagella and maintain butyrate production
745 when host-associated. Moreover, an analysis of bacteria that retain flagella supports the evolution of host
746 control, via toll-like receptor 5, which limits symbiont counter evolution. Our work puts host control
747 mechanisms, including the immune system, at the centre of microbiome evolution.

748 **Costello *et al.*, 2012, The application of ecological theory toward an understanding of**
749 **the human microbiome.**

750 Review of three core scenarios of human microbiome assembly: development in infants, representing
751 assembly in previously unoccupied habitats; recovery from antibiotics, representing assembly after distur-
752 bance; and invasion by pathogens, representing assembly in the context of invasive species.

Hansen Moran, 2013, The impact of microbial symbionts on host plant utilization by herbivorous insects.

Herbivory, defined as feeding on live plant tissues, is characteristic of highly successful and diverse groups of insects and represents an evolutionarily derived mode of feeding. Plants present various nutritional and defensive barriers against herbivory; nevertheless, insects have evolved a diverse array of mechanisms that enable them to feed and develop on live plant tissues. For decades, it has been suggested that insect-associated microbes may facilitate host plant use, and new molecular methodologies offer the possibility to elucidate such roles. Based on genomic data, specialized feeding on phloem and xylem sap is highly dependent on nutrient provisioning by intracellular symbionts, as exemplified by *Buchnera* in aphids, although it is unclear whether such symbionts play a substantive role in host plant specificity of their hosts. Microorganisms present in the gut or outside the insect body could provide more functions including digestion of plant polymers and detoxification of plant-produced toxins. However, the extent of such contributions to insect herbivory remains unclear. We propose that the potential functions of microbial symbionts in facilitating or restricting the use of host plants are constrained by their location (intracellular, gut or environmental), and by the fidelity of their associations with insect host lineages. Studies in the next decade, using molecular methods from environmental microbiology and genomics, will provide a more comprehensive picture of the role of microbial symbionts in insect herbivory.

Zaidman-Rémy *et al.*, 2018, What can a weevil teach a fly, and reciprocally? Interaction of host immune systems with endosymbionts in *Glossina* and *Sitophilus*

Wu *et al.*, 2018, Insect antimicrobial peptides, a mini review.

Skidmore Hansen, 2017, The evolutionary development of plant-feeding insects and their nutritional endosymbionts.

Vavre Kremer, 2014, Microbial impacts on insect evolutionary diversification: from patterns to mechanisms.

Eleftherianos *et al.*, 2013, Endosymbiotic bacteria in insects: guardians of the immune system?

Soucy *et al.*, 2015, Horizontal gene transfer: building the web of life.