Molecular characterization of the *Drosophila* responses towards nematodes

Md. Badrul Arefin

Stockholm University

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Cover picture: A confocal image of nematode inflicted wounds in the *Drosophila* gut where Viking-GFP produces green signal. The black spots show the melanised wounds.

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To my beloved family

Abstract

A sophisticated evolutionary conserved innate immune system has evolved in insects to fight pathogens and to restrict damage in harmful (danger) situations including cancer. A significant amount of knowledge about different infection models in *Drosophila* has been generated in past decades, which revealed functional resemblances and implications for vertebrate systems. However, how *Drosophila* responds towards multicellular parasitic nematodes and in danger situations is still little understood. Therefore, the aim of the thesis was to characterize multiple aspects of the host defense in the two important contexts mentioned above.

We analyzed the transcriptome profiles of nematode-infected *Drosophila* larvae with uninfected samples. For this we employed the entomopathogenic nematode *Heterorhabditis bacteriophora* with its symbiont *Photorhabdus luminescence* to infect *Drosophila* larvae. We found 642 genes were differentially regulated upon infection. Among them a significant portion belonged to immune categories. Further functional analysis identified a thioester containing protein TEP3, a recognition protein GNBP-like 3, the basement membrane component protein Glutactin and several other small peptides. Upon loss or reduced expression of these genes hosts showed mortality during nematode infections. This study uncovers a novel function for several of the genes in immunity.

Furthermore, we investigated the cellular response towards nematodes. When we eliminated hemocytes genetically (referred to as hml-apo) in *Drosophila*, we found hml-apo larvae are resistant to nematodes. Subsequent characterization of hml-apo larvae showed massive lamellocyte differentiation (another blood cell type which is rare in naïve larvae), emergence of melanotic masses, up- and down-regulation of Toll and Imd signaling respectively suggesting a pro-inflammatory response. Moreover, a striking defective leg phenotype in adult escapers from pupal lethality was observed. We identified nitric oxide (NO) as a key regulator of these processes. We also showed that imaginal disc growth factors 3 (IDGF3): (a) protects hosts against nematodes, (b) is a clotting component and (c) negatively regulates Wnt and JAK/STAT signaling. To follow larval behavior in the presence or absence of nematodes we monitored *Drosophila* larval locomotion behaviors using FIMtrack (a recently devised automated method) to elucidate evasive strategies of hosts. Finally, we characterized host defenses in three *Drosophila* leukemia models with and without nematode infection. Taken together, these studies shed light on host responses in two crucial circumstances, nematode infections and danger situations.

List of papers

The thesis is based on the following publications and manuscripts

I. **Arefin, B*.**, Kucerova, L*., Dobes, P., Markus, R., Strnad, H., Wang, Z., Hyrsl, P., Zurovec, M., and Theopold, U. (2014). Genome-wide transcriptional analysis of *Drosophila* larvae infected by entomopathogenic nematodes shows involvement of complement, recognition and extracellular matrix proteins. Journal of Innate Immunity *6*, 192-204. doi: 10.1159/000353734. PubMed PMID: 23988573.

II. **Arefin, B.**, Kucerova, L., Krautz, R., Kranenburg, H., Parvin, F., and Theopold, U. (2015). Apoptosis in Hemocytes Induces a Shift in Effector Mechanisms in the *Drosophila* Immune System and Leads to a Pro-Inflammatory State. PLoS One *10*, e0136593. doi: 10.1371/journal.pone.0136593. PubMed PMID: 26322507

III. Kucerova, L., Broz, V., **Arefin, B.**, Maaroufi, H.O., Hurychova, J., Strnad, H., Zurovec, M., and Theopold, U. (2015). The *Drosophila* Chitinase-Like Protein IDGF3 Is Involved in Protection against Nematodes and in Wound Healing. J Innate Immun. doi: 10.1159/000442351. PubMed PMID: 26694862.

IV. Kunc, M., **Arefin, B.**, Hyrsl, P., and Theopold. U. Monitoring the effect of pathogenic nematodes on locomotion of *Drosophila* larvae **(resubmitted after revision).**

V. **Arefin, B.**, Kunc, M., Krautz, R., and Theopold. U. *Drosophila* models for different grades of leukemia **(manuscript).**

Paper not included in the thesis

VI. Krautz, R., **Arefin, B.,** and Theopold, U. (2014). Damage signals in the insect immune response. *Front. Plant Sci.* **5**:342. doi: 10.3389/fpls.2014.00342. PubMed PMID: 25071815. (Review article)

* These authors contributed equally to this work

Contents

Abbreviations

AMP	Antimicrobial peptides
AP-1	Activator protein 1
ATP	Adenosine triphosphate
CCR	Copper cell region
CLP	Chitinase-like protein
CNS	Central nervous system
CO_2	Carbon dioxide
CZ	Cortical zone
DAMP	Damage-associated molecular patterns
DAP	Diaminopimelic acid
DCV	*Drosophila* C virus
DD	Death domain
Dipt	Diptericin
DNA	Deoxyribonucleic acid
dNOS	*Drosophila* nitric oxide synthase
Drs	Drosomycin
dsRNA	Double stranded ribonucleic acid
e. g.	Exempli gratia (for example)
Ecc	*Erwinia carotovora carotovora*
ECM	Extracellular matrix
EGF	Epidermal growth factor
EPN	Entomopathogenic nematode
ERK	Extracellular signal-regulated kinases
GFP	Green fluorescent protein
GNBPs	Gram-negative binding proteins
GO	Gene ontology
GxE	Genotype and environmental interactions
H_2O_2	Hydrogen peroxide
HDAC	histone deacetylase
HMG	High mobility Group
i. e.	Id est (that is)
IAP	Inhibitor of apoptosis proteins
IDGF	*Drosophila* imaginal disc growth factor
IJ	Infective juvenile
IkB	Inhibitor of kappa B

IKK	IκB kinase
IL	Interleukin
Imd	Immune deficiency
iNOS	Inducible NOS
JAK	Janus kinase
JNK	Jun-N-terminal kinase
JNKK	JNK kinase
kDa	Kilodalton
L-NAME	L-NG-Nitroarginine methyl ester
l2gl	Lethal (2) giant larvae
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
Mcf1	Makes caterpillar floppy 1
Mcr	Macroglobulin complement related
MMP	Matrix metalloproteinase
MZ	Medullary zone
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NI	Nutritional indices
NO	Nitric oxide
NOS	Nitric oxide synthase
PAMP	Pathogen associated molecular patterns
PCD	Programmed cell death
PGN	Peptidoglycan
PGRPs	Peptidoglycan recognition proteins
PPAE	Prophenoloxidase-activating enzyme
PPO	Prophenoloxidase
PRR	Pattern recognition receptor
PS	Phosphatidylserine
PSC	Posterior signaling center
Puc	Puckered
RIP	Receptor Interacting Protein
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxygen species
Scrib	Scribbled
SINV	Sindbis virus
SNAP	S-Nitroso-N-Acetyl-D,L-Penicillamine
SPE	Spätzle Processing Enzyme
spp	Species
STAT	Signal Transducer and Activator of Transcription
TccC	Toxin complex component C
TEP	Thioester-containing protein
TH2	Type 2 helper
TLR	Toll like receptor
TNF	Tumor necrosis factor

TNFR	Tumor necrosis factor receptor
Upd	Unpaired
VDRC	Vienna *Drosophila* RNAi Center
VSV	Vesicular stomatitis virus
μM	Micro molar

Introduction

Drosophila: a model for 100 years and still in use

One day in 1910, when the American geneticist Thomas Hunt Morgan was working with his fruit flies, he discovered a male fly that did not display its regular eye color. Instead of red eyes that were typically found in wild type *Drosophila melanogaster* it had white eyes. This observation led him to pursue what caused this phenotypic difference. His persistence established chromosomal inheritance of a trait long before the discovery of DNA. He named the trait 'white', as it changed the eye color from red to white. Later, he was awarded the Nobel Prize in Physiology or Medicine in 1933 "for his discoveries concerning the role played by the chromosome in heredity" (Morgan, 1910;Bellen et al., 2010;Jennings, 2011). Now we know the '*white*' gene contributes to the eye color (Flybase, 2016).

The first 50 years of *Drosophila* (1910-1960) research were dominated by genetic approaches in which many tools and reagents were generated. Developing 'balancer' chromosomes was the most important. It allows lethal mutations to be maintained in heterozygous condition. All of these tools and reagents were utilized in the next 50 years (1960-present) to allow for the development of additional tools and understanding biological processes. Until now, *Drosophila* is the only multicellular organism that allows the maintenance of more than 95% lethal mutations comfortably (Bellen et al., 2010).

The *Drosophila melanogaster* genome contains approximately 14,000 protein-coding genes. Extensive tools and reagents are available. Collectively both random and site-specific RNAi construct insertions in *Drosophila* cover 91% of all genes for loss of function studies. Importantly, while there are additional stock centers around the world, just one of these stock centers (VDRC) houses more than one independent RNAi line that is available for most of the genes (VDRC, 2016).

Breeding *Drosophila* is easy and inexpensive. It is widely used as a model organism in biology and medicine. Short life cycle and excellent genetics attracted researchers and established it as a very good model organism especially in developmental biology and innate immunity. Two Nobel Prizes were awarded for research performed in *Drosophila* in the respective field

mentioned above in the last two decades (in 1995 and 2011) (Jennings, 2011). Furthermore, three quarters of disease-causing genes in humans have homologs in *Drosophila* (Pandey and Nichols, 2011).

Drosophila: a model for innate immunity

We live in a dangerous world where we are exposed to potential pathogens every day via inhalation, ingestion, or simply direct contact every day. However, we do not get sick often due to the immune system. In vertebrates, there are two types of immunity, i.e., innate and adaptive. Innate immunity is the first line of defense that protects organisms from infection. It is well conserved from plants to humans and eliminates infectious agents. It is activated very quickly within minutes to hours and is less specific to pathogens. Once the innate system fails or is not sufficient, the adaptive immune system sets in. Specialized cells called B and T cells mediate the adaptive immune system and provide immunological memory during subsequent exposure to the same infectious agents. Adaptive immunity mounts pathogen specific immune reactions (Alberts et al., 2002;Akira et al., 2006).

Invertebrate lacks the adaptive immunity of the vertebrate style, however, they have coevolved with microbes for millions of years. Thus they rely entirely on their innate immune system to fight pathogens. Insects like *Drosophila* eat rotten fruits. Despite eating spoiled fruits that contain huge quantities of pathogens, they stay healthy suggesting they have efficient innate immunity (Kingsolver and Hardy, 2012;Broderick, 2016). In the last few decades, significant progress has been made in *Drosophila* immunology. During this time major immune pathways including Toll and Imd were discovered and characterized (Lemaitre and Hoffmann, 2007).

Drosophila immunity and Sweden

A historical perspective: The birthplace of the *Drosophila* immunity

The pioneer in the field of innate immunity, Professor Hans G. Boman (1924-2008) initiated insect and *Drosophila* immunity research at Umeå University, Sweden (1966-1976) (Putsep and Faye, 2009;Kounatidis and Ligoxygakis, 2012). He was the first to provide evidence for an inducible defense system in *Drosophila* against bacterial infection (Boman et al., 1972). Later, it was described as humoral immunity and shown to be mediated by antimicrobial peptides (AMPs) (Faye et al., 1975;Steiner et al., 1981;Hultmark et al., 1983;Putsep and Faye, 2009;Faye and Lindberg, 2016). Since *Drosophila* was too small for biochemical research, he chose a larger insect called *Hyalophora cecropia,* a *Saturniid* moth. Later he moved

to Stockholm University (1976-1997) and purified Cecropin, an AMP from *H. Cecropia* for the first time, which opened the field of AMP-mediated humoral response (Hultmark et al., 1980;Steiner et al., 1981;Putsep and Faye, 2009;Faye and Lindberg, 2016). During his time at the Karolinska Institute (1997-2007) as an emeritus professor, he and his co-workers isolated a vertebrate AMP (LL-37) and reported a case in which a patient with a congenital disease called Kostmann Syndrome lacked IL-37. As a consequence, patients were sensitive to infection. In collaborations, it was also shown that an AMP (PR-39) was involved in wound healing (Putsep and Faye, 2009).

The Drosophila defense

Drosophila depends solely on its innate immune system. It has evolved a very sophisticated system, which can recognize pathogens, activate its defense reactions and effectively eliminate pathogens. *Drosophila* has a multilayered immune system including epithelial barriers, local induction of AMPs, reactive oxygen species (ROS) and nitric oxide (NO) induction both within the epithelia, a potent humoral response and blood cell-mediated cellular reactions. In addition, crosstalk between different immune pathways and ROS-NO interactions that lead to AMPs induction in distant organs were reported (Foley and O'Farrell, 2003;Lemaitre and Hoffmann, 2007;Wu et al., 2012). A recent review summarizes innate immunity in organs and describes a common phenomenon of AMP induction in different *Drosophila* organs. These include the central nervous system (CNS), fat body, respiratory system, digestive system and excretory system. Effector molecules, such as ROS and NO, are produced in these organs (Buchon et al., 2014). *Drosophila* displays immune reactions towards a wide range of pathogens i.e., bacteria, fungi, virus, nematodes, and parasitic wasps (Hallem et al., 2007;Krzemien et al., 2007;Buchon et al., 2014). Moreover, *Drosophila* can elicit immune reactions in aseptic situations including against tumors, clean wounds and against damage (Pastor-Pareja et al., 2008;Razzell et al., 2011).

The systemic immune response

An open question that remained for a long time was how a vertebrate protects itself from bacterial infection being that bacteria multiply very quickly (within minutes) and adaptive immunity takes days to weeks to respond. The discovery of an infection-inducible system in *Drosophila*, and subsequently identification of AMPs inspired the use of biochemistry for further characterization (Steiner et al., 1981;Putsep and Faye, 2009). Similar to moths, when bacteria are injected into the hemocoel of *Drosophila*, AMPs are induced in the fat body (major immune organ in *Drosophila*) and released into

the hemolymph to fight bacteria. This process is highly regulated and is known widely as the 'systemic' or 'humoral' immune response. This activated state of humoral immunity can last several days to protect the host from repeated infections (Lemaitre and Hoffmann, 2007).

A total of 20 AMPs are reported in *Drosophila* and can be categorized into seven groups. By nature, they are cationic and display a wide spectrum of activities against Gram-positive and Gram-negative bacteria, and fungi (Lemaitre and Hoffmann, 2007). AMPs are also induced upon viral and nematode infection but their mechanisms in this context are not as well characterized (Ip, 2005;Castillo et al., 2011;Lamiable and Imler, 2014). AMPs include Defensin, Drosomycin, Metchnikowin, Diptericin, Attacin, Drosocin and Cecropin. These AMPs are very small peptides (less than 10 kDa) with the exception of Attacin (25 kDa). Defensin acts against Gram-positive bacteria. Diptericin, Attacin, Drosocin and Cecropin fight Gram-negative bacteria, and Drosomycin and Metchnikowin are produced upon fungal infection. Once the humoral system is activated, AMPs are released from the fat body into hemolymph in varying concentrations. For instance, the estimated concentrations for Diptericin and Drosomycin in the hemolymph are 0.5 μM and 100 μM after bacterial injection, respectively. The production of AMPs is regulated by NF-kB transcription factors (Dif, Dorsal and Relish) in the fat body (Lemaitre and Hoffmann, 2007).

Immune pathways in Drosophila

Toll and Imd pathways are well characterized which are active against Lys-type containing bacteria and fungi, and DAP-type containing bacteria, respectively. Both pathways are induced via NF-kB transcription factors (Toll/Dif/Dorsal and Imd/Relish) (Lemaitre and Hoffmann, 2007). Upon viral infection, JAK/STAT and RNAi mediated signaling are activated (Ip, 2005;Lemaitre and Hoffmann, 2007;Nayak et al., 2013;Lamiable and Imler, 2014). A recent study provided evidence that JAK/STAT signaling is required in somatic muscles to activate a cellular response towards parasitic wasp infection (Yang et al., 2015). In addition, transcriptional studies demonstrated that genes were differentially regulated via Toll, Imd, JAK/STAT, JNK and IGF-beta signaling in wild type background upon nematode infection (Castillo et al., 2013). However, single and double mutants for Toll and Imd did not show increased mortality of *Drosophila* larvae upon nematode infection indicating that both pathways were dispensable (Hallem et al., 2007). It has been reported that a TGF-beta signaling component (Daw) acts as an anti-nematode factor recently (Eleftherianos et al., 2016).

The Toll pathway

The Toll/NF-kB pathway was discovered initially for its role in setting-up the dorso-ventral axis during early embryogenesis of *Drosophila*. It is an evolutionary conserved signaling pathway (Belvin and Anderson, 1996). Later it was identified as a very potent immune signaling pathway against Gram-positive (Lys-type PGN containing) bacterial and fungal infection where AMPs Defensin and Drosomycin were produced in the fat body of *Drosophila*. Mutant *toll* flies succumb to death during Gram-positive bacterial and fungal infection (Lemaitre et al., 1996;Carvalho et al., 2014). Its activation differs between vertebrates and *Drosophila* upon infection. In vertebrates, Toll-like receptors (TLR) act as pattern recognition receptor (PRR). In contrast, *Drosophila* Toll needs the extracellular cytokine Spätzle for activation (Lemaitre and Hoffmann, 2007). The components identified in this cascade are the cytokine Spätzle, the transmembrane receptor Toll, adaptor molecules Tube and MyD88, the kinase Pelle, the NF-kB transcription factors Dorsal and Dif, and the NF-kB inhibitor Cactus (Belvin and Anderson, 1996;Lemaitre and Hoffmann, 2007).

In *Drosophila*, nine Toll family proteins are encoded in the genome (Valanne et al., 2011) whereas 13 TLRs (TLR 1-13) are reported in vertebrates (Babik et al., 2015). TLR11, TLR12 and TLR13 genes were lost from the human genome (Beutler, 2009). All TLRs play a role in immunity. However, until now, apart from the involvement of Toll-1 or Toll, only Toll-5, and Toll-9 have found involved in immunity (Valanne et al., 2011). In addition, Toll-7 has been reported to mediate the autophagy mediated antiviral response in *Drosophila* (Nakamoto et al., 2012). Furthermore the Toll pathway has been found to be involved in the melanization and the activation of the cellular response (Valanne et al., 2011) and in danger situations (Ming et al., 2014).

The Imd Pathway

The Imd (Immune deficiency) pathway, like the Toll pathway, is evolutionary conserved. It is solely devoted to immune function and it induces AMPs upon Gram-negative bacterial infection. Unlike Toll, the Imd pathway has no role in development. A death domain, which is a part of the Imd protein, shares homology to Receptor Interacting Protein (RIP) of the TNF-receptor (TNFR) pathway. Thus the *Drosophila* Imd pathway is comparable to the mammalian TNFR pathway; however, it also has similarities with TLR signaling (Govind, 2008;Myllymaki et al., 2014).

imd mutant flies show increased mortality upon Gram-negative bacterial infection, however, they resist Gram-positive bacterial and fungal infections.

The well-known read-out for the Imd pathway is Diptericin activation and its induction is regulated by the NF-kB transcription factor Relish (Lemaitre and Hoffmann, 2007;Govind, 2008;Myllymaki et al., 2014). Eight components in the Imd pathway have been identified. These include PGRP-LC receptor, TAK1, TAB2, DIAP2 (inhibitor of apoptosis), IKKβ/IKKγ, dFADD (adaptor), Dredd (caspase) and Relish (NF-kB transcription factor). Upon septic injury, Toll and Imd pathways collectively regulate approximately 80% of total induced genes as a host response (Lemaitre and Hoffmann, 2007;Govind, 2008).

The JAK/STAT pathway

The first documentation of the JAK/STAT pathway was reported in mammals. It was widely found to produce cytokines and different growth factors. Four JAK and seven STAT genes are encoded in the mammalian genome, and they induce more than 30 cytokines and growth factors. JAK is a non-receptor tyrosine kinase that is coupled to cell surface cytokine receptors. Once JAK molecules are activated via trans-phosphorylation by one-another upon ligand binding and receptor dimerization, they activate cytosolic STAT protein by a separate phosphorylation event. As a result, translocation of STAT protein from the cytosol into the nucleus induces target genes (Darnell, 1997;Agaisse and Perrimon, 2004).

JAK/STAT signaling was first reported in the insect mosquito *Anopheles gambiae* where STAT protein was translocated into nucleus upon immune challenge. In the unchallenged condition, STAT protein remained in both cytoplasm and nucleus but after infection, cytoplasmic portions became diminished (Barillas-Mury et al., 1999;Agaisse and Perrimon, 2004). The JAK/STAT pathway was initially studied in flies for its involvement in developmental embryonic segmentation (Binari and Perrimon, 1994), and later for its contribution to immunity (Agaisse et al., 2003;Agaisse and Perrimon, 2004). In *Drosophila*, four components were associated with the JAK/STAT signaling i.e., Upd (ligand), dome (receptor), hop (JAK kinase) and STAT92E (STAT protein). Both humoral and cellular responses are activated by the JAK/STAT pathway (Agaisse and Perrimon, 2004). It was implicated in controlling *Drosophila* C virus (DCV) infections (Dostert et al., 2005), parasitic wasp infections (Yang et al., 2015) and in nematode infections (Castillo et al., 2013).

The JNK pathway

Like the other pathways mentioned above, the JNK (Jun-N-terminal kinase) pathway is remarkably conserved from mammals to insects. JNK is a mito-

gen-activated protein kinase (MAPK) which has diverse physio- and pathological implications such as in cell fate, morphogenesis, stress response, tumorigenesis and neurodegenerative disorders (Ramet et al., 2002;Davies and Tournier, 2012). Its activation is triggered by inflammatory cytokines, apoptosis and environmental stimuli (wounding, radiation) (Chen et al., 1996;Galko and Krasnow, 2004;Dhanasekaran and Reddy, 2008).

In *Drosophila*, the JNK kinase (JNKK) is called Hemipterous (Bakal et al., 2008). Phosphorylation of the transcription factors Jun and Fos (AP-1 complex) by JNK (Basket in *Drosophila*) produces a heterodimer of Jun/Fos. This heterodimer translocates into the nucleus and induces target genes (Kockel et al., 2001;Garver et al., 2013). JNK is negatively regulated by Puckered (Puc), a phosphatase, which dephosphorylates JNK and suppresses signaling. Thus Puc creates a negative feedback loop of the JNK pathway (Martin-Blanco et al., 1998;Garver et al., 2013).

Cross-talk and interplay between pathways

Another very intriguing question in innate immunity is of how a specific immune signal is terminated. Activated immune pathways induce proinflammatory responses and must be precisely controlled to avoid detrimental effects (Myllymaki and Ramet, 2013). There is increasing evidence for cross talk between pathways upon immune challenge. One pathway negatively regulates others and vice versa. JNK signaling triggers both tumor necrosis factor (TNF) mediated apoptosis and proinflammatory response (Deng et al., 2003;Wajant et al., 2003). It was speculated that this proinflammatory response must be regulated in a strict and timely manner. In mammals, it has been reported that activated NF-kB signaling negatively regulates pro-apoptotic JNK signaling (De Smaele et al., 2001;Kim et al., 2005). A similar observation has been reported in *Drosophila* upon lipopolysaccharide (LPS) challenge namely that Relish (NF-kB factor)-induced target genes lead to degradation of TAK1, an upstream MAP kinase kinase kinase involved in the activation of JNK signaling. As a result, JNK signaling was quickly deactivated. Inhibition of the Relish pathway prolonged induction of JNK-dependent target genes. Relish negatively regulates JNK signaling (Park et al., 2004). The opposite scenario has also been reported that JNK signaling down-regulates the NF-kB pathway. Loss-of-function of AP-1 in the JNK pathway boosted expression of NF-kB target genes and gain-of-function diminished NF-kB-dependent target genes. Thus JNK and NF-kB (Relish) pathways have reciprocal interactions (Kim et al., 2005).

Another report showed that NF-kB (Relish) signaling was inhibited by dual input from both JNK and JAK/STAT pathways (AP-1 and STAT complex). The authors provided evidence that the JAK/STAT pathway down regulates

Relish-dependent target genes when bound in a complex containing STAT, AP-1 and Dsp1, a High Mobility Group (HMG) protein. This complex binds to the promoter region that replaces Relish and recruits histone deacetylase (HDAC) which in turn shuts down transcription (Kim et al., 2007).

Genetic analysis provides strong evidences that the Toll and Imd pathways become activated independently upon challenge. However, in some situations when both Toll and Imd pathways show a lower level of induction, they work synergistically. This observation could be useful in the explanation of multiple microbes infecting a host in nature. For instance, during epidermal tissue injury, different microbes could infect *Drosophila* (Tanji et al., 2007). Therefore, the host may protect itself better if there is interplay between the pathways.

Pathogen recognition and activation of immune pathways

Prior to immune signaling, the very first step is to recognize the pathogen in host. Pathogen recognition is achieved via pattern recognition proteins (PRRs) from the host. The *Drosophila* genome encodes several PRR proteins that distinctly activate the Toll and Imd pathway in the fat body through directly binding to microbial ligands or via secretion. In addition, PRRs also activate the proPO (prophenoloxidase) cascade. Two families of PRRs are identified in *Drosophila* called peptidoglycan recognition proteins (PGRPs) and Gram-negative binding proteins (GNBPs). They act upstream of the Toll and Imd pathway. One important distinction between Toll and Imd pathway activation in the fat body is that Toll activation requires secreted PRR whereas Imd is activated by a membrane-coupled PRR (Lee et al., 1996;Kang et al., 1998).

Pathogen associated molecular patterns (PAMP) are pathogen signatures to which PRR proteins specifically bind and elicit immune signaling. Bacterial recognition begins by detecting bacterial cell wall components called peptidoglycans (PGN). PGN is a glucopeptidic polymer localized in the cell wall of both Gram-positive and Gram-negative bacteria. However, its structure from Gram-positive to Gram-negative bacteria differs by a single amino acid residue. Lysine is replaced with meso-diaminopimelic acid (DAP) at the third position of the peptide chain in Gram-negative bacteria. A group of Gram-positive bacteria such as *Bacillus* also possess DAP-type PGN. Therefore, in most cases, Gram-positive and Gram-negative bacteria are recognized via Lys-type PGN and DAP-type PGN, respectively. Biochemical studies showed that purified Lys- and DAP-type PGN activated the Toll and Imd pathways, respectively. Another distinction between Gram-positive and

Gram-negative bacteria is that Gram-negative bacteria contain a single layer of PGN, which is restricted to the periplasmic space. Lipopolysaccharides (LPS) are located outside the PGN layer in Gram-negative bacteria. However, Gram-positive bacteria possess multiple layers of PGN and display them at their surface. LPS is not present in Gram-positive bacteria (Leulier et al., 2003;Lemaitre and Hoffmann, 2007).

Apart from their evolutionary conservation species ranging from insects to mammals, PGRPs also show similarity to bacteriophage T7 lysozyme, a zinc dependent N-acetylmuramoyl-L-alanine amidase in their PGRP domain (160 amino acid long domain) (Werner et al., 2000). Some members of PGRPs (PGRP-SA, PGRP-SD, PGRP-LA, PGRP-LC, PGRP-LD, PRGP-LE and PRGP-LF) are devoid of zinc-binding residues; hence they do not have zinc dependent amidase activity. However, they still bind to PGN and act as PRR. On the other hand, some members of PGRPs (PGRP-SC1, PRGP-LB and PGRP-SB1) have retained their catalytic activity and are called catalytic PGRPs. Although the mode of action of the PGRPs is different depending on whether they contain an active domain or not and the type of microbes they act upon, all members of the family cooperate to minimize the effects of the microbes (Mellroth et al., 2003).

Bacterial PAMP Lys-type PGN or the fungal PAMP glucan activate Toll signaling whereas Imd activation relies on DAP-type peptidoglycans. During Toll activation, recognition molecules (PGRP-SA, PGRP-SD and GNBP1 for Gram-positive bacteria, and GNBP3 for fungi) proteolytically activate an enzyme called Spätzle Processing Enzyme (SPE) for Gram-positive bacteria and a protein called Persephone for fungi. SPE then cleaves the extracellular cytokine Pro-Spätzle into mature Spätzle in the hemolymph, which then binds as a dimer to Toll at the plasma membrane of the fat body cells. Three intra-cellular death domains (DD) containing proteins called dMyD88, Pelle and Tube are recruited to the plasma membrane where Toll associates with the cleaved Spätzle dimers (Weber et al., 2003). A kinase migrates to the downstream complex where it phosphorylates Cactus, which is subsequently removed from the complex and degraded. This causes release of the NF-kB transcription factors Dorsal and Dif, which translocate into the nucleus leading to transcription of the AMP gene Drosomycin in addition to other target genes (Ip et al., 1993;Lemaitre et al., 1995).

In order to activate the Imd pathway in the fat body, the bacterial cell wall component (mono or polymeric DAP type PGN) requires binding to PGRP-LC (membrane-coupled PRR molecule), which in turn, attracts the adaptor molecule Imd (Kaneko et al., 2006). This causes the recruitment of the interactor molecule dFADD and the caspase Dredd (Hu and Yang, 2000). The NF-kB transcription factor Relish is thought to be associated with the caspase Dredd. Once Relish is phosphorylated by the IKK signaling complex, it

causes dissociation of Relish and subsequently Dredd. Phosphorylated Relish translocates into the nucleus leading to the transcription of the AMP gene Diptericin and additional target genes (Hu and Yang, 2000;Stoven et al., 2000).

The Drosophila hematopoietic system as a cellular immunity

Cellular immunity has been a topic for research in invertebrate models for more than a century. Starting in the 1950s, Rizki and Rizki provided insight into *Drosophila* blood cell types, their function and the process of self- and non-self recognition (Rizki, 1956;Rizki and Rizki, 1980b;Evans et al., 2014). *Drosophila* blood cells (hemocytes) devoted to cellular immunity are comprised of two populations, one in the blood (hemolymph) as free-floating cells (termed circulating hemocytes) and another, which attaches to subepidermal tissues called sessile hemocytes. Both populations are comprised of three cell types: plasmatocytes, crystal cells, and lamellocytes but the latter rarely exist in healthy larvae and adults. Lamellocytes appear as large flat cells and are only produced in certain situations, such as parasitoid wasp infestation and during wounding (Lemaitre and Hoffmann, 2007;Markus et al., 2009;Letourneau et al., 2016). They encapsulate large foreign invaders such as parasitic wasp eggs, which are too big to be phagocytosed. In normal wild type larvae, several thousand hemocytes exist. Among these hemocytes, plasmatocytes and crystal cells occupy approximately 95% and 5%, respectively. Plasmatocytes act as phagocytes and remove dead cells and invading microbes. Mature crystal cells contain pro-phenoloxidase and participate in the melanization process by releasing the enzyme (Lanot et al., 2001; Lemaitre and Hoffmann, 2007).

Unlike in vertebrates, *Drosophila* blood cells do not transport oxygen to its tissues; rather, it relies extensively on its tracheal system. *Drosophila* like all insects has an open circulatory system, which bathes tissues and organs. Some circulation is achieved through a contractile ‘heart tube’ structure (dorsal vessel) (Babcock et al., 2008;Gold and Bruckner, 2015).

Hematopoiesis in Drosophila

Drosophila hemocytes resemble the myeloid lineage of the vertebrate system by their functional properties both in development and innate immunity (Hartenstein and Mandal, 2006). Moreover, many striking similarities both at the cellular and molecular level have linked the mammalian innate immune system to hemocytes in the last two decades (Letourneau et al., 2016).

Until very recently, *Drosophila* hematopoiesis was known to occur only in two developmental stages namely the embryo and larval stages. However, a recent study provided evidence that active hematopoietic sites referred to as a "hematopoietic hub", also exist in the abdomen of adults and contribute new blood cells when faced with immune challenge (Ghosh et al., 2015).

The first wave of hematopoiesis begins in the head mesoderm in early embryogenesis. Several very important tasks are carried out by these hemocytes. For instance, hemocytes secrete extracellular matrix (ECM) components and engulf apoptotic corpses to shape various tissues (Fessler and Fessler, 1989). Hemocytes are rich sources for the ECM molecules such as Collagen IV molecules, Laminin A, Glutactin, Tiggrin, Papillin and Peroxidasin. Their recruitments to the central nervous system (CNS) and the gut are crucial for proper morphogenesis for the respective tissues; hence impairment of hemocyte function has fatal consequences in embryogenesis (Brown, 1994;Yarnitzky and Volk, 1995;Sears et al., 2003).

The second wave of hematopoiesis starts once the first paired lobes of the lymph gland are developed during late embryogenesis. The organ size increases at subsequent larval stages by cell proliferation. The lymph gland is considered the major hematopoietic organ for larval stages (Holz et al., 2003;Wood and Jacinto, 2007). Constitutive differentiation of plasmatocytes and crystal cells from precursor cells takes place in the lymph gland. However, in normal conditions, these differentiated cells are not released until metamorphosis (Anderl and Hultmark, 2015;Letourneau et al., 2016). Lamellocytes also differentiate from precursor cells in the lymph gland upon parasitic wasp infection (Anderl and Hultmark, 2015). Only recently, insights have been made on how the plasmatocyte and crystal cell numbers increase in hemolymph circulation and in sessile pockets as lymph glands do not release hemocytes until metamorphosis. They provided evidence that plasmatocytes can differentiate in both circulation and in the sessile clusters (Makhijani et al., 2011;Grigorian and Hartenstein, 2013). Plasmatocytes have self-renewal properties for instance, from 300 plasmatocytes in 1st instar larva to almost 10000 plasmatocytes in late 3rd instar which is 30 times more from its initial larval instar stage. This augmentation suggests a high capacity for self-renewal (Gold and Bruckner, 2015;Sopko et al., 2015). However, currently there is no evidence that crystal cells self-renew (Leitao and Sucena, 2015). Moreover, in the same study Leitao et al. showed that plasmatocytes could transdifferentiate into crystal cells via notch signaling in sessile pockets. Furthermore, the sessile cluster is crucial for this transdifferentiation (Leitao and Sucena, 2015). Beyond differentiation in lymph glands, lamellocytes can be generated in the sessile compartments via mature plasmatocyte trans-differentiation. Lamellocyte generation usually occurs under wasp infection or genetic manipulation (Markus et al., 2009;Honti et al., 2010;Letourneau et al., 2016).

Until very recent, the notion in *Drosophila* hematopoiesis was that embryonic and larval hemocytes were long-lived, and thus persisted to the adult stage. Further, adult plasmatocytes were thought to lack the ability to proliferate whereas they did in larval stages (Makhijani et al., 2011;Honti et al., 2014). Researchers began to challenge this notion that adult flies relied entirely on its earlier-stages hemocyte populations even upon immune defense. The adult stage is much longer than the earlier ones. A careful investigation led to the identification of four active hematopoietic hubs at the dorsal side of the adult fly abdomen. These hubs are webs of Laminin A and Pericardin which house both embryonic and larval hemocyte populations, and precursor cells that give rise to new blood cells (plasmatocytes and crystal cells) *de novo*. Emergence of these new hemocytes depends on Notch signaling and provides an immune defense when challenged. These new hubs are postulated as a simpler version of the vertebrate bone marrow (Ghosh et al., 2015).

Several transcription factors have been shown to contribute to the specification of different hemocyte subtypes in *Drosophila*. These include Serpent, U-shaped, Lozenge and Collier. The GATA transcription factor Serpent has a function during embryonic hemocyte primordium specification in the head mesoderm and maturation in subsequent stages. Similarly, U-shaped and Lozenge have mouse homologues as FOG1 and RUNX1, respectively and both contribute to crystal cell differentiation. Collier has the mouse counterpart EBF1 and functions in lamellocyte differentiation (Tepass et al., 1994;Lebestky et al., 2000;Wood and Jacinto, 2007).

As mentioned above, the lymph gland is a specialized organ for larval hematopoiesis. A single pair of lobes constitutes the embryonic lymph gland positioned along the dorsal vessel and consists of 20 cells in each lobe (Holz et al., 2003;Mandal et al., 2004;Letourneau et al., 2016). In larval stages, additional pairs of lobes emerge in the posterior part of the embryonic lobes (anterior pair) called posterior lobes, and pericardial cells separate each of the posterior pairs (Lanot et al., 2001;Jung et al., 2005). The anterior pair (also known as primary lobe pair) are bigger in size compared to its posterior pairs and contain three regions that are mentioned below (Jung et al., 2005). Progenitor cells remain in the inner side in the medullary zone (MZ). Outside of the MZ, a less characterized intermediate progenitor cell populations exist which is devoid of both progenitor and mature cell markers. The outer or cortical zone (CZ) contains differentiated mature cells that originate from progenitor cells. The 3rd region called posterior signaling center (PSC) is situated in the posterior part of each primary lobe and consists of 30 cells. Its task is to maintain prohemocytes (progenitor cells) in the medullary zone (Sinenko et al., 2009;Krzemien et al., 2010;Tokusumi et al., 2011). The lymph gland disintegrates 8-10 h after pupa formation and releases all hemocytes into circulation (Grigorian et al., 2011).

Phagocytosis, opsonization and encapsulation

One of the earliest immune events was characterized in detail by the Russian zoologist Iyla Ilyich Metchnikov in 1882 in starfish larvae (Prize, 1908). He termed this phenomenon "phagocytosis" which derived from Greek and meant 'cell lysis.' Later he found this event in macrophages living in vertebrates (Kaufmann, 2008). As a consequence, the field of cellular immunity was established (Vikhanski, 2016). Like vertebrate macrophages, *Drosophila* plasmatocytes possess similar properties including the ability to clear dead cells, to generate an extracellular matrix, and to engulf foreign particles. Moreover, they participate in other circumstances, such as tissue injury and tumorous conditions (Gold and Bruckner, 2015). In *Drosophila*, plasmatocytes carry out very crucial functions during development and in innate immunity. Phagocytosis is a process by which cells engulf foreign particles or cell debris inside the host. In larval stages of *Drosophila*, plasmatocytes have immune surveillance function mostly to recognize and eliminate pathogens (Gold and Bruckner, 2015). Later in pupal stages, plasmatocytes have major roles in consuming larval-lysed tissues and in remodeling adult tissue structures. Ecdysone signaling which activates hemocytes to mobilize and perform their functions, is high at the onset of metaphorphosis (Regan et al., 2013).

In *Drosophila*, phagocytosis involves several steps. First, plasmatocytes migrate and then adhere to the imminent particles that are phagocytosed. Next, the cell remodels its cytoskeleton and engulfs particles, which are finally degraded with the help of phagosomes. Within minutes, plasmatocytes can engulf a wide range of particles such as bacteria, yeast and double-stranded RNA (dsRNA) (Lemaitre and Hoffmann, 2007). The proteins involved in this process are different receptor molecules like scavenger receptor family proteins (dSR-CI), the EGF-domain protein Eater and the IgSF-domain protein Dscam. In addition, CD36-like proteins and PGRP family members were suggested to be associated with this process (Pearson et al., 1995;Ramet et al., 2001). Subsequent studies showed that Eater (a transmembrane protein required for phagocytosis) was highly enriched in plasmatocytes and pro-hemocytes (hemocyte progenitor cells). It has been found to bind a wide spectrum of bacteria and aids in engulfing bacteria successfully. Phagocytosis has been found to be severely reduced for both Gram-positive and Gram-negative bacteria in Eater-deficient flies (Ju et al., 2006).

In mammals, complement proteins (C3) label microbial surfaces to enhance phagocytosis in a process called opsonizations. There are six proteins in *Drosophila,* which belong to C3/α2 macroglobulin superfamily. They are glycoproteins, and they contain thioester motifs that attach to microbial surfaces by covalent bonds. They are named thioester containing proteins or TEPs (TEP1-6) (Cherry and Silverman, 2006;Govind, 2008). All TEPs con-

tain signal peptides, this suggests they are secreted. It has been shown that TEP1, TEP2 and TEP4 are induced by immune challenge (Lagueux et al., 2000;Lemaitre and Hoffmann, 2007). In the mosquito *Anopheles gambiae,* TEP1 has been reported to help kill the parasite *Plasmodium* (Blandin et al., 2004). In *in vitro* genetic screens (S2 cell culture), TEPs were found to aid in phagocytosis of different pathogens. For instance, TEP2 and TEP3 were required to effectively phagocytose *E. coli* and *S. aureus* respectively. In addition, in the same study, Mcr (Macroglobulin complement related) protein was identified and was found to have similarities with the four TEPs. Mcr bound to the surface of the fungus, *C. albicans* which aided in phagocytosis (Stroschein-Stevenson et al., 2006).

When phagocytosis is not sufficient enough to engulf foreign intruders in the hemolymph, another important cellular reaction occurs. While plasmatocytes patrol the hemolymph, they activate upon detection of an invader and send signals to the lymph gland to produce lamellocytes (Russo et al., 1996). Later, mature lamellocytes develop a multilayer capsule around the invader and kill it by locally activating cytotoxic ROS and melanization. A protein identified in *Drosophila* to be involved in the melanization is Myospheroid, a member of the integrin family, which is generally required for adherence. Mutants in *myospheroid* showed reduced encapsulation efficiency (Irving et al., 2005). The encapsulation reactions can also be initiated by a damaged basement membrane (Rizki and Rizki, 1980a).

The Coagulation system

The clotting system in invertebrate models has been well studied in horseshoe crabs and in crayfish. Research on horseshoe crabs dates back to 1885, when William H. Howell at Johns Hopkins University used them scientifically for experiments in hemolymph coagulation. He concluded that similar events allowed clotting in mammals and in horseshoe crabs alike, and that blood cells rapidly produces clots (Kawabata and Muta, 2010;Cerenius and Soderhall, 2011). Two very important discoveries led to further work on the underlying mechanisms (low concentrations of LPS induce clotting, and serine proteinase cascades mediate the conversion of soluble clotting factors into gel-like structure) (Cerenius and Soderhall, 2011). In recent years, *Drosophila* has started to be used as a model organism, due to its excellent genetics to study hemolymph coagulation. For example, the enzyme Transglutaminase has been found to play a key role during clotting in *Drosophila* (Wang et al., 2010).

Physical damage to the insect cuticle leads to hemolymph loss and creates a potential entry route for pathogens, both which may lead to fly death. However, coagulation cascades and a prompt response to restrict both hemo-

lymph loss and pathogen entry into insects are important defense reactions of insects. Moreover, coagulation immobilizes and eventually kills the microbes at the physical injury site (Wang et al., 2010). In *Drosophila*, both hemocytes and fat body-derived proteins in the hemolymph contribute to coagulation. Upon injury, the hemocyte-specific protein Hemolectin provides an initial major clotting fiber component and successive cross-linking of the protein Fondue by the enzyme Transglutaminase which produces a soft clot. Later proPO, which is released by the crystal cells, activates the melanization cascade leading to hardening of the soft clot. However, the initial coagulation process is independent of melanization as the former occurs in proPO mutants (Goto et al., 2003;Scherfer et al., 2004).

The process of melanization, which involves the deposition of melanin and causes a blackening reaction, is another facet of immune defenses in insects. Melanization is an immediate reaction and occurs at the end of both encapsulation and coagulation reactions and is speculated to kill the microorganisms by producing toxic intermediates (Nappi and Vass, 1993;Lemaitre and Hoffmann, 2007). The melanization reaction is initiated through the cleavage of the inactive enzyme proPO into the active form, PO by a serine protease called prophenoloxidase-activating enzyme (PPAE). Active PO catalyzes the oxidation of mono- and diphenols to orthoquinones, and then non-enzymatic polymerization produces melanin. The cascade is initially amplified by physical injury or by binding to microbial elicitors such as PGN, β (1, 3) glucan, and LPS by recognition molecules (PRRs) based on studies in other insects (Ma and Kanost, 2000). There are three proPO-coding genes in *Drosophila*; PPO1, PPO2 and PPO3. PPO1 and PPO2 are expressed in crystal cells and PPO3 in lamellocytes (Dudzic et al., 2015). Crystal cell and lamellocyte-mediated melanization reactions occur at the injury site and during encapsulation respectively (Irving et al., 2005).

Gut microbiota and host response

Most organisms harbor non-pathogenic commensal microbes in their guts shortly after birth (Wong et al., 2016). From then until death, the gut microbiota has diverse functions, from regulating host physiology to immunological tasks (Smith et al., 2007). An imbalance of microbial communities (dysbiosis) can lead to a wide range of diseases. For instance, dysbiosis contributes to the development of chronic metabolic diseases such as diabetes and obesity (Wong et al., 2016) and in worse scenarios, it causes severe pathological states including colorectal cancer (Yang and Jobin, 2014). Due to the complexity of the mammalian system, simpler model systems have been used for understanding host-microbes interactions at the molecular and genetic level. The *Drosophila* model has become an apt choice due to its

genetic tools, simplicity, and functional resemblance to the mammalian intestine (Wong et al., 2016).

A striking difference between fly and human microbiota is that wild type flies harbor only 5-30 bacterial taxa (Wong et al., 2011;Broderick and Lemaitre, 2012) whereas more than 500 taxa are found in the human intestine flora (Consortium, 2012;Wong et al., 2016). The *Drosophila* gut is partly micro-aerobic and spatially variable due to different oxygen tensions. Two genera are predominant in the fly gut such as *Acetobacter* and *Lactobacillus*. *Acetobacter* relies on molecular oxygen for colonization but *Lactobacillu*s is sensitive to a fully oxic environment. This suggests that the fly gut may harbor an inhospitable and heterogeneous environment thus allowing only a few species to grow (Wong et al., 2011;Staubach et al., 2013;Matos and Leulier, 2014). A recent report showed that, compared to wild type an increased number of microbes and a different taxonomic composition of the gut was found when the POU transcription factor Pdm1/nubbin was mutated. Mutant animals have a reduced life span (Dantoft et al., 2016). Another elegant study from Heinrich Jasper's lab provided evidence that the stomach, like the copper cell region (CCR) regulates distribution and composition of the gut microbiota. In aging guts, chronic activation of JAK/STAT signaling caused dysbiosis and epithelial dysplasia in the gut intestine (Li et al., 2016). Several other studies suggest that the gut microbiota contributes to host mortality in a pathological situation (Charroux and Royet, 2009;Defaye et al., 2009). On the contrary, beneficial effects have been observed in the administration of *Lactobacillus plantarum* to the *Drosophila* gut, which promote host development by activating intestinal proteases (Erkosar et al., 2015).

Foods and immunity

Food, the gut microbiota, metabolism and immunity are all intertwined with the host's physiological processes. Here the gut provides a platform to connect all of these (Hooper and Macpherson, 2010;Lee and Brey, 2013;Broderick, 2016). In *Drosophila*, foods are a rich source for microbes because flies feed on rotten fruits. Joint efforts from the commensal flora and innate immunity are efficient to eliminate pathogenic microbes and maintain the physiological function of the microbiota (Bonnay et al., 2013;Broderick, 2016). Removal of harmful microbes from the gut is mediated mostly by locally producing reactive oxygen species and AMPs (Bonnay et al., 2013).

'Nutritional immunology' is a field in which researchers are keen to understand how diet interferes with immunity. It's not a 'black and white' matter since a phenotype varies depending on the genotype and the environmental interactions (GxE) (Ponton et al., 2011;Unckless et al., 2015). It was reported that increased glucose levels correlated with a higher pathogen burden.

The immunological status was altered too but varied within sampled populations suggesting that GxE interactions contribute to this variation. In the same study it was also hypothesized that each metabolic profile might have an association with immunity. Nutritional indices (NI) demonstrate overall nutritional status of an individual or the assessment of nutritional variables such as free glucose, glycogen, total triglycerides, free glycerol, soluble protein and wet mass. When NI were measured, it was found that free glucose level correlated with high pathogen load (Unckless et al., 2015). In another study, caterpillars, when reared on 20 different diets were shown to display different immune traits depending on macronutrient (protein and carbohydrates) uptake (Cotter et al., 2011;Ponton et al., 2011). The gut microbial composition is affected by diet being that specific food ingredients may support certain microbes. For instance, flies reared on food supplemented with casein shifted the gut flora towards more *Lactobacillus* species (Galenza et al., 2016).

Nutrient responsive ERK signaling is activated by insulin in mosquito guts after blood meals (Surachetpong et al., 2009). However, the role of ERK signaling in the gut remains unclear. Transcriptome profiling of insects upon oral infection with viral pathogens differentially affects immune signaling pathways like Toll, JAK/STAT and JNK (Souza-Neto et al., 2009;Ramirez and Dimopoulos, 2010;Xu et al., 2013). Thus, the question remains if there is a direct role for these pathways that restrict viral pathogens. An *in vitro* study in Aag2 cells from *Aedes* mosquitoes showed that down-regulation of ERK signaling made Aag2 cells sensitive to vesicular stomatitis virus (VSV) and Sindbis virus (SINV) infection. This study was confirmed by an *in vivo* set up in *Drosophila*. Flies fed with insulin restricted viral infection in the gut, which supported the concept that blood-meal-driven ERK activation promotes *Plasmodium* replication in the mosquito gut. Inhibition of viral propagation in the mosquito midgut via ERK activated by blood meals demonstrates that nutrients are connected with host immunological status (Xu et al., 2013).

Nitric oxide signaling in host defense

Nitric oxide (NO) has emerged as a crucial determinant for many physiological processes ranging from expansion of blood vessels to neurological functions in mammals. Its involvement in immune signaling is reported to provide host defense (Bredt and Snyder, 1994;Kuzin et al., 1996;Foley and O'Farrell, 2003). NO is a short-lived, highly soluble second messenger that freely travels within and to nearby cells. Diffusion of NO can be as long as 100μm (Lancaster, 1997;Iwakiri et al., 2006). The intracellular function of NO is comprised of effects on protein trafficking via targeted S-nitrosylation (Iwakiri et al., 2006); in this way it can interfere with immunity too. For

example, NO is reported to suppress the inhibitory kappaB kinase (IKK) via S-nitrosylation in lung epithelia and in Jurkat T cells. On the other hand, activation of IKK phosphorylates IkB, which then dissociates IkB from the NF-kB transcription factor in the cytoplasm. As a result, NF-kB translocates into the nucleus and induces AMPs, and thus NO contributes to host immunity (Reynaert et al., 2004;Davies and Dow, 2009).

Nitric oxide synthase (NOS) utilizes the substrate arginine to produce NO in almost every cell type (Kuzin et al., 1996). There are three NOS genes that can produce many isoforms in mammals (Knowles and Moncada, 1994;Kuzin et al., 1996). In *Drosophila*, only a single NOS (dNOS) gene is encoded in the genome, which can generate several transcripts by alternative splicing (Regulski and Tully, 1995;Stasiv et al., 2001).

NO was reported to be involved in the defense against Gram-negative bacterial infections in *Drosophila*. Further, it was found to affect Imd/Rel signaling. Earlier work in mammals has shown that one of the NOS isoforms, inducible NOS (iNOS) is elevated in macrophages upon immune challenge or treatment with lipopolysaccharide (LPS). To check whether NOS was induced in hemocytes too, larvae were fed with the Ecc pathogen. An elevated expression of NOS was detected in hemocytes (from infected larvae) by a NOS specific antibody (universal anti-NOS) compared to hemocytes from uninfected controls.

In *Drosophila domino* mutants, in which hemocytes are missing, Dipt induction was blocked in the fat body suggesting a role for hemocytes upon infection, while Drs remained unchanged. Interestingly in uninfected larval fat body of *domino* mutants, the NO donor SNAP induces Drs induction but does not induce Dipt. This indicates a requirement of hemocytes for Dipt induction in the fat body (Foley and O'Farrell, 2003). On the other hand, it was shown that there is an interplay between NO and ROS, and both NO and hemocytes acted together to induce Dipt in the fat body (Wu et al., 2012). For example, ROS production in the larval gut either by feeding the pathogen orally, chemically or genetically triggers Relish dependent global AMPs production in the fat body in a wild type background. Larvae fed with H_2O_2 trigger ROS generation in the gut. Endogenous Dipt and Dipt-LacZ reporter expression were blocked in the fat body when both H_2O_2 and L-NAME (NOS inhibitor) were co-administered orally. However, this co-administration did not block Drs and Drs-reporter expression. This observation suggests a role for NO in Dipt induction in the fat body. Similarly, in a *l(3)hem* mutant where hematopoiesis is blocked, Dipt induction in the fat body by ROS stimulation is diminished implying a role for hemocytes in relaying signal from gut to fat body (Wu et al., 2012). Moreover, hemocytes are known to relay signals from the gut to the fat body (organ to organ communication) (Foley and O'Farrell, 2003). A recent report on different labora-

tory *Drosophila* strains showed NOS mutant strains generated in different genetic backgrounds were more sensitive to bacterial infection. *Drosophila* sensitivity to bacterial infection supports NO's role in host immunity (Eleftherianos et al., 2014).

An anti-parasitic role for NO through direct killing has also been reported in other insects (Rivero, 2006). NOS was induced in the midgut of *Anopheles mosquitoes* and *Glossina morsitans,* the Tsetse fly, upon *Plasmodium* and *Trypanosome* infections respectively suggesting NO plays a role in restricting parasite colonization (Luckhart and Li, 2001;Hao et al., 2003). A role for NO in controlling intracellular parasite *Leishmania* and *Toxoplasma gondii* infestation has also been reported (Silva et al., 2009;Wink et al., 2011).

Nematode infection in Drosophila: a natural infection system

Parasitic nematodes infect a wide range of hosts such as humans, domestic animals and plants. Almost half of the human population is infected by nematodes worldwide, causing diseases leading to morbidity and in some cases to death. Nematode infections are asymptomatic in many cases, thus preventative measures are scarce. Despite their role in developing diseases, nematode infections have not been studied as widely as many other pathogens (Krecek and Waller, 2006;Nicol et al., 2007;Bockarie et al., 2009;Castillo et al., 2011).

Much of our knowledge of *Drosophila* immunity results from studies of bacteria and fungal infections (Ferrandon et al., 2007). However, bacterial infection by injecting causes wounds in *Drosophila*. Epithelial wounds require tissue remodeling and other factors to seal and heal the wound, and these induce immune responses (Theopold et al., 2004;Lesch et al., 2010). Conversely, feeding the fly bacteria is often times not sufficient enough to breach multiple host immune layers. For instance, feeding bacteria *Photorhabdus luminescence* alone (without its carrier *Heterorhabditis bacteriophora*) does not breach the epithelial layer of the *Drosophila melanogaster* gut. Of note, *Heterorhabditis bacteriophora* is an entomopathogenic nematode and it contains its symbiont *Photorhabdus luminescence*. Moreover, the *Drosophila* gut efficiently clears the bacteria and subsequently, survives as non-infected wild type *Drosophila* (Hauling et al., 2014). However, the same bacterium causes septicemia to wild type larvae upon infecting the host with the nematode containing its symbionts. Incubating *Drosophila* larvae with nematodes is sufficient to infect the fruit fly larvae, thus nematodes are used as a natural infection system (Hallem et al., 2007;Dobes et al., 2012).

Sensing and invasion strategy of the nematodes

In order to enter into the insect host, entomopathogenic nematodes (EPNs) utilize their dorsal tooth or "hook" to burrow both the cuticle and the epithelial layer to reach the hemocoel (if they first use host's body openings e. g., the mouth, anus and posterior spiracles it lets them end up temporarily in the lumen of the respective tubular organs such as the gut and the trachea). Once nematodes have access to the hemocoel, they regurgitate their symbiotic bacteria to cause septicemia and kill the host by generating various toxins. Nematodes then feed on both bacterial biomass and digested tissues of the host, and reinitiate their developmental program in order to become adults. They replicate 2-3 times inside the host producing approximately 100,000 nematodes from a single insect host (Ciche, 2007;Castillo et al., 2011;Omkar, 2016). EPN *Heterorhabditis bacteriophora* has four larval stages (juvenile larval stages 1-4) before becoming an adult. Infective juvenile (IJ) stage 2 (J2) is able to survive without a host and does not require food, hence it is known as the non-feeding stage (Ciche, 2007).

Successful parasitism is essential for the nematode's survival in nature. Thus detection of hosts is imperative. It was reported that nematodes sense dead, already infected and live healthy insect hosts using various cues (Griffin, 2012). For hosts already infected by the same species of nematodes, second or subsequent entrance into the host varies depending on the level of nematode crowding (Lewis et al., 2006;Griffin, 2012) and the time since first occupation. Later entrance into the same infected host was either inhibited or declined for *Steinernema spp.* (Glazer, 1997;Griffin, 2012). Two paradigms have been described for foraging strategies of EPNs and they differ between species. For example, nearly all *Heterorhabditis spp.* and *Steinernema carpocapsae* display cruiser and ambusher strategies to infect insect hosts respectively. However, *Steinernema feltiae* is thought to employ an intermediate foraging strategy. Since cruising behavior requires active efforts it detects both immobile and mobile hosts (Ciche, 2007;Griffin, 2012). In contrast, ambusher strategy is less suited for finding immobile hosts (Gaugler et al., 1997) because ambusher nematodes are less active and less mobile. The cruiser strategy utilizes carbon dioxide (CO_2) and other volatile cues to search for hosts whereas the ambusher strategy involves body lifting to reach passing insects (Ciche, 2007;Griffin, 2012). However, *Steinernema carpocapsae* that employ the ambusher strategy have also been reported to respond to CO_2 emissions from viable hosts (Hallem et al., 2011).

Several strategies employed by nematodes are thought to provide an escape from the host's immune responses. These include modulation of both the cellular and humoral immune response in *Drosophila* by interference and/or disruption (Castillo et al., 2011). Encapsulation in *Drosophila* is a successful cellular response against big particles such as parasitic wasp eggs (Lanot et

al., 2001). A similar cellular defense (encapsulation) is found in the mosquito *Anopheles quadrimaculatus* when it is infected by the microfilariae *Brugia pahangi* (filarial nematode). However, the encapsulation reaction is not induced in *Drosophila* during EPN infection. The mechanism for suppressing encapsulation is not known. In addition, the symbiotic *Photorhabdus luminescence* of the EPNs has anti-phagocytic activity that prevents the *Drosophila* hemocytes from engulfing them (Castillo et al., 2011).

Several toxins of *Photorhabdus luminescence* have been shown to interfere with phagocytosis and induce apoptosis. For instance, toxins TccC3 and TccC5 affect ADP-ribosylation (a process which involves adding one or more ADP-ribose moieties to a protein) of actin, and Rho GTPases (RhoA and Rac), respectively. Since actin, RhoA and Rac are involved in cell cytoskeleton organization, interfering with ADP-ribosylation alters the cytoskeleton and blocks phagocytosis (Eleftherianos et al., 2010;Lang et al., 2010). Another toxin called "Makes Caterpillar Floppy 1" (Mcf1) causes destruction of the hemocytes and the gut by inducing apoptosis (Daborn et al., 2002).

Immune responses towards nematodes

From a mammalian perspective, helminth infections have immunomodulatory features, which allow helminthes to persist in the host. This has both advantages and disadvantages. For instance, helminth infection suppresses the immune system and subsequently some uncontrolled immune reactions are prohibited, like autoimmune, allergic and inflammatory responses. Thus this is beneficial for the host. However, most nematode infections are a huge burden for human health. They may render the host less sensitive to vaccines, more susceptible to coinfection and at a higher risk for tumor progression due to less immunosurveillance (Hotez et al., 2008;Maizels and McSorley, 2016). Chronic malnutrition and morbidity are associated with helminth infections. Asymptomatic or subclinical chronic infections are also common, therefore, treating the infections or preventative measures are difficult (Hall et al., 2009;Moreau and Chauvin, 2010). Innate immune cells like eosinophils, basophils, mast cells and innate lymphoid cells are involved during helminth infections in humans and mice. Recently it has been shown that neutrophil assisted macrophage development was involved in clearing helminth from mice (Chen et al., 2014). In addition, type 2 immunity sets in to reduce parasite numbers or kill them directly. Type 2 helper T (T_H2) cells mediate induction of cytokines IL-4, IL-5, IL-9 and IL-13 which are released to restrict parasite burden (Allen and Sutherland, 2014;Chen et al., 2014).

Invertebrate *Drosophila* mounts immune reactions against *Heterorhabditis* nematodes by producing AMPs. Further characterization demonstrated that

Heterorhabditis's symbiotic bacteria, *Photorhabdus* elicited AMPs production when administered separately. In addition, axenic *Heterorhabditis* (devoid of symbionts) alone failed to induce AMPs. Major immune pathways like Toll and Imd were found dispensable for controlling nematode infections since single or double mutants died at the same rate as wild type control. This study was conducted in larvae (Hallem et al., 2007). A somewhat contrasting result was observed in adult flies upon nematode infection. There it was shown that axenic and symbiotic *Heterorhabditis* could induce AMPs but not by their symbiont *Photorhabdus, which* might argue for distinct mechanisms in larvae and adults (Castillo et al., 2013). An RNA-Seq study was performed recently in adult flies and showed involvement of immune pathways such as Toll, Imd, JAK/STAT, and TNF upon nematode infection with/without symbiont. In addition, transcription was affected for a big group of genes engaged in translation suppression upon *Photorhabdus* infection (Castillo et al., 2015). Moreover, TGF-β signaling was found to have an anti-nematode role in *Drosophila* (Eleftherianos et al., 2016). Several clotting components such as Fondue, Transglutaminase, and eicosanoids were identified as anti-nematode factors in fly larvae. Inhibition of these clotting components left *Drosophila* larvae sensitive to nematodes infection (Wang et al., 2010;Hyrsl et al., 2011).

Immune responses towards nematodes have not been characterized in great detail in other insects but in a broad sense, like at humoral and cellular level. In addition, hemolymph coagulation was found to be associated with different insect hosts upon *Heterorhabditis bacteriophora* infection (Castillo et al., 2011). In the filarial nematode *Brugia malayi* associated with mosquito *Armigeres subalbatus,* recognition proteins (PGRP), C-type lectins and calreticulin were recognized during anti-filarial reactions (Aliota et al., 2007;Castillo et al., 2011). In *Galleria*, the phenol oxidase (PO) system was found to be inhibited upon *Steinernema feltiae* infection. This PO inhibitory activity was suggested to prevent molecules from being sequestered to nematodes from the hemolymph thus evading an encapsulation reaction (Brivio et al., 2004).

Wound closure

Wound healing appears as the first line of defense to protect hosts from the environment, and is crucial for survival (Campos et al., 2010). The process involves tissue repair and regeneration in a timely manner (Belacortu and Paricio, 2011). There are differences between vertebrate and the invertebrate *Drosophila* model for wound healing. However, in both cases, both models share several cellular and molecular events, which are orchestrated in the wound healing process. In vertebrates, the key events are inflammation, proliferation and remodeling. Inflammation involves hemostasis, aggregating

platelets and secreting cytokines. In the proliferation phase, angiogenesis, hyperplasia, collagen deposition and reepithelization are major events. In the last step tissues are remodeled via removing unnecessary cells through apoptosis (Martin, 1997;Midwood et al., 2004;Hinz, 2007;Belacortu and Paricio, 2011). In *Drosophila*, two processes, inflammation and remodeling are conserved however, proliferation is not, at least not in cuticular wounds. Another distinction is that wounded cells fuse to form syncytia in *Drosophila* whereas in mammals, they remain as distinct cells (Belacortu and Paricio, 2011). The pathways and factors that are identified in the vertebrate system are all conserved in *Drosophila* too e.g., JNK, Wnt, Notch, JAK/STAT, and transcription factors Fos and Grainy head (Grh) (Belacortu and Paricio, 2011;Razzell et al., 2011). An elegant study from the Krasnow lab established a *Drosophila* larval wounding protocol and identified several cellular and genetic factors. They also recorded the timing of the events, that were required for wound healing process such as "bleeding, scab formation, JNK activation, cell orientation, cell fusion, cell spreading, cuticle synthesis, basal lamina synthesis and phagocytosis". JNK activation was required for the spreading and the migration of cells around the wound (Galko and Krasnow, 2004). Wnt and Notch signaling are required for fate determination of the cells during wound healing, for instance, during epithelial differentiation (Shi et al., 2015). In both vertebrate and invertebrates, Wnt signaling is involved in cell shape alteration and cell polarity during wound healing. Notch was reported to be involved in "tissue repair, regeneration and stem cell turn over" (Belacortu and Paricio, 2011). The JAK/STAT pathway has cell a proliferative role during wound healing. In addition, activated JAK/STAT induces cytokines, stimulates inflammatory reactions, degrades ECM and aids during cell migration (Hirano et al., 2000;Yoshimura et al., 2007;Belacortu and Paricio, 2011).

Nematode infections lead to wounds due to nematode penetration either at the cuticle or the gut epithelium to get into the hemolymph of *Drosophila* larvae. When pathways were mapped in transcriptome profiling of *Drosophila* larvae upon nematode infection, it was found that several pathways, which are active during wound responses such as Wnt, JAT/STAT, Hedgehog and ECM receptor interactions were also activated (Paper 1 in this thesis). Wound healing is considered an important aspect during nematode infections in *Drosophila*.

Apoptosis and necrosis

Apoptosis is a key event during development and disease progression. For example, initially all our fingers, and toes are connected. Later, separation of the fingers and toes from each other occurs by apoptosis to form individual digits. Too little or too much apoptosis may lead to pathological conditions,

such as in cancer and atrophy, respectively, and thus, this process is tightly regulated (Thompson, 1995;Alberts et al., 2008).

The term “apoptosis” is derived from a Greek word, which means “falling off” (Alberts et al., 2002). The term was first described in a classic paper where it was described as “a morphologically distinct form of cell death” (Kerr et al., 1972;Elmore, 2007). In multicellular organisms, cells are sometimes no longer needed in the developmental process, they destroy themselves by committing cell suicide. This process of self-destruction is activated by an intracellular death machinery called programmed cell death (PCD) or more commonly, apoptosis. The morphological features of apoptosis are cell shrinkage, a reduction in size, chromatin and cytoplasm condensation, tightly packed organelles and nuclear DNA breaks or fragmentation (Alberts et al., 2002;Elmore, 2007).

Another type of programmed cell death is called “necrosis” and it differs significantly from apoptosis. In necrosis, cells die due to cellular injury and sometimes it is described as traumatic cell death. It starts by cell swelling, followed by a burst and then the release of cytoplasmic contents, which triggers an inflammatory response. In contrast to necrosis, apoptosis is an immunologically silent process that does not induce inflammation (Thompson, 1995;Alberts et al., 2002). Phagocytes recognize apoptotic cells when they show “eat me signals” on their surface. Phosphatidylserine (Thompson, 1995) is one of several “eat me signals” released by apoptotic cells (Garg et al., 2010).

In *Drosophila*, the transcriptionally activated pro-apoptotic genes hid, grim or reaper trigger apoptosis by activating caspases (Steller, 2008). Endoproteolytic caspases cleave peptide bonds when they contain catalytic cysteine residues at the active site and the substrate possesses aspartic acid residues (McIlwain et al., 2013). Conversion from procaspases to active caspases is crucial for apoptosis induction. Generally, procaspases are expressed in living cells at low amounts but possess a significant enzymatic activity. Inhibitor of apoptosis proteins (IAPs) inhibit caspases by direct binding (Steller, 2008). In recent years apoptosis has been employed as a tool to make hemocyte-deficient *Drosophila* by inducing apoptosis genetically in hemocytes and investigating the role of the hemocytes during different infection models (Charroux and Royet, 2009;Defaye et al., 2009).

Immunity in danger situations including in cancer

In the last decades, *Drosophila* immunity has been studied extensively for pathogenic infection. However, *Drosophila* elicits immune responses other than by pathogens and this is crucial for defense. For instance, damaged

tissues (i.e., wounding or epithelial injury), necrotic cells and tumorous organs/tissues are sufficient to induce immune reactions (Shaukat et al., 2015). Various intracellular molecules are exposed on the cell surface or released from traumatic cells due to injury, or from dead cells that are due to different cell death pathways. These signals are collectively regarded as "damage-associated molecular patterns (DAMPs) or alarmins or simply, a "danger signal" (Bianchi, 2007;Garg et al., 2010).

DAMPs vary to a great extent depending on the cell type and the causative factors that injure or kill the cells. Therefore, they might be categorized into several classes such as 1) exposed DAMPs on plasma membrane (e.g., calreticulin and heat shock proteins, 2) secreted extracellular DAMPs (e.g., High Mobility Group Box-1 protein and uric acid), 3) degraded products (e.g., DNA, RNA and ATP) and 4) extracellular matrix (e.g., hayluronan, heparan sulphate and matrix components) (Spisek and Dhodapkar, 2007;Garg et al., 2010). DAMPs linked to necrosis have been relatively well studied, however, other cell death pathways associated with DAMPs, such as apoptosis and autophagic cell death, have started to emerge themselves as a new source of DAMPs (Garg et al., 2010). Moreover, necrotic cells or damaged tissues trigger generation of reactive oxygen species (ROS), which is an immediate danger signal. Subsequently ROS-mediated cellular and humoral reactions are mounted as host defense (Moreira et al., 2010;Yang et al., 2013;Shaukat et al., 2015). Tumor necrosis factor alpha (TNFα, Eiger in *Drosophila*) is released from necrotic neuronal cells and stimulates JNK activation in surrounding cells and recruits hemocytes (Yang et al., 2013;Shaukat et al., 2015).

In tumorous situations, DAMPs engage in both tumor progression and restrict metastasis. For example, degradation of basement membrane by activation of matrix metalloproteinases (MMPs) leads to tumor metastasis, simultaneously recruits hemocytes and induces inflammatory reactions presumably to restrict tumor progression (Hauling et al., 2014;Shaukat et al., 2015). In addition, loss of apicobasal polarity turns benign tumor growth into malignant outgrowth. Mutation of tumor suppressors *scribbled (scrib)* and *lethal (2) giant larvae (l2gl)* genes affect apicobasal polarity (Bilder, 2004;Parisi et al., 2014). Encapsulation reactions and different AMPs are induced in early stage of tumor development presumably to restrict tumor progression (Hauling et al., 2014;Shaukat et al., 2015)

Aims of the thesis

The aim of the thesis was to

I. Identify the *Drosophila* genes that function during nematode infection

II. Dissect the role of the hemocytes towards nematodes

III. Understand how danger signals contribute to the *Drosophila* defense

IV. Identify clotting factors and their implications for host defense

V. Monitor the evasive strategies of hosts during nematodes encounter

VI. Characterize host responses in different leukemic models

Summary of the papers

Paper I

Genome-wide transcriptional analysis of Drosophila larvae infected by entomopathogenic nematodes shows involvement of complement, recognition and extracellular matrix proteins

Powerful genetic tools have established *Drosophila* as an invertebrate model to study host responses towards pathogens. Beyond their devastating consequences in vertebrate systems, nematodes pose a serious threat to insects in nature and can be utilized as a useful tool for pest management. For example, the entomopathogenic nematode *Heterorhabditis bacteriophora* is used as a bio-control agent. Moreover, the striking similarities of several pathways and genes between invertebrate and vertebrate systems facilitate the use of *Drosophila* as a model to decipher host-pathogen interactions. Bacterial, fungal and even viral infections have been relatively well characterized in *Drosophila*, however, other pathogens like nematodes, have not been used until recently. Previously only a few reports showed that *Drosophila* mounts an immune reaction upon nematode infection.

In order to get insight into the complete response of *Drosophila* towards nematodes, we performed a genome wide analysis, a microarray on infected and non-infected larvae. Three independent samples for each condition (infected vs non-infected) were analyzed. We set the fold change ≥ 2 (either induction or repression) and the q value was <0.05. We found a total of 642 transcripts that were significantly differentially regulated in infected larvae compared to non-infected. Among them, a majority of transcripts (540) were induced upon nematode infection. Gene ontology (GO) analysis of the top 100 up-regulated genes identified a quarter of them to be immune genes suggesting a strong immune defense had been initiated against nematodes. In addition, we found 17 immune genes that overlap in the transcriptome of nematode infected *Drosophila* larvae with other-pathogen infections in *Drosophila* such as infection with a mixture of Gram-positive and Gram-negative bacteria, Gram-negative bacterial infection alone, and parasitic wasp infection. Closer inspection of these 17 genes showed that all were immune genes. We also observed a large quantity of the transcripts (485), which were differentially regulated only upon nematode infection and not in other infection situations. In line with the fact that nematodes inflict wounds

as part of their invasion strategy, mapping of all differentially regulated genes showed a strong signature of a wound response. Moreover, several pathways were found to be mapped to wounding, including, Wnt, Hedgehog and ECM-receptor interactions signaling. This implies that if host are deficient for the gene functions involved in wound closure or development of wound associated tissues, this compromises responses to nematode infection. Subsequently, we performed functional analysis of select candidate genes that were identified with the array and other genes that were known to have function against other pathogens and not found in the array. For these genes, we employed tissue-specific knock-downs and mutant analysis. Fat body and hemocytes were chosen to knock down genes. With this combined approach, we identified two categories of genes: 1) several genes that belonged to gene families with known immune functions but lacking such functions in *Drosophila,* 2) several genes, which had not been implicated in immune responses. These molecules include homologs of thioester-containing proteins (TEP3), an extracellular matrix protein (Glutactin), a recognition protein (GNBP-like3) and some small peptides that protect *Drosophila* larvae from nematodes.

This study covers nematode infection response in *Drosophila* on a genome wide scale. Our transcriptome analysis focused mostly on early-host responses (6h after infection). In functional studies, mortality was scored after 48h incubation period. In addition, other events such as nematodes entry via cuticle penetration or hindgut, melanization, septicemia and wound edges were followed. Hemocyte dispersal from sessile compartments (hemocytes attached to tissues) and Glutactin's function via hemocytes illustrate previously uncharacterized cellular events upon infection. One important notion with this model is that it causes epithelial injury, which might provide multiple accesses to other nematodes and thus seriously dampen host protection. Taken together, this study recorded early host responses and identified several genes that are specific to the nematobacterial complex. Furthermore, this provides a rich source of unexplored genes and pathways, and analyzing them might open new insights into host responses against multicellular parasites.

Paper II

Apoptosis in hemocytes induces a shift in effector mechanisms in the Drosophila immune system and leads to a pro-inflammatory state

Paper I showed that upon nematode infection, sessile hemocytes migrate into the hemolymph circulation and knock down Glutactin in hemocytes making *Drosophila* larvae sensitive to nematodes. These observations implicated

hemocytes in the fight against nematode infection and encouraged us to decipher the hemocytes' role. We first asked whether hemocytes were recruited to the nematode inflicted wound sites. The rationale behind this was that phagocytosis is blocked upon nematode infection, and we asked whether migration was inhibited too. Using pan-hemocyte specific antibody revealed hemocyte recruitment to wound edges. Further subtype specific antibodies detected plasmatocytes (95% of the total population) and lamellocytes (rarely seen in healthy larvae) at wound edges but not crystal cells. However, crystal cells were found in vicinity to the wound site. We observed melanization in nematode inflicted wounds. Crystal cells contain PPO1 and PPO2, which are major contributors for melanization reaction and are released when the crystal cells rupture. Therefore, we speculate that crystal cells move to the wound site prior to bursting. We cannot rule out the possibility that lamellocytes contribute to melanization via PPO3.

Next, we employed a previously established method to deplete hemocytes by expressing the pro-apoptotic gene hid in hemocytes using Hml-GAL4 driver (initially referred to as hemoless larvae but we termed it as hml-apo as few hemocytes remained) and tested larval survival after nematodes infection. Hml-GAL4 drives expression in plasmatocytes and crystal cells which remain in naïve larvae. GFP was co-expressed in hemocytes to identify hemocytes that had undergone apoptosis (GFP negative). In hml-apo larvae, most of the hemocytes were removed except for a few GFP positive cells. To our surprise, larvae lacking most hemocytes were not found to be more sensitive to nematodes. This observation was in contrast to bacterial infections reported earlier. Moreover, it contradicted our paper 1 result i.e., knocking down a single gene (e.g., Glutactin) using the same driver led to a higher mortality upon nematode infection compared to control. Therefore, to study this in more detail, we bled hml-apo larvae. Unexpectedly, we found massive proliferation of lamellocytes. We also detected more hemocytes in hml-apo larvae although most of them were GFP-negative suggesting they had proliferated prior to their death. To confirm this phenotype upon induction of apoptosis, we included another pro-apoptotic gene (grim) and this led to a similar phenotype. To detect lamellocyte differentiation in the lymph gland, we stained lymph glands with the lamellocytes specific antibody L1. Extensive L1 staining was observed in the lymph gland in hml-apo samples but not in control. We detected melanotic masses in hml-apo larvae, and we found a correlation between lamellocyte numbers and the frequency of larvae that contained melanotic masses. Previous reports showed that melanotic masses activate the Toll pathway, thus we tested whether Toll was induced in hml-apo larvae. We found Toll activation and Imd suppression.

Increased mortality in hml-apo pupae was observed during metamorphosis. Feeding antibiotics rescued this lethality suggesting a connection between the gut flora and lethality. Co-expressing anti-apoptotic P35 with grim res-

cued pupal lethality implicating that apoptosis contributes to lethality. A deletion of the terminal leg segments (mild phenotype) or even a complete absence of leg (strong phenotype) was associated with adults that escaped pupal lethality. Feeding antibiotics or co-expression of an anti-apoptotic gene rescued the defective leg phenotype suggested the defective leg phenotype was linked to the gut flora. Previous reports showed both gut epithelial cells and lamellocytes contribute to NO production. The defective leg phenotype is alleviated by administering L-NAME (inhibitor of NOS) or by a different food source indicating NO involvement. The rescuing effect of L-NAME was consistent with a previous observation where over-expression of NOS led to the size reduction of the leg segment at the 3rd posterior pair. Finally, NO contribution to melanotic mass and lamellocyte differentiation was found in pro-tumor conditions by feeding the NO donor arginine. Taken together, these responses indicate a proinflammatory shift had occurred in the immune system and that NO serves as a key regulator.

Paper III

The Drosophila chitinase-like protein IDGF3 is involved in protection against nematodes and in wound healing

Coagulation is a crucial factor during wounding which requires tissue repair and remodeling. Efficient tissue remodeling and regeneration begin once the initial wound sealing takes place. Many factors at a cellular and molecular level contribute to this initial sealing. In *Drosophila*, blood cells (hemocytes), and secreted proteins in hemolymph from hemocytes and other organs (e.g., fat body) are devoted to activate the clotting cascades. In this study, we analyzed a conserved chitinase-like protein (CLP) in *Drosophila* called IDGF3. Improper activity of CLPs interferes with inflammation and tissue remodeling which in turn causes many diseases such as asthma, rheumatoid arthritis, cancer, diabetes and atherosclerosis (Lee et al., 2011).

In order to investigate IDGF3 function in immunity, we first generated an *idgf3* mutant by mobilizing P-element (imprecise excision). *Idgf3* mutants were viable but showed a wing defect. This observation supports its original contribution as a growth factor. *Idgf3* mutants also had immune defects and showed higher mortality rates compared to controls which imply that IDGF3 has a protective function. IDGF3 serves as a growth factor and acts during the anti-nematode response. We thus analyzed transcriptome profiles of *idgf3* mutants and controls upon nematode infection. While comparing transcripts by gene ontology (GO) annotations of the infected control and mutants, we found infected groups to induce immune genes associated with the infection suggesting that overall immunity was not affected in the mutant. Interestingly, Wnt and JAK/STAT signaling were inhibited in controls but

not in mutants implying that IDGF3 acts upstream of these signaling pathways. Since general immunity was not affected in *idgf3* mutant, we wondered if there was a clotting defect. We found that mutants did indeed have a clotting defect and over-expression of IDGF3 in a mutant background rescued this phenotype suggesting that IDGF3's plays a role in the clotting reaction. In addition, mutant larvae had a delayed development in puparium formation upon epidermal wound by a tungsten needle. This observation further implied a requirement for IDGF3 in wound healing. Using IDGF3 antibody and endogenous IDGF3-GFP assays, we established that IDGF3 was incorporated in clot fiber and that overexpression of IDGF3 led to formation of a thicker clot. We also observed that oviposition was reduced and melanization was increased. Altogether these results from an invertebrate model demonstrate an overlapping function for CLPs when compared with vertebrate models. Further molecular characterization of CLPs has the potential to broaden our insight into inflammatory responses.

Paper IV

Monitoring the effect of pathogenic nematodes on locomotion of Drosophila larvae

The host defense is partly influenced by its efforts not to be infected. Avoiding contact with pathogens or evasive behaviors evolve as one of many successful defense strategies. Here, with the aim of capturing the locomotion patterns of *Drosophila* on nematodes we used a recently devised automated equipment called FIMtrack, which is coupled with a computer. This technique is based on frustrated total internal (FI) reflection where the animal is illuminated with infrared light and the reflected light is recorded and analyzed with the FIM software. This method allows us to monitor and quantify different parameters such as distance in movement, total trajectory of the traveling host, velocity, 'go' phase, bending, and its preferences, which are among the key responses from the host. We analyzed two control lab strains, w1118 and Canton S (which are frequently used in different behavioral experiments) in our first attempts to assess *Drosophila* locomotion. Interestingly, these control strains behave differently in the presence and absence of two different nematodes, *Heterorhabditis bacteriophora* and *Steinernema feltiae.* In contrast to its counterpart w1118, Canton S displays higher movement distance, higher velocity and less bending in presence of nematodes suggesting visual ability concerted with locomotion as w1118 has an impaired vision. Moreover, Canton S was more successful in reaching a target food source than w1118. To eliminate the impaired visual ability of w1118 in the different parameters mentioned above, offspring from the cross Canton S x w1118 had improved ability to find food over w1118 alone implying that the genetic background made a difference. In conclusion, FIM

facilitates screening for genetic background differences in host behavior and may allow screening for mutants that engage in avoidance behavior.

Paper V

Drosophila models for different grades of leukemia

Drosophila blood cells (hemocytes) have crucial roles both in development and in immunity. An imbalance of blood cell numbers at different stages of the life cycle affects physiology and may contribute to different diseases. In vertebrates, abnormal proliferation of blood cells causes leukaemia where the function of the remaining normal blood cells is hampered in certain cases due to overcrowding and/or inability of the leukaemic blood cells to perform their normal function. Despite intense efforts, our understanding of the progression of leukemia and how it modulates the immune status is still scarce.

Here we established three *Drosophila* leukaemia models by driving expression of dominant active Ras^{v12} alone or in combination with knockdown of tumor suppressors (leading to stronger leukaemia) in the hemocytes. We studied immune competence at the cellular and humoral level, and organismal homeostasis at different stages of *Drosophila* development. Our results suggest that phagocytosis which is crucial for development and immunity is not affected by the leukemic state even though we observed a massive increase in hemocyte numbers compared to wild type. Hemocyte migration is another important factor to perform its cellular reactions. We observed that leukemic hemocytes were able to recruit themselves to the wound site and that wound sealing in leukemic situations was as good as wild type. We determined if humoral responses are affected in leukemic larvae and found that Toll signaling is activated but Imd suppressed. We also observed a similar response (Toll activation and Imd suppression) in another pathophysiological set up without infection (Paper II). Moreover, Toll signaling was found to be activated in *Drosophila* when DAMPs are in the hemolymph or in stress situation (Ming et al., 2014), thus the above observation implies that Toll activation is a danger signal. We found that, in contrast to other tumor models, leukemic larvae with a strong phenotype were sensitive to nematode infections. Since hemocyte migration to the wound site and wound sealing were not affected, we checked if leukemic larvae have reduced locomotion on nematodes. We recorded that leukemic larvae had a reduced ability to move on nematodes, which thus enhanced the possibility for nematodes to infect more. Formation of adult tissues is blocked during metamorphosis and erosion of cell masses is seen in leukaemia which is similar to the state of cancer-dependent cachexia (a cancer-dependent wasting syndrome of weight loss). Indirect approaches suggest this occurred independent of ecdysone signaling. Taken together, this work establishes new

Drosophila models which will aid in the study of the physiological- and immune consequences of leukemia.

Conclusion

We provide new insights into *Drosophila* defense towards nematodes and into the contributions of damage (danger) signals to *Drosophila* immune reactions. In paper I, we analyzed for the first time the complete transcriptome response of *Drosophila* larvae upon nematode infection. We further analyzed a group of genes based on the microarray as well as those of importance outside of array results, in survival assays after nematode infection. These included a complement like protein (Tep3), a basement membrane component (Glutactin), a recognition molecule (GNBP like 3) and several small peptides. Few of the identified genes had previously been analyzed *in vivo* for their role in *Drosophila* immune defense. We also observed that hemocytes are released into circulation upon nematode infection, which stimulated further studies on the function of hemocytes towards nematodes. Our analysis at the genome-wide level also provides an extensive source for future investigations of the *Drosophila* response towards nematodes and other parasites. Of note, the model nematode is already in use as a biocontrol agent to restrict insect pests.

In paper II, we addressed the function of hemocytes towards nematodes. We discovered several surprising phenotypes that contribute to understanding the *Drosophila* defense towards danger signals. We found that plasmatocytes and lamellocytes migrate to nematode-inflicted wound sites. We then observed significantly increased lamellocytes numbers (generally rare in healthy larvae) after expressing pro-apoptotic genes hid or grim in plasmatocytes and crystal cells. Moreover, total hemocyte numbers also increased before the death of the hemocytes. We found that lamellocyte differentiation had occurred in the lymph gland concurrent with the appearance of melanotic masses, the activation of the Toll pathway and defective legs in adult escapers from pupal lethality. Finally we showed most of the phenotypes are alleviated by blocking NO production and thus, NO emerged as a key regulator in these processes. This study further adds evidences to the importance of NO and danger signals during immune responses.

In Paper III, we identified IDGF3 as a clotting component and provide evidence for its role upstream of Wnt and Jak/STAT signaling as a negative regulator. *Idgf3* mutants had a severely damaged ability to defend themselves towards nematodes. In Paper IV, we employed a recent method (FIMtrack) to monitor larval locomotion with different parameters to assess

their evasive strategy when encountering nematodes. In Paper V, we established three leukemic *Drosophila* models to evaluate their defense reactions with and without infection. Although hemocyte numbers increased dramatically in different leukemic models, their phagocytic ability and migration to wound sites were not affected. Further characterization of leukemic larvae showed activated Toll while Imd was suppressed. Stronger leukemic models produced increased mortality upon nematode infection presumably due to leukemic larvae having reduced movement. Finally, a cancer cachexia-like phenotype in which no adult tissues were formed in metamorphosis was associated with leukemic larvae models.

In conclusion, this thesis characterizes *Drosophila* responses in two situations e.g., during multicellular nematode infection and in danger situations. Many sequential events of the *Drosophila* defenses during nematode infection were unraveled between nematode entries and host death. Similarly, beyond infection, *Drosophila* responses were monitored in two independent danger models in great detail. In addition, a methodological study was presented to capture *Drosophila* larval behavioral patterns on nematodes. Altogether, these studies from invertebrate models address some of the mysteries of host defenses in two important contexts (infection and danger situation), which also exist in vertebrate systems. Thus our results have the potential for a broader impact.

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References

Agaisse, H., and Perrimon, N. (2004). The roles of JAK/STAT signaling in Drosophila immune responses. *Immunol Rev* 198**,** 72-82.

Agaisse, H., Petersen, U.M., Boutros, M., Mathey-Prevot, B., and Perrimon, N. (2003). Signaling role of hemocytes in Drosophila JAK/STAT-dependent response to septic injury. *Dev Cell* 5**,** 441-450.

Akira, S., Uematsu, S., and Takeuchi, O. (2006). Pathogen recognition and innate immunity. *Cell* 124**,** 783-801.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P. (2002). Molecular Biology of the Cell. *Garland Science, Taylor and Francis Group, New York* 4th edition.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P. (2008). *Molecular Biology of the Cell.*

Aliota, M.T., Fuchs, J.F., Mayhew, G.F., Chen, C.C., and Christensen, B.M. (2007). Mosquito transcriptome changes and filarial worm resistance in Armigeres subalbatus. *BMC Genomics* 8**,** 463.

Allen, J.E., and Sutherland, T.E. (2014). Host protective roles of type 2 immunity: parasite killing and tissue repair, flip sides of the same coin. *Semin Immunol* 26**,** 329-340.

Anderl, I., and Hultmark, D. (2015). New ways to make a blood cell. *Elife* 4.

Babcock, D.T., Brock, A.R., Fish, G.S., Wang, Y., Perrin, L., Krasnow, M.A., and Galko, M.J. (2008). Circulating blood cells function as a surveillance system for damaged tissue in Drosophila larvae. *Proc Natl Acad Sci U S A* 105**,** 10017-10022.

Babik, W., Dudek, K., Fijarczyk, A., Pabijan, M., Stuglik, M., Szkotak, R., and Zielinski, P. (2015). Constraint and adaptation in newt toll-like receptor genes. *Genome Biol Evol* 7**,** 81-95.

Bakal, C., Linding, R., Llense, F., Heffern, E., Martin-Blanco, E., Pawson, T., and Perrimon, N. (2008). Phosphorylation networks regulating JNK activity in diverse genetic backgrounds. *Science* 322**,** 453-456.

Barillas-Mury, C., Han, Y.S., Seeley, D., and Kafatos, F.C. (1999). Anopheles gambiae Ag-STAT, a new insect member of the STAT family, is activated in response to bacterial infection. *EMBO J* 18**,** 959-967.

Belacortu, Y., and Paricio, N. (2011). Drosophila as a model of wound healing and tissue regeneration in vertebrates. *Dev Dyn* 240**,** 2379-2404.

Bellen, H.J., Tong, C., and Tsuda, H. (2010). 100 years of Drosophila research and its impact on vertebrate neuroscience: a history lesson for the future. *Nat Rev Neurosci* 11**,** 514-522.

Belvin, M.P., and Anderson, K.V. (1996). A conserved signaling pathway: the Drosophila toll-dorsal pathway. *Annu Rev Cell Dev Biol* 12**,** 393-416.

Beutler, B.A. (2009). TLRs and innate immunity. *Blood* 113**,** 1399-1407.

Bianchi, M.E. (2007). DAMPs, PAMPs and alarmins: all we need to know about danger. *J Leukoc Biol* 81**,** 1-5.

Bilder, D. (2004). Epithelial polarity and proliferation control: links from the Drosophila neoplastic tumor suppressors. *Genes Dev* 18**,** 1909-1925.

Binari, R., and Perrimon, N. (1994). Stripe-specific regulation of pair-rule genes by hopscotch, a putative Jak family tyrosine kinase in Drosophila. *Genes Dev* 8**,** 300-312.

Blandin, S., Shiao, S.H., Moita, L.F., Janse, C.J., Waters, A.P., Kafatos, F.C., and Levashina, E.A. (2004). Complement-like protein TEP1 is a determinant of vectorial capacity in the malaria vector Anopheles gambiae. *Cell* 116**,** 661-670.

Bockarie, M.J., Pedersen, E.M., White, G.B., and Michael, E. (2009). Role of vector control in the global program to eliminate lymphatic filariasis. *Annu Rev Entomol* 54**,** 469-487.

Boman, H.G., Nilsson, I., and Rasmuson, B. (1972). Inducible antibacterial defence system in Drosophila. *Nature* 237**,** 232-235.

Bonnay, F., Cohen-Berros, E., Hoffmann, M., Kim, S.Y., Boulianne, G.L., Hoffmann, J.A., Matt, N., and Reichhart, J.M. (2013). big bang gene modulates gut immune tolerance in Drosophila. *Proc Natl Acad Sci U S A* 110**,** 2957-2962.

Bredt, D.S., and Snyder, S.H. (1994). Nitric oxide: a physiologic messenger molecule. *Annu Rev Biochem* 63**,** 175-195.

Brivio, M.F., Mastore, M., and Moro, M. (2004). The role of Steinernema feltiae body-surface lipids in host-parasite immunological interactions. *Mol Biochem Parasitol* 135**,** 111-121.

Broderick, N.A. (2016). Friend, foe or food? Recognition and the role of antimicrobial peptides in gut immunity and Drosophila-microbe interactions. *Philos Trans R Soc Lond B Biol Sci* 371.

Broderick, N.A., and Lemaitre, B. (2012). Gut-associated microbes of Drosophila melanogaster. *Gut Microbes* 3**,** 307-321.

Brown, N.H. (1994). Null mutations in the alpha PS2 and beta PS integrin subunit genes have distinct phenotypes. *Development* 120**,** 1221-1231.

Buchon, N., Silverman, N., and Cherry, S. (2014). Immunity in Drosophila melanogaster--from microbial recognition to whole-organism physiology. *Nat Rev Immunol* 14**,** 796-810.

Campos, I., Geiger, J.A., Santos, A.C., Carlos, V., and Jacinto, A. (2010). Genetic screen in Drosophila melanogaster uncovers a novel set of genes required for embryonic epithelial repair. *Genetics* 184**,** 129-140.

Carvalho, L., Jacinto, A., and Matova, N. (2014). The Toll/NF-kappaB signaling pathway is required for epidermal wound repair in Drosophila. *Proc Natl Acad Sci U S A* 111**,** E5373-5382.

Castillo, J.C., Creasy, T., Kumari, P., Shetty, A., Shokal, U., Tallon, L.J., and Eleftherianos, I. (2015). Drosophila anti-nematode and antibacterial immune regulators revealed by RNA-Seq. *BMC Genomics* 16**,** 519.

Castillo, J.C., Reynolds, S.E., and Eleftherianos, I. (2011). Insect immune responses to nematode parasites. *Trends Parasitol* 27**,** 537-547.

Castillo, J.C., Shokal, U., and Eleftherianos, I. (2013). Immune gene transcription in Drosophila adult flies infected by entomopathogenic nematodes and their mutualistic bacteria. *J Insect Physiol* 59**,** 179-185.

Cerenius, L., and Soderhall, K. (2011). Coagulation in invertebrates. *J Innate Immun* 3**,** 3-8.

Charroux, B., and Royet, J. (2009). Elimination of plasmatocytes by targeted apoptosis reveals their role in multiple aspects of the Drosophila immune response. *Proc Natl Acad Sci U S A* 106**,** 9797-9802.

Chen, F., Wu, W., Millman, A., Craft, J.F., Chen, E., Patel, N., Boucher, J.L., Urban, J.F., Jr., Kim, C.C., and Gause, W.C. (2014). Neutrophils prime a long-lived effector macrophage phenotype that mediates accelerated helminth expulsion. *Nat Immunol* 15**,** 938-946.

Chen, Y.R., Meyer, C.F., and Tan, T.H. (1996). Persistent activation of c-Jun N-terminal kinase 1 (JNK1) in gamma radiation-induced apoptosis. *J Biol Chem* 271**,** 631-634.

Cherry, S., and Silverman, N. (2006). Host-pathogen interactions in drosophila: new tricks from an old friend. *Nat Immunol* 7**,** 911-917.

Ciche, T. (2007). The biology and genome of Heterorhabditis bacteriophora. *WormBook***,** 1-9.

Consortium, T.H.M.P. (2012).

Cotter, S.C., Simpson, S.J., Raubenheimer, D., and Wilson, K. (2011). Macronutrient balance mediates trade-offs between immune function and life history traits. *Functional Ecology*.

Daborn, P.J., Waterfield, N., Silva, C.P., Au, C.P., Sharma, S., and Ffrench-Constant, R.H. (2002). A single Photorhabdus gene, makes caterpillars floppy (mcf), allows Escherichia coli to persist within and kill insects. *Proc Natl Acad Sci U S A* 99**,** 10742-10747.

Dantoft, W., Lundin, D., Esfahani, S.S., and Engstrom, Y. (2016). The POU/Oct Transcription Factor Pdm1/nub Is Necessary for a Beneficial Gut Microbiota and Normal Lifespan of Drosophila. *J Innate Immun* 8**,** 412-426.

Darnell, J.E., Jr. (1997). STATs and gene regulation. *Science* 277**,** 1630-1635.

Davies, C., and Tournier, C. (2012). Exploring the function of the JNK (c-Jun N-terminal kinase) signalling pathway in physiological and pathological processes to design novel therapeutic strategies. *Biochem Soc Trans* 40**,** 85-89.

Davies, S.A., and Dow, J.A. (2009). Modulation of epithelial innate immunity by autocrine production of nitric oxide. *Gen Comp Endocrinol* 162**,** 113-121.

De Smaele, E., Zazzeroni, F., Papa, S., Nguyen, D.U., Jin, R., Jones, J., Cong, R., and Franzoso, G. (2001). Induction of gadd45beta by NF-kappaB downregulates pro-apoptotic JNK signalling. *Nature* 414**,** 308-313.

Defaye, A., Evans, I., Crozatier, M., Wood, W., Lemaitre, B., and Leulier, F. (2009). Genetic ablation of Drosophila phagocytes reveals their contribution to both development and resistance to bacterial infection. *J Innate Immun* 1**,** 322-334.

Deng, Y., Ren, X., Yang, L., Lin, Y., and Wu, X. (2003). A JNK-dependent pathway is required for TNFalpha-induced apoptosis. *Cell* 115**,** 61-70.

Dhanasekaran, D.N., and Reddy, E.P. (2008). JNK signaling in apoptosis. *Oncogene* 27**,** 6245-6251.

Dobes, P., Wang, Z., Markus, R., Theopold, U., and Hyrsl, P. (2012). An improved method for nematode infection assays in Drosophila larvae. *Fly (Austin)* 6**,** 75-79.

Dostert, C., Jouanguy, E., Irving, P., Troxler, L., Galiana-Arnoux, D., Hetru, C., Hoffmann, J.A., and Imler, J.L. (2005). The Jak-STAT signaling pathway is required but not sufficient for the antiviral response of drosophila. *Nat Immunol* 6**,** 946-953.

Dudzic, J.P., Kondo, S., Ueda, R., Bergman, C.M., and Lemaitre, B. (2015). Drosophila innate immunity: regional and functional specialization of prophenoloxidases. *BMC Biol* 13**,** 81.

Eleftherianos, I., Castillo, J.C., and Patrnogic, J. (2016). TGF-beta signaling regulates resistance to parasitic nematode infection in Drosophila melanogaster. *Immunobiology*.

Eleftherianos, I., Ffrench-Constant, R.H., Clarke, D.J., Dowling, A.J., and Reynolds, S.E. (2010). Dissecting the immune response to the entomopathogen Photorhabdus. *Trends Microbiol* 18**,** 552-560.

Eleftherianos, I., More, K., Spivack, S., Paulin, E., Khojandi, A., and Shukla, S. (2014). Nitric oxide levels regulate the immune response of Drosophila melanogaster reference laboratory strains to bacterial infections. *Infect Immun* 82**,** 4169-4181.

Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicol Pathol* 35**,** 495-516.

Erkosar, B., Storelli, G., Mitchell, M., Bozonnet, L., Bozonnet, N., and Leulier, F. (2015). Pathogen Virulence Impedes Mutualist-Mediated Enhancement of Host Juvenile Growth via Inhibition of Protein Digestion. *Cell Host Microbe* 18**,** 445-455.

Evans, C.J., Liu, T., and Banerjee, U. (2014). Drosophila hematopoiesis: Markers and methods for molecular genetic analysis. *Methods* 68**,** 242-251.

Faye, I., and Lindberg, B.G. (2016). Towards a paradigm shift in innate immunity-seminal work by Hans G. Boman and co-workers. *Philos Trans R Soc Lond B Biol Sci* 371.

Faye, I., Pye, A., Rasmuson, T., Boman, H.G., and Boman, I.A. (1975). Insect immunity. 11. Simultaneous induction of antibacterial activity and selection synthesis of some hemolymph proteins in diapausing

pupae of Hyalophora cecropia and Samia cynthia. *Infect Immun* 12**,** 1426-1438.
Ferrandon, D., Imler, J.L., Hetru, C., and Hoffmann, J.A. (2007). The Drosophila systemic immune response: sensing and signalling during bacterial and fungal infections. *Nat Rev Immunol* 7**,** 862-874.
Fessler, J.H., and Fessler, L.I. (1989). Drosophila extracellular matrix. *Annu Rev Cell Biol* 5**,** 309-339.
Flybase (2016). http://flybase.org/.
Foley, E., and O'farrell, P.H. (2003). Nitric oxide contributes to induction of innate immune responses to gram-negative bacteria in Drosophila. *Genes Dev* 17**,** 115-125.
Galenza, A., Hutchinson, J., Campbell, S.D., Hazes, B., and Foley, E. (2016). Glucose modulates Drosophila longevity and immunity independent of the microbiota. *Biol Open* 5**,** 165-173.
Galko, M.J., and Krasnow, M.A. (2004). Cellular and genetic analysis of wound healing in Drosophila larvae. *PLoS Biol* 2**,** E239.
Garg, A.D., Nowis, D., Golab, J., Vandenabeele, P., Krysko, D.V., and Agostinis, P. (2010). Immunogenic cell death, DAMPs and anticancer therapeutics: an emerging amalgamation. *Biochim Biophys Acta* 1805**,** 53-71.
Garver, L.S., De Almeida Oliveira, G., and Barillas-Mury, C. (2013). The JNK pathway is a key mediator of Anopheles gambiae antiplasmodial immunity. *PLoS Pathog* 9**,** e1003622.
Gaugler, R., Lewis, E., and Stuart, R.J. (1997). Ecology in the service of biological control: the case of entomopathogenic nematodes. *Oecologia* 109:483–489.
Ghosh, S., Singh, A., Mandal, S., and Mandal, L. (2015). Active hematopoietic hubs in Drosophila adults generate hemocytes and contribute to immune response. *Dev Cell* 33**,** 478-488.
Glazer, I. (1997). Effects of infected insects on secondary invasion of steinernematid entomopathogenic nematodes. *Parasitology* 114 (Pt 6)**,** 597-604.
Gold, K.S., and Bruckner, K. (2015). Macrophages and cellular immunity in Drosophila melanogaster. *Semin Immunol* 27**,** 357-368.
Goto, A., Kadowaki, T., and Kitagawa, Y. (2003). Drosophila hemolectin gene is expressed in embryonic and larval hemocytes and its knock down causes bleeding defects. *Dev Biol* 264**,** 582-591.
Govind, S. (2008). Innate immunity in Drosophila: Pathogens and pathways. *Insect Sci* 15**,** 29-43.
Griffin, C.T. (2012). Perspectives on the behavior of entomopathogenic nematodes from dispersal to reproduction: traits contributing to nematode fitness and biocontrol efficacy. *J Nematol* 44**,** 177-184.
Grigorian, M., and Hartenstein, V. (2013). Hematopoiesis and hematopoietic organs in arthropods. *Dev Genes Evol* 223**,** 103-115.
Grigorian, M., Mandal, L., and Hartenstein, V. (2011). Hematopoiesis at the onset of metamorphosis: terminal differentiation and dissociation of the Drosophila lymph gland. *Dev Genes Evol* 221**,** 121-131.

Hall, A., Horton, S., and De Silva, N. (2009). The costs and cost-effectiveness of mass treatment for intestinal nematode worm infections using different treatment thresholds. *PLoS Negl Trop Dis* 3**,** e402.

Hallem, E.A., Dillman, A.R., Hong, A.V., Zhang, Y., Yano, J.M., Demarco, S.F., and Sternberg, P.W. (2011). A sensory code for host seeking in parasitic nematodes. *Curr Biol* 21**,** 377-383.

Hallem, E.A., Rengarajan, M., Ciche, T.A., and Sternberg, P.W. (2007). Nematodes, bacteria, and flies: a tripartite model for nematode parasitism. *Curr Biol* 17**,** 898-904.

Hao, Z., Kasumba, I., and Aksoy, S. (2003). Proventriculus (cardia) plays a crucial role in immunity in tsetse fly (Diptera: Glossinidiae). *Insect Biochem Mol Biol* 33**,** 1155-1164.

Hartenstein, V., and Mandal, L. (2006). The blood/vascular system in a phylogenetic perspective. *Bioessays* 28**,** 1203-1210.

Hauling, T., Krautz, R., Markus, R., Volkenhoff, A., Kucerova, L., and Theopold, U. (2014). A Drosophila immune response against Ras-induced overgrowth. *Biol Open* 3**,** 250-260.

Hinz, B. (2007). Formation and function of the myofibroblast during tissue repair. *J Invest Dermatol* 127**,** 526-537.

Hirano, T., Ishihara, K., and Hibi, M. (2000). Roles of STAT3 in mediating the cell growth, differentiation and survival signals relayed through the IL-6 family of cytokine receptors. *Oncogene* 19**,** 2548-2556.

Holz, A., Bossinger, B., Strasser, T., Janning, W., and Klapper, R. (2003). The two origins of hemocytes in Drosophila. *Development* 130**,** 4955-4962.

Honti, V., Csordas, G., Kurucz, E., Markus, R., and Ando, I. (2014). The cell-mediated immunity of Drosophila melanogaster: hemocyte lineages, immune compartments, microanatomy and regulation. *Dev Comp Immunol* 42**,** 47-56.

Honti, V., Csordas, G., Markus, R., Kurucz, E., Jankovics, F., and Ando, I. (2010). Cell lineage tracing reveals the plasticity of the hemocyte lineages and of the hematopoietic compartments in Drosophila melanogaster. *Mol Immunol* 47**,** 1997-2004.

Hooper, L.V., and Macpherson, A.J. (2010). Immune adaptations that maintain homeostasis with the intestinal microbiota. *Nat Rev Immunol* 10**,** 159-169.

Hotez, P.J., Brindley, P.J., Bethony, J.M., King, C.H., Pearce, E.J., and Jacobson, J. (2008). Helminth infections: the great neglected tropical diseases. *J Clin Invest* 118**,** 1311-1321.

Hu, S., and Yang, X. (2000). dFADD, a novel death domain-containing adapter protein for the Drosophila caspase DREDD. *J Biol Chem* 275**,** 30761-30764.

Hultmark, D., Engstrom, A., Andersson, K., Steiner, H., Bennich, H., and Boman, H.G. (1983). Insect immunity. Attacins, a family of antibacterial proteins from Hyalophora cecropia. *EMBO J* 2**,** 571-576.

Hultmark, D., Steiner, H., Rasmuson, T., and Boman, H.G. (1980). Insect immunity. Purification and properties of three inducible bactericidal proteins from hemolymph of immunized pupae of Hyalophora cecropia. *Eur J Biochem* 106**,** 7-16.

Hyrsl, P., Dobes, P., Wang, Z., Hauling, T., Wilhelmsson, C., and Theopold, U. (2011). Clotting factors and eicosanoids protect against nematode infections. *J Innate Immun* 3**,** 65-70.

Ip, Y.T. (2005). Drosophila innate immunity goes viral. *Nat Immunol* 6**,** 863-864.

Ip, Y.T., Reach, M., Engstrom, Y., Kadalayil, L., Cai, H., Gonzalez-Crespo, S., Tatei, K., and Levine, M. (1993). Dif, a dorsal-related gene that mediates an immune response in Drosophila. *Cell* 75**,** 753-763.

Irving, P., Ubeda, J.M., Doucet, D., Troxler, L., Lagueux, M., Zachary, D., Hoffmann, J.A., Hetru, C., and Meister, M. (2005). New insights into Drosophila larval haemocyte functions through genome-wide analysis. *Cell Microbiol* 7**,** 335-350.

Iwakiri, Y., Satoh, A., Chatterjee, S., Toomre, D.K., Chalouni, C.M., Fulton, D., Groszmann, R.J., Shah, V.H., and Sessa, W.C. (2006). Nitric oxide synthase generates nitric oxide locally to regulate compartmentalized protein S-nitrosylation and protein trafficking. *Proc Natl Acad Sci U S A* 103**,** 19777-19782.

Jennings, B.H. (2011). Drosophila – a versatile model in biology & medicine. *Materials Today* 14**,** 190-195.

Ju, J.S., Cho, M.H., Brade, L., Kim, J.H., Park, J.W., Ha, N.C., Soderhall, I., Soderhall, K., Brade, H., and Lee, B.L. (2006). A novel 40-kDa protein containing six repeats of an epidermal growth factor-like domain functions as a pattern recognition protein for lipopolysaccharide. *J Immunol* 177**,** 1838-1845.

Jung, S.H., Evans, C.J., Uemura, C., and Banerjee, U. (2005). The Drosophila lymph gland as a developmental model of hematopoiesis. *Development* 132**,** 2521-2533.

Kaneko, T., Yano, T., Aggarwal, K., Lim, J.H., Ueda, K., Oshima, Y., Peach, C., Erturk-Hasdemir, D., Goldman, W.E., Oh, B.H., Kurata, S., and Silverman, N. (2006). PGRP-LC and PGRP-LE have essential yet distinct functions in the drosophila immune response to monomeric DAP-type peptidoglycan. *Nat Immunol* 7**,** 715-723.

Kang, D., Liu, G., Lundstrom, A., Gelius, E., and Steiner, H. (1998). A peptidoglycan recognition protein in innate immunity conserved from insects to humans. *Proc Natl Acad Sci U S A* 95**,** 10078-10082.

Kaufmann, S.H. (2008). Immunology's foundation: the 100-year anniversary of the Nobel Prize to Paul Ehrlich and Elie Metchnikoff. *Nat Immunol* 9**,** 705-712.

Kawabata, S., and Muta, T. (2010). Sadaaki Iwanaga: Discovery of the lipopolysaccharide- and beta-1,3-D-glucan-mediated proteolytic cascade and unique proteins in invertebrate immunity. *J Biochem* 147**,** 611-618.

Kerr, J.F., Wyllie, A.H., and Currie, A.R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 26**,** 239-257.

Kim, L.K., Choi, U.Y., Cho, H.S., Lee, J.S., Lee, W.B., Kim, J., Jeong, K., Shim, J., Kim-Ha, J., and Kim, Y.J. (2007). Down-regulation of NF-kappaB target genes by the AP-1 and STAT complex during the innate immune response in Drosophila. *PLoS Biol* 5**,** e238.

Kim, T., Yoon, J., Cho, H., Lee, W.B., Kim, J., Song, Y.H., Kim, S.N., Yoon, J.H., Kim-Ha, J., and Kim, Y.J. (2005). Downregulation of lipopolysaccharide response in Drosophila by negative crosstalk between the AP1 and NF-kappaB signaling modules. *Nat Immunol* 6**,** 211-218.

Kingsolver, M.B., and Hardy, R.W. (2012). Making connections in insect innate immunity. *Proc Natl Acad Sci U S A* 109**,** 18639-18640.

Knowles, R.G., and Moncada, S. (1994). Nitric oxide synthases in mammals. *Biochem J* 298 (Pt 2)**,** 249-258.

Kockel, L., Homsy, J.G., and Bohmann, D. (2001). Drosophila AP-1: lessons from an invertebrate. *Oncogene* 20**,** 2347-2364.

Kounatidis, I., and Ligoxygakis, P. (2012). Drosophila as a model system to unravel the layers of innate immunity to infection. *Open Biol* 2**,** 120075.

Krecek, R.C., and Waller, P.J. (2006). Towards the implementation of the "basket of options" approach to helminth parasite control of livestock: emphasis on the tropics/subtropics. *Vet Parasitol* 139**,** 270-282.

Krzemien, J., Crozatier, M., and Vincent, A. (2010). Ontogeny of the Drosophila larval hematopoietic organ, hemocyte homeostasis and the dedicated cellular immune response to parasitism. *Int J Dev Biol* 54**,** 1117-1125.

Krzemien, J., Dubois, L., Makki, R., Meister, M., Vincent, A., and Crozatier, M. (2007). Control of blood cell homeostasis in Drosophila larvae by the posterior signalling centre. *Nature* 446**,** 325-328.

Kuzin, B., Roberts, I., Peunova, N., and Enikolopov, G. (1996). Nitric oxide regulates cell proliferation during Drosophila development. *Cell* 87**,** 639-649.

Lagueux, M., Perrodou, E., Levashina, E.A., Capovilla, M., and Hoffmann, J.A. (2000). Constitutive expression of a complement-like protein in toll and JAK gain-of-function mutants of Drosophila. *Proc Natl Acad Sci U S A* 97**,** 11427-11432.

Lamiable, O., and Imler, J.L. (2014). Induced antiviral innate immunity in Drosophila. *Curr Opin Microbiol* 20**,** 62-68.

Lancaster, J.R., Jr. (1997). A tutorial on the diffusibility and reactivity of free nitric oxide. *Nitric Oxide* 1**,** 18-30.

Lang, A.E., Schmidt, G., Schlosser, A., Hey, T.D., Larrinua, I.M., Sheets, J.J., Mannherz, H.G., and Aktories, K. (2010). Photorhabdus luminescens toxins ADP-ribosylate actin and RhoA to force actin clustering. *Science* 327**,** 1139-1142.

Lanot, R., Zachary, D., Holder, F., and Meister, M. (2001). Postembryonic hematopoiesis in Drosophila. *Dev Biol* 230**,** 243-257.

Lebestky, T., Chang, T., Hartenstein, V., and Banerjee, U. (2000). Specification of Drosophila hematopoietic lineage by conserved transcription factors. *Science* 288**,** 146-149.

Lee, C.G., Da Silva, C.A., Dela Cruz, C.S., Ahangari, F., Ma, B., Kang, M.J., He, C.H., Takyar, S., and Elias, J.A. (2011). Role of chitin and chitinase/chitinase-like proteins in inflammation, tissue remodeling, and injury. *Annu Rev Physiol* 73**,** 479-501.

Lee, W.J., and Brey, P.T. (2013). How microbiomes influence metazoan development: insights from history and Drosophila modeling of gut-microbe interactions. *Annu Rev Cell Dev Biol* 29**,** 571-592.

Lee, W.J., Lee, J.D., Kravchenko, V.V., Ulevitch, R.J., and Brey, P.T. (1996). Purification and molecular cloning of an inducible gram-negative bacteria-binding protein from the silkworm, Bombyx mori. *Proc Natl Acad Sci U S A* 93**,** 7888-7893.

Leitao, A.B., and Sucena, E. (2015). Drosophila sessile hemocyte clusters are true hematopoietic tissues that regulate larval blood cell differentiation. *Elife* 4.

Lemaitre, B., and Hoffmann, J. (2007). The host defense of Drosophila melanogaster. *Annu Rev Immunol* 25**,** 697-743.

Lemaitre, B., Meister, M., Govind, S., Georgel, P., Steward, R., Reichhart, J.M., and Hoffmann, J.A. (1995). Functional analysis and regulation of nuclear import of dorsal during the immune response in Drosophila. *EMBO J* 14**,** 536-545.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M., and Hoffmann, J.A. (1996). The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell* 86**,** 973-983.

Lesch, C., Jo, J., Wu, Y., Fish, G.S., and Galko, M.J. (2010). A targeted UAS-RNAi screen in Drosophila larvae identifies wound closure genes regulating distinct cellular processes. *Genetics* 186**,** 943-957.

Letourneau, M., Lapraz, F., Sharma, A., Vanzo, N., Waltzer, L., and Crozatier, M. (2016). Drosophila hematopoiesis under normal conditions and in response to immune stress. *FEBS Lett*.

Leulier, F., Parquet, C., Pili-Floury, S., Ryu, J.H., Caroff, M., Lee, W.J., Mengin-Lecreulx, D., and Lemaitre, B. (2003). The Drosophila immune system detects bacteria through specific peptidoglycan recognition. *Nat Immunol* 4**,** 478-484.

Lewis, E.E., Campbell, J., Griffin, C., Kaya, H., and Peters, A. (2006). Behavioral ecology of entomopathogenic nematodes. *Biological Control* 38:66–79.

Li, H., Qi, Y., and Jasper, H. (2016). Preventing Age-Related Decline of Gut Compartmentalization Limits Microbiota Dysbiosis and Extends Lifespan. *Cell Host Microbe* 19**,** 240-253.

Luckhart, S., and Li, K. (2001). Transcriptional complexity of the Anopheles stephensi nitric oxide synthase gene. *Insect Biochem Mol Biol* 31**,** 249-256.

Ma, C., and Kanost, M.R. (2000). A beta1,3-glucan recognition protein from an insect, Manduca sexta, agglutinates microorganisms and activates the phenoloxidase cascade. *J Biol Chem* 275**,** 7505-7514.

Maizels, R.M., and Mcsorley, H.J. (2016). Regulation of the host immune system by helminth parasites. *J Allergy Clin Immunol* 138**,** 666-675.

Makhijani, K., Alexander, B., Tanaka, T., Rulifson, E., and Bruckner, K. (2011). The peripheral nervous system supports blood cell homing and survival in the Drosophila larva. *Development* 138**,** 5379-5391.

Mandal, L., Banerjee, U., and Hartenstein, V. (2004). Evidence for a fruit fly hemangioblast and similarities between lymph-gland hematopoiesis in fruit fly and mammal aorta-gonadal-mesonephros mesoderm. *Nat Genet* 36**,** 1019-1023.

Markus, R., Laurinyecz, B., Kurucz, E., Honti, V., Bajusz, I., Sipos, B., Somogyi, K., Kronhamn, J., Hultmark, D., and Ando, I. (2009). Sessile hemocytes as a hematopoietic compartment in Drosophila melanogaster. *Proc Natl Acad Sci U S A* 106**,** 4805-4809.

Martin, P. (1997). Wound healing--aiming for perfect skin regeneration. *Science* 276**,** 75-81.

Martin-Blanco, E., Gampel, A., Ring, J., Virdee, K., Kirov, N., Tolkovsky, A.M., and Martinez-Arias, A. (1998). puckered encodes a phosphatase that mediates a feedback loop regulating JNK activity during dorsal closure in Drosophila. *Genes Dev* 12**,** 557-570.

Matos, R.C., and Leulier, F. (2014). Lactobacilli-Host mutualism: "learning on the fly". *Microb Cell Fact* 13 Suppl 1**,** S6.

Mcilwain, D.R., Berger, T., and Mak, T.W. (2013). Caspase functions in cell death and disease. *Cold Spring Harb Perspect Biol* 5**,** a008656.

Mellroth, P., Karlsson, J., and Steiner, H. (2003). A scavenger function for a Drosophila peptidoglycan recognition protein. *J Biol Chem* 278**,** 7059-7064.

Midwood, K.S., Williams, L.V., and Schwarzbauer, J.E. (2004). Tissue repair and the dynamics of the extracellular matrix. *Int J Biochem Cell Biol* 36**,** 1031-1037.

Ming, M., Obata, F., Kuranaga, E., and Miura, M. (2014). Persephone/Spatzle pathogen sensors mediate the activation of Toll receptor signaling in response to endogenous danger signals in apoptosis-deficient Drosophila. *J Biol Chem* 289**,** 7558-7568.

Moreau, E., and Chauvin, A. (2010). Immunity against helminths: interactions with the host and the intercurrent infections. *J Biomed Biotechnol* 2010**,** 428593.

Moreira, S., Stramer, B., Evans, I., Wood, W., and Martin, P. (2010). Prioritization of competing damage and developmental signals by migrating macrophages in the Drosophila embryo. *Curr Biol* 20**,** 464-470.

Morgan, T.H. (1910). Sex Limited Inheritance in Drosophila. *Science* 32**,** 120-122.

Myllymaki, H., and Ramet, M. (2013). Transcription factor zfh1 downregulates Drosophila Imd pathway. *Dev Comp Immunol* 39**,** 188-197.

Myllymaki, H., Valanne, S., and Ramet, M. (2014). The Drosophila imd signaling pathway. *J Immunol* 192**,** 3455-3462.

Nakamoto, M., Moy, R.H., Xu, J., Bambina, S., Yasunaga, A., Shelly, S.S., Gold, B., and Cherry, S. (2012). Virus recognition by Toll-7 activates antiviral autophagy in Drosophila. *Immunity* 36**,** 658-667.

Nappi, A.J., and Vass, E. (1993). Melanogenesis and the generation of cytotoxic molecules during insect cellular immune reactions. *Pigment Cell Res* 6**,** 117-126.

Nayak, A., Tassetto, M., Kunitomi, M., and Andino, R. (2013). RNA interference-mediated intrinsic antiviral immunity in invertebrates. *Curr Top Microbiol Immunol* 371**,** 183-200.

Nicol, J.M., Elekcioglu, I.H., Bolat, N., and Rivoal, R. (2007). The global importance of the cereal cyst nematode (Heterodera spp.) on wheat and international approaches to its control. *Commun Agric Appl Biol Sci* 72**,** 677-686.

Omkar (2016). *Ecofriendly Pest Management for Food Security.* Academic Press.

Pandey, U.B., and Nichols, C.D. (2011). Human disease models in Drosophila melanogaster and the role of the fly in therapeutic drug discovery. *Pharmacol Rev* 63**,** 411-436.

Parisi, F., Stefanatos, R.K., Strathdee, K., Yu, Y., and Vidal, M. (2014). Transformed epithelia trigger non-tissue-autonomous tumor suppressor response by adipocytes via activation of Toll and Eiger/TNF signaling. *Cell Rep* 6**,** 855-867.

Park, J.M., Brady, H., Ruocco, M.G., Sun, H., Williams, D., Lee, S.J., Kato, T., Jr., Richards, N., Chan, K., Mercurio, F., Karin, M., and Wasserman, S.A. (2004). Targeting of TAK1 by the NF-kappa B protein Relish regulates the JNK-mediated immune response in Drosophila. *Genes Dev* 18**,** 584-594.

Pastor-Pareja, J.C., Wu, M., and Xu, T. (2008). An innate immune response of blood cells to tumors and tissue damage in Drosophila. *Dis Model Mech* 1**,** 144-154; discussion 153.

Pearson, A., Lux, A., and Krieger, M. (1995). Expression cloning of dSR-CI, a class C macrophage-specific scavenger receptor from Drosophila melanogaster. *Proc Natl Acad Sci U S A* 92**,** 4056-4060.

Ponton, F., Wilson, K., Cotter, S.C., Raubenheimer, D., and Simpson, S.J. (2011). Nutritional immunology: a multi-dimensional approach. *PLoS Pathog* 7**,** e1002223.

Prize, N. (1908). Nobel Prizes and Laureates. http://www.nobelprize.org/nobel_prizes/medicine/laureates/1908/mechnikov-facts.html.

Putsep, K., and Faye, I. (2009). Hans G Boman (1924-2008): pioneer in peptide-mediated innate immune defence. *Scand J Immunol* 70**,** 317-319.

Ramet, M., Lanot, R., Zachary, D., and Manfruelli, P. (2002). JNK signaling pathway is required for efficient wound healing in Drosophila. *Dev Biol* 241**,** 145-156.

Ramet, M., Pearson, A., Manfruelli, P., Li, X., Koziel, H., Gobel, V., Chung, E., Krieger, M., and Ezekowitz, R.A. (2001). Drosophila scavenger receptor CI is a pattern recognition receptor for bacteria. *Immunity* 15**,** 1027-1038.

Ramirez, J.L., and Dimopoulos, G. (2010). The Toll immune signaling pathway control conserved anti-dengue defenses across diverse Ae. aegypti strains and against multiple dengue virus serotypes. *Dev Comp Immunol* 34**,** 625-629.

Razzell, W., Wood, W., and Martin, P. (2011). Swatting flies: modelling wound healing and inflammation in Drosophila. *Dis Model Mech* 4**,** 569-574.

Regan, J.C., Brandao, A.S., Leitao, A.B., Mantas Dias, A.R., Sucena, E., Jacinto, A., and Zaidman-Remy, A. (2013). Steroid hormone signaling is essential to regulate innate immune cells and fight bacterial infection in Drosophila. *PLoS Pathog* 9**,** e1003720.

Regulski, M., and Tully, T. (1995). Molecular and biochemical characterization of dNOS: a Drosophila Ca2+/calmodulin-dependent nitric oxide synthase. *Proc Natl Acad Sci U S A* 92**,** 9072-9076.

Reynaert, N.L., Ckless, K., Korn, S.H., Vos, N., Guala, A.S., Wouters, E.F., Van Der Vliet, A., and Janssen-Heininger, Y.M. (2004). Nitric oxide represses inhibitory kappaB kinase through S-nitrosylation. *Proc Natl Acad Sci U S A* 101**,** 8945-8950.

Rivero, A. (2006). Nitric oxide: an antiparasitic molecule of invertebrates. *Trends Parasitol* 22**,** 219-225.

Rizki, R.M., and Rizki, T.M. (1980a). Hemocyte Responses to Implanted Tissues in Drosophila-Melanogaster Larvae. *Roux Arch Dev Biol* 189 207-213.

Rizki, T.M. (1956). Blood cells of Drosophila as related to metamorphosis. *In: Campbell FL, editor. Physiology of Insect Development. Chicago University Press; Chicago, IL.***,** pp. 91–94.

Rizki, T.M., and Rizki, R.M. (1980b). Properties of the larval hemocytes of Drosophila melanogaster. *Experientia* 36**,** 1223–1226.

Russo, J., Dupas, S., Frey, F., Carton, Y., and Brehelin, M. (1996). Insect immunity: early events in the encapsulation process of parasitoid (Leptopilina boulardi) eggs in resistant and susceptible strains of Drosophila. *Parasitology* 112 (Pt 1)**,** 135-142.

Scherfer, C., Karlsson, C., Loseva, O., Bidla, G., Goto, A., Havemann, J., Dushay, M.S., and Theopold, U. (2004). Isolation and characterization of hemolymph clotting factors in Drosophila melanogaster by a pullout method. *Curr Biol* 14**,** 625-629.

Sears, H.C., Kennedy, C.J., and Garrity, P.A. (2003). Macrophage-mediated corpse engulfment is required for normal Drosophila CNS morphogenesis. *Development* 130**,** 3557-3565.

Shaukat, Z., Liu, D., and Gregory, S. (2015). Sterile inflammation in Drosophila. *Mediators Inflamm* 2015**,** 369286.

Shi, Y., Shu, B., Yang, R., Xu, Y., Xing, B., Liu, J., Chen, L., Qi, S., Liu, X., Wang, P., Tang, J., and Xie, J. (2015). Wnt and Notch signaling

pathway involved in wound healing by targeting c-Myc and Hes1 separately. *Stem Cell Res Ther* 6**,** 120.

Silva, N.M., Vieira, J.C., Carneiro, C.M., and Tafuri, W.L. (2009). Toxoplasma gondii: the role of IFN-gamma, TNFRp55 and iNOS in inflammatory changes during infection. *Exp Parasitol* 123**,** 65-72.

Sinenko, S.A., Mandal, L., Martinez-Agosto, J.A., and Banerjee, U. (2009). Dual role of wingless signaling in stem-like hematopoietic precursor maintenance in Drosophila. *Dev Cell* 16**,** 756-763.

Smith, K., Mccoy, K.D., and Macpherson, A.J. (2007). Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota. *Semin Immunol* 19**,** 59-69.

Sopko, R., Lin, Y.B., Makhijani, K., Alexander, B., Perrimon, N., and Bruckner, K. (2015). A systems-level interrogation identifies regulators of Drosophila blood cell number and survival. *PLoS Genet* 11**,** e1005056.

Souza-Neto, J.A., Sim, S., and Dimopoulos, G. (2009). An evolutionary conserved function of the JAK-STAT pathway in anti-dengue defense. *Proc Natl Acad Sci U S A* 106**,** 17841-17846.

Spisek, R., and Dhodapkar, M.V. (2007). Towards a better way to die with chemotherapy: role of heat shock protein exposure on dying tumor cells. *Cell Cycle* 6**,** 1962-1965.

Stasiv, Y., Regulski, M., Kuzin, B., Tully, T., and Enikolopov, G. (2001). The Drosophila nitric-oxide synthase gene (dNOS) encodes a family of proteins that can modulate NOS activity by acting as dominant negative regulators. *J Biol Chem* 276**,** 42241-42251.

Staubach, F., Baines, J.F., Kunzel, S., Bik, E.M., and Petrov, D.A. (2013). Host species and environmental effects on bacterial communities associated with Drosophila in the laboratory and in the natural environment. *PLoS One* 8**,** e70749.

Steiner, H., Hultmark, D., Engstrom, A., Bennich, H., and Boman, H.G. (1981). Sequence and specificity of two antibacterial proteins involved in insect immunity. *Nature* 292**,** 246-248.

Steller, H. (2008). Regulation of apoptosis in Drosophila. *Cell Death Differ* 15**,** 1132-1138.

Stoven, S., Ando, I., Kadalayil, L., Engstrom, Y., and Hultmark, D. (2000). Activation of the Drosophila NF-kappaB factor Relish by rapid endoproteolytic cleavage. *EMBO Rep* 1**,** 347-352.

Stroschein-Stevenson, S.L., Foley, E., O'farrell, P.H., and Johnson, A.D. (2006). Identification of Drosophila gene products required for phagocytosis of Candida albicans. *PLoS Biol* 4**,** e4.

Surachetpong, W., Singh, N., Cheung, K.W., and Luckhart, S. (2009). MAPK ERK signaling regulates the TGF-beta1-dependent mosquito response to Plasmodium falciparum. *PLoS Pathog* 5**,** e1000366.

Tanji, T., Hu, X., Weber, A.N., and Ip, Y.T. (2007). Toll and IMD pathways synergistically activate an innate immune response in Drosophila melanogaster. *Mol Cell Biol* 27**,** 4578-4588.

Tepass, U., Fessler, L.I., Aziz, A., and Hartenstein, V. (1994). Embryonic origin of hemocytes and their relationship to cell death in Drosophila. *Development* 120**,** 1829-1837.

Theopold, U., Schmidt, O., Soderhall, K., and Dushay, M.S. (2004). Coagulation in arthropods: defence, wound closure and healing. *Trends Immunol* 25**,** 289-294.

Thompson, C.B. (1995). Apoptosis in the pathogenesis and treatment of disease. *Science* 267**,** 1456-1462.

Tokusumi, T., Tokusumi, Y., Hopkins, D.W., Shoue, D.A., Corona, L., and Schulz, R.A. (2011). Germ line differentiation factor Bag of Marbles is a regulator of hematopoietic progenitor maintenance during Drosophila hematopoiesis. *Development* 138**,** 3879-3884.

Unckless, R.L., Rottschaefer, S.M., and Lazzaro, B.P. (2015). The complex contributions of genetics and nutrition to immunity in Drosophila melanogaster. *PLoS Genet* 11**,** e1005030.

Valanne, S., Wang, J.H., and Ramet, M. (2011). The Drosophila Toll signaling pathway. *J Immunol* 186**,** 649-656.

Vdrc (2016). Vienna Drosophila Resource Center.

Vikhanski, L. (2016). Immunity: How Elie Metchnikoff Changed the Course of Modern Medicine. *Chicago Review Press, 2016*.

Wajant, H., Pfizenmaier, K., and Scheurich, P. (2003). Tumor necrosis factor signaling. *Cell Death Differ* 10**,** 45-65.

Wang, Z., Wilhelmsson, C., Hyrsl, P., Loof, T.G., Dobes, P., Klupp, M., Loseva, O., Morgelin, M., Ikle, J., Cripps, R.M., Herwald, H., and Theopold, U. (2010). Pathogen entrapment by transglutaminase--a conserved early innate immune mechanism. *PLoS Pathog* 6**,** e1000763.

Weber, A.N., Tauszig-Delamasure, S., Hoffmann, J.A., Lelievre, E., Gascan, H., Ray, K.P., Morse, M.A., Imler, J.L., and Gay, N.J. (2003). Binding of the Drosophila cytokine Spatzle to Toll is direct and establishes signaling. *Nat Immunol* 4**,** 794-800.

Werner, T., Liu, G., Kang, D., Ekengren, S., Steiner, H., and Hultmark, D. (2000). A family of peptidoglycan recognition proteins in the fruit fly Drosophila melanogaster. *Proc Natl Acad Sci U S A* 97**,** 13772-13777.

Wink, D.A., Hines, H.B., Cheng, R.Y., Switzer, C.H., Flores-Santana, W., Vitek, M.P., Ridnour, L.A., and Colton, C.A. (2011). Nitric oxide and redox mechanisms in the immune response. *J Leukoc Biol* 89**,** 873-891.

Wong, A.C., Vanhove, A.S., and Watnick, P.I. (2016). The interplay between intestinal bacteria and host metabolism in health and disease: lessons from Drosophila melanogaster. *Dis Model Mech* 9**,** 271-281.

Wong, C.N., Ng, P., and Douglas, A.E. (2011). Low-diversity bacterial community in the gut of the fruitfly Drosophila melanogaster. *Environ Microbiol* 13**,** 1889-1900.

Wood, W., and Jacinto, A. (2007). Drosophila melanogaster embryonic haemocytes: masters of multitasking. *Nat Rev Mol Cell Biol* 8**,** 542-551.

Wu, S.C., Liao, C.W., Pan, R.L., and Juang, J.L. (2012). Infection-induced intestinal oxidative stress triggers organ-to-organ immunological communication in Drosophila. *Cell Host Microbe* 11**,** 410-417.

Xu, J., Hopkins, K., Sabin, L., Yasunaga, A., Subramanian, H., Lamborn, I., Gordesky-Gold, B., and Cherry, S. (2013). ERK signaling couples nutrient status to antiviral defense in the insect gut. *Proc Natl Acad Sci U S A* 110**,** 15025-15030.

Yang, H., Kronhamn, J., Ekstrom, J.O., Korkut, G.G., and Hultmark, D. (2015). JAK/STAT signaling in Drosophila muscles controls the cellular immune response against parasitoid infection. *EMBO Rep* 16**,** 1664-1672.

Yang, Y., Hou, L., Li, Y., Ni, J., and Liu, L. (2013). Neuronal necrosis and spreading death in a Drosophila genetic model. *Cell Death Dis* 4**,** e723.

Yang, Y., and Jobin, C. (2014). Microbial imbalance and intestinal pathologies: connections and contributions. *Dis Model Mech* 7**,** 1131-1142.

Yarnitzky, T., and Volk, T. (1995). Laminin is required for heart, somatic muscles, and gut development in the Drosophila embryo. *Dev Biol* 169**,** 609-618.

Yoshimura, A., Naka, T., and Kubo, M. (2007). SOCS proteins, cytokine signalling and immune regulation. *Nat Rev Immunol* 7**,** 454-465.