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An analysis of the structural and functional similarities of insect hemocytes and mammalian phagocytes

Niall Browne, Michelle Heelan, and Kevin Kavanagh*

Department of Biology; NUI Maynooth; Maynooth, Co. Kildare Ireland

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The insect immune response demonstrates a number of structural and functional similarities to the innate immune system of mammals. As a result of these conserved features insects have become popular choices for evaluating the virulence of microbial pathogens or for assessing the efficacy of antimicrobial agents and give results which are comparable to those that can be obtained using mammals. Analysis of the cellular component of the insect and mammalian immune systems demonstrates many similarities. Insect hemocytes recognize pathogens and phagocytose material in a similar manner to neutrophils. The killing of ingested microbes is achieved in both cell types by the production of superoxide and by the release of enzymes in the process of degranulation. Insect hemocytes and mammalian neutrophils are sensitive to the same inhibitors. This review highlights the strong similarities between the phagocytic cells of both groups of animals and demonstrates the potential benefits of using selected insects as in vivo screening systems.

Insects as In Vivo Model Systems

Insects are a very successful group of invertebrates, with approximately one million species that inhabit all ecological niches, apart from the sea.[1,2] The ability of insects to inhabit such a wide variety of environments indicates a highly efficient and versatile immune response.[3-5] Insects and vertebrates diverged approximately 500 million years ago, and while vertebrates have developed an adaptive immune response, the vertebrate innate immune system still retains strong structural and functional similarities to the insect immune system.[6-9] As a result of these conserved similarities a wide range of insects has been employed to study the virulence of bacterial and fungal pathogens and give results that are comparable with those obtained using vertebrates (e.g., mice).[8,10-13] Insects such as *Galleria mellonella* (greater wax moth), *Bombyx mori* (silkworm), *Manduca sexta* (tobacco hornworm or Goliath worm), and *Drosophila melanogaster* (fruit fly) are now widely employed as model organisms and provide results comparable to those that can be obtained with mammals.[14] *G. mellonella* larvae are useful as insect models because it is possible to accurately quantify the inoculum which is injected directly into the hemocoel.[15] *G. mellonella* larvae are cost effective, widely available and results can be obtained within 2 or 3 d (**Fig. 1**).[16] *G. mellonella* larvae can be incubated at 37 °C, thus allowing the study of temperature-dependent microbial virulence factors.[15] In addition to monitoring larval survival following infection, a variety of end-points can be used to monitor the response of *Galleria* larvae to specific pathogens. Changes in the density of circulating hemocytes, alteration in the phagocytosing ability of hemocytes, or alterations in the expression of antimicrobial peptides or immune-related proteins have been employed to quantify the response of larvae to pathogens or antimicrobial agents.[15]

The virulence of bacteria such as *Pseudomonas aeruginosa*,[17] *Campylobacter jejuni*,[18] *Listeria monocytogenes*,[19,20] *Burkholderia cepacia* complex,[21] *Yersinia pseudotuberculosis*,[22] and *Staphylococcus aureus*,[11] and fungi such as *Candida albicans*,[10] *Aspergillus fumigatus*,[23] and *Cryptococcus neoformans*[24] has been determined in a variety of insect systems.[6,14,25,26] Insects have also been utilized to assess the in vivo efficacy of antimicrobial agents.[27-30] *Galleria* larvae have recently been employed to study the effect of *Listeria* infection on the brain and show similar pathologies to those observed in mammals infected with this bacterium.[31]

The use of insects in place of mammals for assessing the virulence of microbial pathogens or for determining the efficacy of novel antimicrobial drugs exploits the similarities between the immune system of insects and the innate immune response of mammals. Central to both of these immune systems is the action of phagocytic cells which display strong structural and functional similarities.

The Insect Immune System

The insect immune response consists of two tightly interconnected components, the cellular and the humoral responses.[1,32] The cellular response is mediated by hemocytes and involves responses such as phagocytosis, encapsulation, and clotting.[33] The humoral defenses are composed of soluble effector molecules such as anti-microbial peptides, complement-like proteins, melanin, and products created by proteolytic cascades, such as the phenoloxidase (PO) pathway, which immobilize or kill pathogens in the insect.[34]

*Correspondence to: Kevin Kavanagh; Email: kevin.kavanagh@nuim.ie
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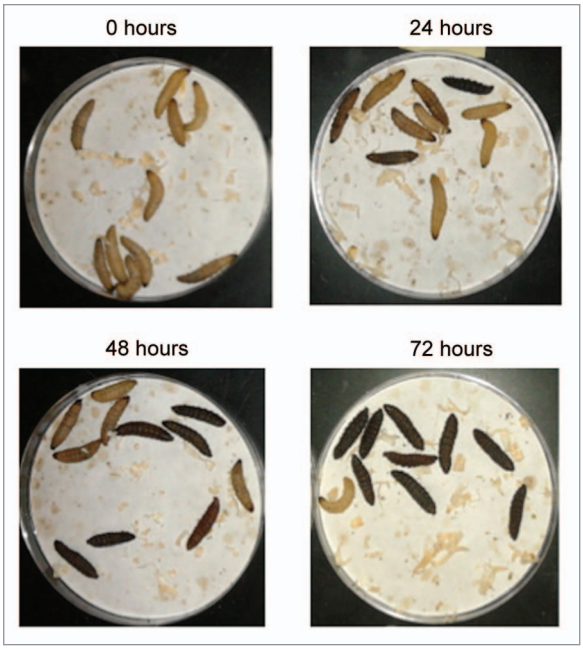

Figure 1. Effect of *Staphylococcus aureus* on viability of *G. mellonella* larvae. Larvae were inoculated with *S. aureus* and incubated at 37 °C. A mortality rate of 90% is observed at 72 h. The dark color is due to the process of melanization in response to the growth of the pathogen in the insect. Note that the larvae are supplied with a food source (wood shavings).

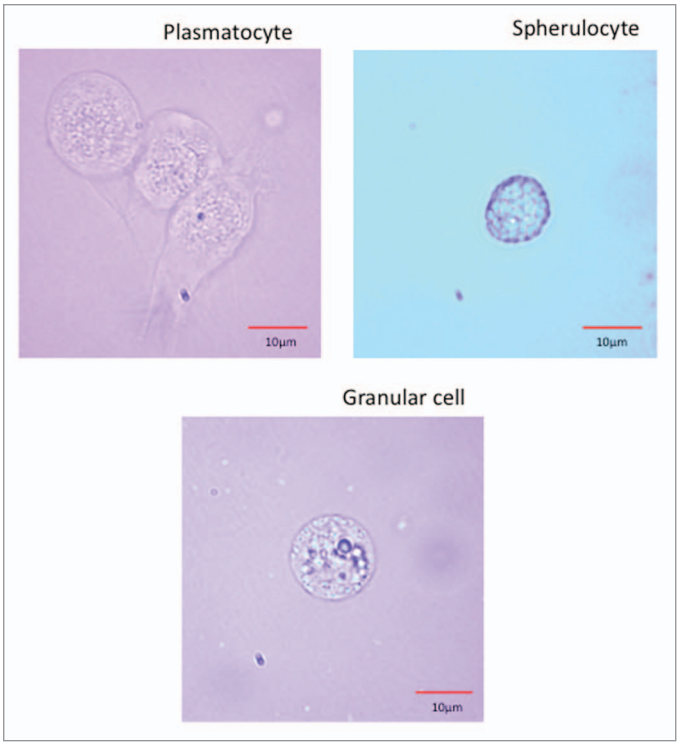

Figure 2. Hemocytes of *G. mellonella* larvae. Hemocytes were recovered from hemolymph of *G. mellonella* larvae and viewed using an Olympus Microscope. Images show plasmatocytes, spherulocyte, and granular cell.

Insect hemocytes: structure, function, and diversity. There at least eight types of hemocytes found in insects: prohemocytes, plasmatocytes, granular cells, coagulocytes, crystal cells, spherulocytes, oenocytoids, and thrombocytoids;[5] however, the majority of insects do not possess all types of hemocytes (**Fig. 2**). Hemocytes are found circulating freely in the hemolymph or adhering to internal organs such as the fat body or the digestive tract of the insect.[6] The density of hemocytes in the hemolymph varies during the life of the insect and also in response to the introduction of pathogens.[35]

Prohemocytes are small circular cells with a large nucleus and a basophilic cytoplasm that can differentiate into a number of cell types. Plasmatocytes are the most common hemocyte, they are leaf like in shape and their cytoplasm contains lysosomal enzymes. They are usually larger than granular cells and are involved in capsule formation.[33] Granular cells have a small nucleus and many granules in the cytoplasm. Spherulocytes display a variety of differing shapes, with numerous small spherical inclusions (**Fig. 2**). Oenocytoids are large, bi-nucleate, non-phagocytic cells. Coagulocytes participate in the clotting process. Hemocytes can recognize foreign material and also distinguish self from non-self during the cellular immune response in a similar manner to human immune cells.[33,36] In Lepidopteran insects (e.g., *G. mellonella*) plasmatocytes and granular cells are involved in most cellular defense responses, whereas in *Drosophila* these responses include primarily plasmatocytes and lamellocytes.[33]

Hemocyte-mediated phagocytosis. As in mammalian neutrophils, the activity of the phagocytic cell in insects (the plasmatocyte) is enabled by opsonization of microorganisms with complement-like proteins.[8,37] When a pathogen has been engulfed by a phagocytic cell, a membrane-bound enzyme system is activated which causes the translocation of electrons from cytosolic NADPH to extracellular oxygen, resulting in superoxide production (**Fig. 3**).[38] The respiratory burst is necessary for killing pathogens but also results in local tissue damage and inflammatory responses in the area of infection due to the release of enzymes following degranulation.[38,39]

Phagocytosis depends upon the recognition of the target as foreign which activates downstream signaling and effector responses.[6] Some pathogens are recognized by humoral pattern recognition molecules that bind to a target which increases its recognition by other receptors on the surface of hemocytes. Alternatively pathogens can be recognized directly by hemocyte surface receptors, e.g., calrecticulin or apolipophorin. The phagocytic process itself is generally non-destructive to hemocytes, unlike melanization, coagulation, and encapsulation, which are terminal events in the lifespan of hemocyte.[40]

In order for phagocytosis to occur, opsonic ligands bind to molecules on the outside of the pathogen. Once the ligands are recognized, an intracellular cascade occurs resulting in the pathogen being internalized in the cell.[6] The process of phagocytosis is lectin-mediated and lectins are found in the insect hemolymph, along with lysozyme.[6] Lysozyme is an anti-microbial peptide that has been found within hemocytes. The presence of gram-negative bacteria causes binding of N-acetylglucosamine (GlcNa)-specific lectins (e.g., BDL-2 lectins and PGBP 1/2) to peptidoglycans on

the bacterial cell wall. After the lectins have bound to the surface, they then bind to plasmatocytes and induce phagocytosis of the bacterial cell.[6] Lectins and lysozyme act simultaneously. While the lectins bind to the sugars on the bacterial cell surface, lysozyme breaks down the peptidoglycan layer, causing a release of sugars, revealing technoic acid and lipomannan, which in turn are recognized by BDL-1 lectins.[6]

Hemocyte-mediated nodulation. Nodulation occurs when multiple hemocytes bind to clusters of bacteria. This is the main insect cellular defense reaction to infection and allows large numbers of bacteria to be cleared from the hemolymph. Hemocytes begin to bind together, causing an overlapping sheath to form around the pathogen.[33] Nodulation is completed with the activation of prophenoloxidase and melanization of mature nodules. An equivalent nodulization process does not occur in human phagocytes but complement displays a similar cascade event to melanization.

Hemocyte-mediated encapsulation. Encapsulation occurs in response to the entry of large structures such as protozoa, nematodes and eggs or larvae of parasitic insects into the hemolymph.[41] There are two different types of encapsulation in insects: cellular encapsulation mainly in *Lepidoptera* and humoral encapsulation mainly in *Diptera*. Humoral encapsulation can occur with or without hemocytes, and is always associated with phenoloxidase, cellular encapsulation can occur without melanization. The encapsulation process in *G. mellonella* is led by the recognition of a foreign body by granular cells which upon contact lyse or degranulate releasing material that promotes plasmatocytes to attach.[42] The attachment of multiple layers of plasmatocytes forms around the foreign body resulting in a smooth capsule of overlaid cells.[43,44] Granular cells and plasmatocytes are commonly observed in capsules in *Lepidoptera*, and in *Drosophila* lamellocytes were observed most frequently in capsules.[45]

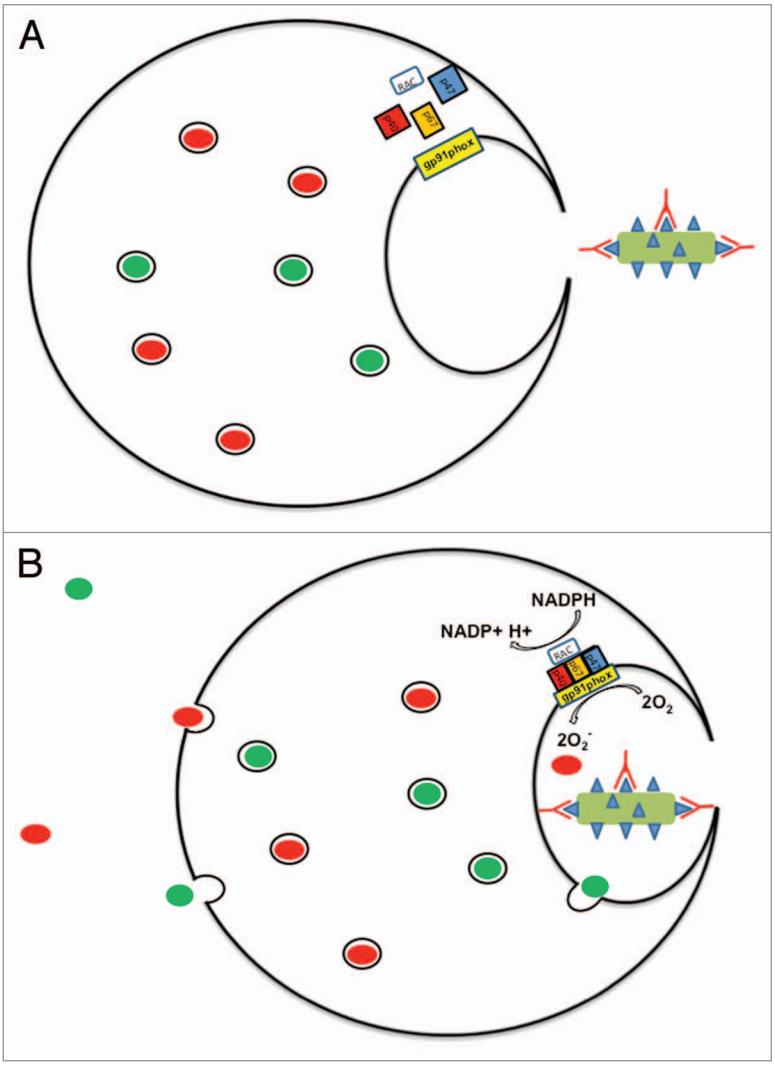

Figure 3. Mode of action of neutrophil. (**A**) Unactivated neutrophil: Opsonized bacterium is about to be phagocytosed, NADPH oxidase is not functional and all enzymes are retained on primary (red) and secondary (green) granules within cytoplasm. (**B**) Activated neutrophil: Opsonized bacterium has been phagocytosed, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and rac have translocated from the cytoplasm to join $gp91^{phox}$ on the cell membrane and as a result superoxide is produced from oxygen with the consequent conversion of NADPH to $NADP^+$ and H^+. Degranulation has commenced releasing enzymes (red, green) from granules into the phagocytic vacuole and outside the cell.

Vertebrate Phagocytes

The innate immune system of mammals is very similar to the immune system of insects, and contains phagocytes which function in a similar manner to plasmatocytes and granular cells in insects by reacting rapidly and defending against infection.[46] There are various functions of the innate immune system in mammals such as recruiting immune cells to the site of infection through the production of cytokines, activation of the complement cascade, recognition and removal of foreign substances by specialized phagocytic cells, presenting antigens on the cell surface to activate the adaptive immune system and acting as a physical and a chemical barrier to pathogens.

Phagocytic cells of the innate immune system include macrophages, neutrophils and dendritic cells. Phagocytic cells have receptors which include Fc receptors, complement receptors, scavenger receptors, and Toll-like receptors on their cell membrane. In order for a phagocyte to engulf a particle or pathogen it extends pseudopods around the particle until it is surrounded and this action eventually results in the particle being engulfed. Once the pathogen has been engulfed it is retained inside an endosome which merges with a lysosome. The lysosome contains enzymes and acids which kill and digest the particle or organism.[47] It has been noted in studies on mammalian phagocytes that O_2^- is not strongly anti-microbial on its own, but is involved in innate defense systems by interacting with nitric oxide (NO) to generate peroxynitrite ($ONOO^-$).[48]

Macrophages. Macrophages are large phagocytic leukocytes capable of moving outside the vascular system by crossing the cell membrane of capillary vessels. Macrophages are the most efficient

phagocytes and can phagocytose large numbers of pathogens. When pathogenic molecules bind to receptors on the surface of the macrophage it triggers the cell to engulf and destroy the pathogen through the activation of a respiratory burst, which causes the release of reactive oxygen species.[49] Encountering pathogens stimulates the macrophage to produce chemokines which causes migration of immune cells to the site of infection. Macrophages have a distinct kidney-shaped nucleus, and can be identified by CD14 cell surface marker. Macrophages differ to neutrophils in that they do not contain distinct granules; instead, they have many lysosomes which are similar in content to the neutrophil granules.

Neutrophils. Neutrophils have numerous granules in their cytoplasm, so are known as granular cells. These immune cells have similar phagocytic characteristics to granular cells and plasmatocytes of insects.[5] Neutrophil production occurs in the bone marrow of normal healthy adults, this production increases when there is infection; similarly in insects, hemocyte production also increases when infection is present.[50] Neutrophil contain different types of granules. The primary granules contain cationic proteins and defensins which kill bacteria, proteolytic enzymes like elastase, cathepsin G to degrade protein, and lysozyme to degrade cell walls (**Fig. 3**).[39] The secondary granules contain lysozyme, components of NADPH oxidase which produce superoxide, lactoferrin, an iron-chelating protein, and B12-binding protein.[50]

Neutrophils, like insect hemocytes, attack pathogens by the activation of a respiratory burst. The main products of neutrophil respiratory burst are powerful oxidizing agents which include hydrogen peroxide, free oxygen radicals, and hypochlorite (**Fig. 3**).[48] Neutrophils are the most common phagocyte and are usually first to the site of an infection. Neutrophils are essential for destroying microorganism but phagocytosis and degranulation can also damage cells and tissues of the host.[50]

Dendritic cells. Dendritic cells (DCs) are central antigen presenting cells (APCs) that present antigen by exogenous and endogenous routes to naïve cells. DCs are derived from progenitor cells in the bone marrow that can mature to the myeloid or lymphoid pathway. The main role of a DC is to capture and process antigens (Ag), before migrating to the site of naïve T and B cells localized in the lymph nodes where they initiate a specific immune response.[51]

DCs utilize receptors to detect microbial components in their immediate environment which enables activation of the adaptive immune response acting to interlink the innate and adaptive immune systems. The recognition of danger signals by immature DCs triggers localization to the site of danger where Ag uptake initiates changes in the phenotype signaled by the expression of co-stimulatory molecules on the DC surface in conjunction with altered homing and chemokine receptors that facilitate migration to the lymph tissues.[52,53]

DCs can phagocytose particles and even cells but this is mostly attributed to less differentiated or immature DCs that have selective phagocytic activity.[54-56] The immature stage of DCs with their phagocytic capability, in contrast to their mature stage, is similar to the innate immune phagocytosing cells such as macrophages and granular cells. Pinocytosis is another means of sampling small soluble molecules in the environment around a DC that involves the uptake of Ag in the fluid phase.[57]

Dendritic cells express similar cell surface molecules to the innate immune cells of mammalian macrophages and polymorphonuclear cells (granulocytes), these have complex carbohydrate binding ability through C-type lectins as macrophage mannose receptor (MMR).[57-60] Similarly insects have C-type lectins that act as pattern recognition receptors including LPS-specific immulectin-2 of *Manduca sexta*, which facilitates phagocytosis of bacteria by selected hemocytes.[61]

Insect Hemocytes and Mammalian Phagocytes: Similarities and Differences

Several aspects of insect and human immune responses exhibit functional similarities which suggests they both use similar effector and receptors, and have similar regulation of gene expression.[7] Various immune proteins in insects demonstrate a high degree of homology to proteins found in mice, such as insect proteins malvolio and dSR-C1 which are similar to mouse natural resistance associated macrophage protein-1 (NRAMP-1).[6]

The phagocytic cells in insects, the plasmatocytes and granular cells have receptors (e.g., calrecticulin) on the surface which are similar to receptors on mammalian neutrophils.[6,9] The phagocytic cells in both insects and humans engulf and kill pathogens and produce superoxide using similar p47 and p67 proteins.[9] Neutrophils require the translocation of proteins $p47^{phox}$ and $p67^{phox}$ from the cytosol to the plasma membrane for the generation of a functional NADPH oxidase complex.[9] This translocation event occurs when the cell is stimulated by exposure to a pathogen and activates flavocytochrome b_{558} which is the redox center of this enzyme system. Hemocytes of *G. mellonella* have been shown to have proteins of 67 and 47kDa homologous to $p67^{phox}$ and $p47^{phox}$ proteins of the superoxide-forming NADPH oxidase complex of neutrophils (**Fig. 3**). The mode of superoxide production is so similar in insect hemocytes and human phagocytes that phorbol 12-myristate 13 acetate (PMA) induced superoxide generation in both. The translocation of hemocyte 47 and 67 kDa proteins from the cytosol to the plasma membrane of *G. mellonella* hemocytes can be suppressed by the fungal secondary metabolite gliotoxin.[9] Gliotoxin causes the same inhibition of translocation in human neutrophils, and thus inhibits the production of superoxide.[62,63] Fumagillin is also produced by *A. fumigatus* and was demonstrated to inhibit phagocytosis, superoxide production and degranulation in human neutrophils and *G. mellonella* hemocytes.[64,65] Its primary mode of action was the inhibition of the conversion of G- to F-actin in both cell types.[64,65]

In order for the phagocytic cells to engulf and kill pathogens, they need to be able to recognize them. Human immune cells express several Toll-like receptors (TLR), which directly recognize lipopolysaccharide and other components of the pathogen. These TLR are considered cellular pattern recognition receptors (PRRs). The systems that mediate *Drosophila* Toll and mammalian IL-1 receptor-mediated signaling are very similar in structure and function.[66] Toll induces the production of antimicrobial

Table 1. Summary of similarities between insect hemocytes and human neutrophils

	Hemocytes	Neutrophils
Phagocytosis	Lectin-mediated	Lectin-mediated
ROS	O_2^-, H_2O_2, NO^-	O_2^-, H_2O_2, NO^-
Degranulation	Yes	Yes
AMPs	Peroxynectin, transferrin, lysozyme, defensin	MPO, transferrin, lysozyme, defensin
Receptors	TLRs, B-1,3-glucan, IL-IR	TLRs, B-1,3-glucan, IL-IR
Transcription factors	NFκB, IκB	NFκB, IκB
Cascades	IMD, JNK, JAK-STAT	IMD, JNK, JAK-STAT
Kinases	p38 MAPK, ERK, PKC, PKA	p38 MAPK, ERK, PKC, PKA
Neutrophil extracellular nets (NET)	NET-like structures present	NETs present

peptides (e.g., drosomycin), an essential component of the insect immune response.[14] The Toll and immune deficiency (IMD) pathways in insects activate two distinct NFκB-like transcription factors, which is attributed to the production of AMPs.

Human Toll, like *Drosophila* Toll protein (Toll), is a type I transmembrane protein that has an extracellular domain consisting of a leucine-rich repeat (LRR) domain and an intracellular domain that is homologous to the cytoplasmic domain of the human interleukin IL-1 receptor.[66] *Drosophila* Toll and the IL-1 receptor signal through the NFκB pathway. Depending on the stage of the development in *Drosophila* the Toll protein serves different functions. In the embryonic stage it controls dorsal–ventral patterning and the activation of transcription factor Dorsal when it is bound to its ligand Spätzle. In the adult *Drosophila* the Toll/Dorsal signaling pathway is involved in antifungal immune response.[66]

The pathway induced by the IL-1 receptor (IL-IR) in mammalian cells mirrors the signaling pathway through Toll: IL-IR signals through the NFκB pathway and Dorsal and its inhibitor Cactus are homologous to NFκB and IκB proteins, respectively.[66] A pattern recognition molecule in insects, apolopophorin III (apoLp-III) is similar to apolipoprotein E (apoE) found in mammals which is involved in LPD detoxification and phagocytosis.[67] The production of a wide range of antimicrobial peptides, which are crucial in combating infection, is similar in vertebrates and invertebrates (**Table 1**).[2]

Neutrophils produce extracellular traps (NETs) containing nucleic acids and proteins to immobilize and kill pathogens.[68] A similar process has been observed to occur in hemocytes of *Galleria* in response to infection and has the effect of increasing the survial of the host.[69] Interestingly the introduction of bacteria into *Galleria* larvae expressing nucleic acid hydrolysing enzymes allows the pathogen to escape entrapment and thus reduce the survival of the host.

Human neutrophils and insect granular cells respond to inhibitors in a similar manner thus highlighting another layer of similarities between the two cell types. The addition of the NADPH oxidase inhibitor diphenyleneiodonium chloride inhibited superoxide production and halted microbial killing in both hemocytes of *G. mellonella* and in human neutrophils.[9] Cytochalasin b and nocodazole affect the activity and function of both neutrophils and hemocytes. In neutrophils these inhibitors disrupt the F-actin assembly which affects phagocytosis, the formation of NADPH-oxidase and degranulation.[70-72] Exposure of *G. mellonella* larvae to these inhibitors increased their susceptibility to infection, inhibited the phagocytosing ability of hemocytes and decreased the rate of F-actin formation in hemocytes, however the viability of the hemocytes was unaffected.[73]

Structurally insect granular cells are slightly larger than neutrophils and do not have a multi-lobed nucleus. Hemocytes have a very granular cytoplasm compared with neutrophils.[74] There is a major difference between mammalian Toll-like receptors (TLRs) and the *Drosophila* Toll protein, in that *Drosophila* Toll is not a pattern recognition receptor (PRR); instead, it binds to the endogenous messenger Spätzle for the signaling cascade to occur.[8] Recognition of the pathogen occurs upstream of Toll by secreted receptors which bind to their ligand and cause a cascade of serine proteases and cleavage of Spätzle; this forms dimers that bind to and activate Toll.[8] In mammals, the expression of TLR is limited to immune-responsive cells; however, in insects expression is not only limited to immune cells.[8] *Drosophila* has nine Toll-like genes; only Toll has known immune functions. The expression of these genes is regulated in different tissues during embryogenesis, suggesting most Toll-like genes have developmental roles.[8,75]

Conclusion

The use of insects in place of mammals as in vivo screening systems has dramatically changed the speed with which in vivo data on microbial pathogens or novel antimicrobial agents can be generated. While this is a very welcome development and has facilitated the rapid and cost-effective characterization of the virulence of microbial pathogens it is only possible because of the strong similarities between the insect and mammalian innate immune responses. One effect of this development has been the increasing interest in insect immunity and in the evolutionary relationships between it and the innate immune system of vertebrates. Recent work has begun to extend the use of *Galleria* larvae, in particular, beyond being only an effective alternative in vivo screening system to mammals and may represent a novel application with great potential for modeling disease processes. Mukherjee et al. have demonstrated similar brain pathologies in *Galleria* larvae as

occur in mammals infected with *Listeria* and have suggested that *Galleria* could be a model for studying microbially induced brain disease pathologies.[31] The potential now exists to further explore the immunological and developmental similarities between insects and mammals and utilize *Galleria* larvae as a model for studying a range of neurological diseases.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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