Cell Line Support Arthropod Immunity

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Many insect and tick species are serious pests, because insects damage crop plants and, along with ticks, transmit a wide range of human and animal diseases. One way of controlling these pests is by impairing their immune system, which protects them from bacterial, fungal, and viral infections. An important tool for studying immunity is using long-lasting cell cultures, known as cell lines. Cell line research is a powerful method for understanding how invertebrates mount defenses against pathogenic organisms and testing hypotheses on how these responses occur. In particular, immortal arthropod cell lines are valuable tools, providing a tractable, high-throughput, cost-effective, and consistent platform to investigate the mechanisms underpinning insect and tick immune responses. Here we review how cell line research results inform the controls of medically and agriculturally important insects and ticks.

Keywords: innate immunity ; antimicrobial peptide ; RNAi ; lysozyme ; pathogen ; signaling pathway

1. Pathogen-Associated Molecular Patterns (PAMPs), Pattern Recognition Receptors (PRRs), and Opsonins

Invertebrate cell lines have been used to identify pathogen-associated molecular patterns (PAMPS) (**Table 1**). Ha Lee et al. [1] reported that bacterial peptidoglycans (PGN) were more potent activators of the antimicrobial protein (AMP) gene cecropin B (CecB) than lipopolysaccharides (LPS) in the *Bombyx mori* cell line NISES-BoMo-Cam1. They found that PGN from *Escherichia coli* stimulated the expression of several antibacterial peptide genes and other genes, whereas PGN from *Micrococcus luteus* activated a few genes. They showed that *E. coli* PGN or cells elicited a higher expression of the peptidoglycan recognition protein gene involved in the prophenoloxidase activation pathway compared to *M. luteus* PGN or cells. Using the mosquito cell line C6/36, Mizutani et al. [2] reported the constitutive expression of low levels of two AMPs, cecropin and defensin, which were upregulated by exposure to lipopolysaccharides.

Shaw et al. [3] found that two lipids isolated from bacteria-infected cells, 1-palmitoyl-2-oleoyl-snglycero-3-phosphoglycerol (POPG) and 1-palmitoyl-2-oleoyl diacylglycerol (PODAG), stimulated the IMD pathway in ISE6 tick cells (**Table 2**). They also reported that exposing the cells to these lipids protected the tick cells from infection by two rickettsia-related bacteria, *Anaplasma phagocytophilum* and *A. marginale*.

Table 1. Examples of the insect cell lines used in immune-related studies.

Order	Species of Origin	Stage/Tissue of Origin	Cell Line Designation	Research Focus	References
Coleoptera	***Anthonomus grandis***	**Embryo**	**BRL-AG-1**	**Humoral Responses**	[4]
	A. grandis	**Embryo**	**BRL-AG-3A**	**Humoral Responses**	[4]
	A. grandis	**Embryo**	**BRL-AG-3C**	**Humoral Responses**	[4]
	Tribolium castaneum	**Pupa/Adult**	**BCIRL-TcA-CLG1**	**Signaling Pathways**	[5]

Order	Species of Origin	Stage/Tissue of Origin	Cell Line Designation	Research Focus	References
Diptera	*Aedes aegypti*	Neonate larva	Aag-2	Signaling Pathways, Cellular Responses, Humoral Responses	[4][6][7][8][9][10][11][12][13][14][15]
	Ae. aegypti	Neonate larva	AF5 and subline AF319	Signaling Pathways	[10][12]
	Ae. aegypti	Neonate larva	ATC-10 (CCL-125)	Signaling Pathways, Humoral Responses	[9][12][16][17]
	Aedes albopictus	Neonate larva	C6/36	PAMPs, Signaling Pathways, Humoral Responses	[2][9][11][12][13][18][19][20][21][22][23][24]
	Ae. albopictus	Neonate larva	C6/36 HT	Signaling Pathways	[6]
	Ae. albopictus	Neonate larva	C7-10	Signaling Pathways, Cellular Responses, Humoral Responses	[4][9][12][25][26][27]
	Ae. albopictus	Neonate larva	U4.4	Signaling Pathways	[9][11][12][28]
	Anophelesgambiae	Neonate larva	4a-2	Humoral Responses	[4]
	A. gambiae	Neonate larva	4a-3A	Humoral Responses	[4][29]
	A. gambiae	Neonate larva	4a-3B	Humoral Responses	[4]
	A. gambiae	Neonate larva	Sua1B	Signaling Pathways, Cellular Responses, Humoral Responses	[4][29][30]
	A. gambiae	Neonate larva	Sua5.1*	Opsonins, Signaling Pathways	[31]
	Anopheles stephensi	1st Instar larva	4a-3A	Signaling Pathways	[29]
	A. stephensi	1st Instar larva	4a-3B	Signaling Pathways	[32]
	A. stephensi	1st Instar larva	LSTM-AS-43 (MSQ43)	Signaling Pathways, Humoral Responses	[29]
	Culex quinquefasciatus	Ovary (adult)	Hsu	Signaling Pathways	[23]
	Culex tarsalis	Embryo	CT	Signaling Pathways	[23]
	Lutzomyia longipalpis	Embryo	LL5	Signaling Pathways	[33][34]
	Sarcophaga peregrina	Embryo	NIH-Sape-4	Humoral Responses	[4][35]
Hemiptera	*Anasa tristis*	Embryo	BCIRL-AtE-CLG15A	Signaling Pathways	[5]

Order	Species of Origin	Stage/Tissue of Origin	Cell Line Designation	Research Focus	References
Lepidoptera	*Opodiphthera (Antheraea) eucalypti*	Pupal ovaries	Ae	Cellular Responses	[36]
	Bombyx mori	Ovary (larval)	Bm5	Signaling Pathways	[22][37][38]
	B. mori	Ovary	BmN	Signaling Pathways	[39]
	B. mori	Ovary	BmN4	Signaling Pathways	[22]
	B. mori	Ovary (larval)	BmN-SWU1	Signaling Pathways	[40]
	B. mori	Ovary	NISES-BoMo-Cam1	PAMPs, Signaling Pathways	[1]
	Choristoneurafumiferana	Midgut (larval)	IPRI-CF-1	Signaling Pathways	[41]
	C. fumiferana	Midgut	FPMI-CF-203	Signaling Pathways	[22]
	Chrysodeixis (Pseudoplusia) includens	Embryo	UGA-CiE1	Cellular Responses, Humoral Responses	[42]
	Estigmene acraea	Hemocyte (larval)	BTI-EA-1174-A	Cellular Responses, Humoral Responses	[4][43]
	Helicoverpa zea	Ovary (pupal)	BCIRL-HzAM1	Signaling Pathways, Humoral Responses	[44][45][46][47]
	H. zea	Midgut (larval)	RP-HzGUT-AW1	Signaling Pathways	[22]
	Helithis virescens	Ovary (pupal)	BCIRL-HvAM1	Signaling Pathways	[5]
	Lymantria dispar	Ovary (pupal)	IPLB-Ld-652Y	Cellular Responses	[48]
	Malacosoma disstria	Hemocyte (larval)	IPRI-Md-66	Signaling Pathways, Cellular Responses, Humoral Responses	[49]
	M. disstria	Hemocyte (larval)	IPRI-Md-108	Signaling Pathways, Cellular Responses, Humoral Responses	[49]
	Manduca sexta	Embryo	MRRL-CHE-20	Signaling Pathways	[41]
	Perina nuda	Ovary (pupal)	NTU-Pn-HH	Cellular Responses	[48]
	Plodia interpunctella	Unspecified	KSU-P15.3	Signaling Pathways	[41]
	Spodoptera exigua	Embryo/neonate larva	Se301	Cellular Responses	[36]
	S. exigua	Hemocyte (larval)	SeHe920-1a	Cellular Responses	[36]
	S. exigua	Neonate larva	UCR-Se-1	Cellular Responses	[48]
	Spodoptera frugiperda	Ovary (pupal)	IPLB-Sf-5-5C	Cellular Responses	[48]
	S. frugiperda	Ovary (pupal)	Sf21	Signaling Pathways	[22]
	S. frugiperda	Ovary (pupal)	Sf9	Signaling Pathways, Humoral Responses	[50][51][52][53][54]
	S. frugiperda	Ovary (pupal)	Sf9-SF (serum-free)	Signaling Pathways	[22]
	Spodoptera littoralis	Ovary	Sl2	Signaling Pathways	[22]
	Spodoptera litura	Ovary (pupal)	IBL-Sl-1A	Cellular Responses	[48]
	Trichoplusia ni	Embryo	High Five (BTI-TN-5B1-4)	Signaling Pathways, Cellular Responses	[22][36][38][54][55]
	T. ni	Embryo	High Five-SF (serum-free)	Signaling Pathways, Humoral Responses	[22]

Table 2. Examples of the tick cell lines used in immune-related studies.

Order	Species of Origin	Stage/Tissue of Origin	Cell Line Designation	Research Focus	References
Parasitiformes	*Amblyomma americanum*	Embryo	AAE2	Cellular Responses	[56]
	Amblyomma variegatum	Molting larva	AVL/CTVM17	Cellular Responses	[57]
	Dermacentor andersoni	Embryo	DAE15	Cellular Responses	[58][59]
	D. andersoni	Embryo	DAE100	Humoral Responses	[59]
	Hyalomma anatolicum	Embryo	HAE/CTVM8	Cellular Responses	[57]
	Ixodes ricinus	Embryo	IRE/CTVM19	Cellular Responses	[57][60][61]
	Ixodes scapularis	Embryo	IDE8	Cellular Responses, Humoral Responses	[57][61]
	I. scapularis	Embryo	IDE12	Cellular Responses	[56][58]
	I. scapularis	Embryo	ISE6	PAMPs, Signaling Pathways, Cellular Responses	[3][57][58][62]
	Ixodes ricinus	Embryo	IRE/CTVM19	Humoral Responses	[60][61]
	Rhipicephalus appendiculatus	Molting nymph	RA243	Cellular Responses	[57]
	R. appendiculatus	Embryo	RAE/CTVM1	Cellular Responses	[57]
	Rhipicephalus (Boophilus) microplus	Embryo	BME/CTVM2	Cellular Responses	[57]
	R. microplus	Embryo	BME26	Cellular Responses, Humoral Responses	[63]

A key attribute of arthropod innate immunity is to recognize foreign threats. Pattern recognition receptors (PRRs) are a class of receptors that recognize PAMPs [64][65][66]. Consequently, PRRs initiate the intracellular signaling cascades central to the organism's defense to pathogenic incursions [67][68]. *D. melanogaster* cell lines have been primarily used for the identification and study of pattern recognition receptors (PRRs) [66][69]. Moita et al. [31] investigated the role of integrins (specifically, the Arg.Gly.Asp-recognizing receptors) in phagocytosis using cell line 5.1* from *Anopheles gambiae*. Growing these cells in suspension elevated their ability to engulf bacteria compared to the attached cultures, suggesting that the adhesion and phagocytosis processes share receptors. This helped identify a new integrin gene involved in phagocytosis, BINT2. Their study confirmed that the glycoprotein TEP1 (thiol-ester motif-containing protein-1) is not a phagocytic receptor but an opsonin that indirectly promotes phagocytosis.

2. Signaling Pathways and Signaling Molecules Involved in Antimicrobial and Antiviral Responses

2.1. Phagocytosis Related Signaling Pathways

The recognition of foreign PAMPs activates several signaling pathways that stimulate cellular and humoral responses. Mizutani et al. [2] investigated a phagocytosis signaling pathway in the mosquito cell line C6/36 using fluorescein-labeled spheres or bacteria. They reported that exposing the cells to the JNK-specific inhibitor SP600125 led to reduced sphere/bacteria uptake. Similarly, incubating cells with the same inhibitor led to a reduced accumulation of acridine

orange, as well as the uptake of the West Nile virus. Hence, the JAK/STAT signaling pathway acts in phagocytosis, endocytosis, and virus entry in mosquito cells.

Trujillo-Ocampo [6] used the cell lines C6/36 HT from *Aedes albopictus* and Aag-2 from *Ae. aegypti* to investigate the 14-3-3ε and 14-3-3ζ protein actions in phagocytosis. The 14-3-3 proteins interact with protein partners as adapters, activators, and repressors, and they are involved in regulating signaling pathways and other cellular processes. Using RNAi to decrease protein expression in Aag-2 cells, they recorded changes in the cytoskeleton organization and decreased phagosome maturation and phagocytosis.

2.2. Signaling Pathways Associated with Antimicrobial Humoral Responses

Using the mosquito cell lines Sua1B and 4a3a and MSQ43, combined with RNAi, Luna et al. [29] showed that the expression of two antimicrobial peptide (AMP) genes gambicin (*gam1*) and defensin (*def1*) were regulated by the IMD pathway. They determined that overexpression of the NF-κB transcription factors involved in the IMD pathway (designated as Relish 2 or REL2) and the Toll pathway (designated as REL1) stimulated the expression of cecropin (*cec1*), *gam1*, and *def1*. These findings suggest there is crosstalk between the Toll and IMD pathways within the cells.

Barletta et al., 2012 [7] incubated Asg-2 cells with a variety of immune stimuli, including Gram-positive or Gram-negative heat-inactivated bacteria, fungal zymosan, or the Sindbis virus, and used qPCR to quantify the expression of genes specific in the Toll, IMD, and JAK/STAT pathways. Gram-positive/negative bacteria and zymosan stimulated the expression of key markers of both the Toll (cactus) and IMD (REL2) pathways. For the JAK/STAT pathway, bacteria increased the expression of a thiol-ester motif-containing protein, TEP, and the virus upregulated expression of the transcription factor STAT in Aag-2 cells.

Zhang et al. [8] exposed the Aag2 cell line to a variety of microbes, including Gram-negative and Gram-positive bacteria and fungi, and found an upregulation of a number of AMPS (including three defensins, six cecropins, and gambicin). They identified the specific pathways involved in AMP induction. Most AMP genes activated by the Gram-negative bacterium were regulated by the IMD pathway. Gambicin was controlled by the combination of all three pathways (IMD, Toll, and JAK/STAT). Their findings differed from *D. melanogaster*, which regulate AMP expression primarily through the Toll and IMD pathways.

The embryonic cell line LL5 was used to investigate the Toll and IMD pathways in the sandfly *Lutzomyia longipalpis* [33]. The investigators silenced the repressor genes for the Toll and IMD pathways (*cactus* or *dorsal* for Toll and *caspar* or *relish* for IMD), then exposed the cell line to heat-killed bacteria, yeast, or live protozoa. The cells exhibited increased expression of AMP genes after each pathway repressor was silenced. After the cells were incubated with *E. coli*, the authors noted increases in the mRNAs encoding *cactus, caspar*, *cecropin*, and *defensin 2*, (but not *attacin*) and decreases in the mRNAs encoding *dorsal* and *relish*. Similar findings followed exposure to two other bacteria and a yeast. Likewise, live protozoa challenges led to the upregulation of *cactus* with no change in *caspar* and increased the expression in both the *dorsal* and *relish* levels.

The Toll pathway was studied using the lepidopteran cell line BmN-SWU1, as well as the dipteran cell line S2 [40]. The S2 cells were co-transfected with vectors from the Toll-interleukin-1 receptor domains from the *Bombyx mori* Toll family members, which showed that BmToll11 and BmToll9–1 can activate the *drosomycin* and *diptericin* promoters. The overexpression of the Toll-interleukin-1 receptor domains in *B. mori*, BmN-SWU1, cells resulted in the upregulation of a variety of AMPs, and their silencing led to the inhibition of AMP expression. In vivo experiments confirmed that BmToll11, BmToll9–1, and five Spätzle genes were upregulated in *B. mori* larvae after an infection by *E. coli* and *Staphylococcus aureus*. One of the *BmSpz* genes, *BmSpz2*, interacted with BmToll11 and BmToll9–1. This study provided evidence that *Toll* and *Spz* act in the *B. mori* innate immunity.

Shaw et al. [3] reported that the x-linked inhibitor of the apoptosis protein (XIAP), which discourages rickettsia bacterium infections, is linked to the IMD pathway in a tick cell line. They also showed that Bendless directly interacts with XIAP. Silencing the *uev1a* and *bendless* genes led to an increased bacterial load. The authors conducted a detailed phylogenic analysis, from which they proposed two functionally distinct IMD pathways in ticks and insects.

Rao et al., 2011 [50] described how the activities of the AMP gene promoters from different species regulate the expression of these genes in a species-specific manner in S2 and Sf9 cells (from *Spodoptera frugiperda)*. They suggested that transcription complexes using common nuclear factors are combined with species-related coregulators and that these are responsible for the species-specific regulation of AMP gene expression.

Muller et al. [32] reported that the 4a-3B line from *A. gambiae* constitutively expressed six pathway (PPO) genes. LaPointe et al. [49] reported that two lepidopteran cell lines derived from hemocytes Md-66 and Md-108 generated intracellular melanin in response to the bacterium *Bacillus subtilis*. The cells did not release PO into the medium during the early stages of infection, but longer incubations led to the formation of a melanotic coagulum around the granular-like cells.

Braconid wasps transmit immunosuppressive bracoviruses when they parasitize their hosts. Beck and Strand [55] used the lepidopteran cell line High Five to elucidate the mechanism of the *Microplitis demolitor* bracovirus. They reported that a conditioned medium from virus-infected High Five cells blocked the melanization of bacteria-challenged *Manduca sexta* plasma. After the *egf1.0/1.5* (a putative melanization inhibitor based on a sequence analysis) expression was silenced, the conditioned medium no longer inhibited the melanization. Additionally, High Five cells were used to determine the virus viability. The bracovirus viability declined in the presence of PO but was unaffected when Egf1.0 was present. The authors concluded that activated PO is directly responsible for reducing the virus and parasitoid viability.

A functional PO cascade occurred in a mosquito cell line U4.4 from *Ae. albopictus* [28]. Exposing the cells to *E. coli* or the arbovirus Semliki Forest virus (SFV) led to an increased medium melanization, which was correlated with a reduced virus viability. They produced a recombinant SFV that expressed the PO pathway blocker Egf1.0 and noted that its expression enhanced the spread of the virus. U4.4 cells are morphologically similar to oenocytoids, the primary source of PO in mosquito plasma.

2.3. Eicosanoid-Related Signaling

Prostaglandins (PGs), a group within eicosanoids, mediate the immune responses and other physiological processes in arthropods [70][71]. PGs are synthesized in vertebrates by cyclooxygenases, although, in insects, the enzymes have been recently identified as peroxidases [72][73]. Barbosa and Rebello [18] reported that prostaglandin A1 (PGA_1) mediates the synthesis of stress proteins during cells' lag phase (specifically, HSPs 27, 29, 70, 80, and 87 kDa) in the C6/36 *Ae. albopictus* cell line. When the cells were in the exponential and stationary phases, PGA_1 induced fewer HSPs and in lower quantities. Similarly, de Meneses et al. [19] confirmed PGA_1 mediates the increased HSP synthesis and reported that PGA_1 also increases the synthesis of HSPs 23 and 57. These two HSPs are primarily regulated by PGA_1, not heat shock. Treating the cells with an inhibitor of cyclooxygenase, aspirin, did not influence the HSP70 levels when the cells were maintained at their standard growth temperature (28 °C) but upregulated their synthesis at a higher temperature of 37 °C. HSPs act in a variety of immunoregulatory roles at the intracellular and extracellular levels [74][75].

Later studies showed that a variety of PGs influence the up- and downregulation of proteins of differing functionalities, including those involved in defensive responses [44][45]. The lepidopteran cell line BCIRL-HzAM1 was treated with either PGA_1 or PGE_1, followed by a proteomic and bioinformatic analysis [44]. The authors reported changes in the expression of 34 proteins, with functionalities involving the protein action, lipid metabolism, signal transduction, protection, cell functions, and metabolism. Of these, significant changes in the HSP levels were influenced by both PGs, including the up- and downregulation of the HSP70 levels. The expression of the proteins involved in cell defense included the antioxidants superoxide dismutases (Mn and Cu/Zn) and glutathione-S-transferase. These enzymes act in the cellular host defense against reactive oxygen species, which are often elevated as part of the innate immune response [76]. A follow-up study working with the two-series PGs was performed using the same cell line [45]. The incubation with PGA_2 influenced the expression of 60 proteins, whereas the PGE_2 and PGF_{2a} treatments influenced a few proteins. The expression of the antioxidant proteins were altered—specifically, thiol peroxiredoxin; glutathione S-transferase; and the heat shock-related proteins (including heat shock cognate 70, HSP20.7, and HSP60). Other immune-related proteins affected included the growth blocking peptide-binding protein and lipopolysaccharide-binding protein.

PGs also influence the post-translational modification of selected proteins [46]. The HzAM1 cells were again exposed to PGs (PGA_2, PGE_1, or $PGF_{2\alpha}$) for shorter time periods (20–40 min). Significant modifications in the phosphorylation levels occurred in 31 proteins, with decreased levels in 15, increased levels in another 15, and one protein with either increased or decreased phosphorylation, depending on the specific PG treatment. Changes in the phosphorylation of five HSPs were recorded (e.g., HSP60, HSP70, and HSP90), as well as that of two proteins that regulate HSP activities (Hsc-70-interacting-protein and DnaJ homolog shv).

Johnson and Howard [41] recorded the impacts of selected eicosanoid synthesis inhibitors on the responses of three lepidopteran cell lines (MRRL-CHE-20 from *M. sexta*, IPRI-CF-1 from *Choristoneura fumiferana*, and KSU-P15.3 from *Plodia interpunctella*) to the *Bacillus thuringiensis* cryproteins, CryIA(c) and CryIC. They documented the influence of the inhibitors on the cell viability. This was similar to Li et al. [5], who reported that PG synthesis inhibitors, as well as PGs themselves, lead to either reduced cell numbers or cell death in a concentration-dependent manner in cell lines from three insect orders. Johnson and Howard [41] reported that the inhibitors that decreased the cell viability lessened the toxicity of

the endotoxins. They performed in vivo experiments with these compounds and found that they were nontoxic to larvae and that the lipoxygenase inhibitor, nordihydroguaiaretic acid, decreased the Cry1Ac toxicity. The authors suggested that the antioxidant activities, potentially involving eicosanoids, are part of the insect responses to endotoxins.

Burlandy et al. [20] showed the involvement of PGA_1 on virus replication in the mosquito cell line C6/36. They reported a dose-dependent reduction of the vesicular stomatitis virus (VSV) in this cell line, recording up to a 95% decrease with 8-µg PGA_1/mL. PG-treated cells increased the expression of HSP70, although VSV plus PG-treated cells exhibited reduced HSP70 levels. These studies strongly support the findings that PGs act in insect immunity at the whole-animal level [70][71].

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