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# INSIGHTS FROM MODEL SYSTEMS
# *Drosophila* Immune Responses as Models for Human Immunity

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Why do insects need immune defenses? They are so small and multiply so rapidly that it is understandable to think that, although infected individuals might die, the species could survive by sheer reproductive capacity. The fallacy of this reasoning is in the numbers. Under optimal conditions, bacteria double every 20 min or so, whereas even the rapidly maturing (and favorite model) insect, the fruit fly *Drosophila melanogaster,* requires close to 2 wk to reproduce. If insects were unable to fight off bacterial infections, they would never survive to reproductive maturity. Study of insect immunity has revealed striking similarities with human immunity, making insects—and *Drosophila,* in particular—useful model systems.

## Infection: Ancient Problem, Ancient Solution

The origins of immunity may go back to the earliest competition for limited resources; even bacteria release antibiotic peptides that kill other species of bacteria. With the development of multicellularity came the need to recognize and respond to pathogenic microbes, and the defense mechanisms that arose early in metazoan evolution have persisted, since the threat of attack and infection has never subsided. Since evolution conserves both functional molecules and biochemical pathways, it is not surprising that animal genera as widely separated as *Homo* and *Drosophila* share the basic signaling events and molecular machinery to respond to injury and microbial infections. Even plants use similar mechanisms to detect and signal injury and infection (Yang et al. 1997), a finding supporting the ancient origin of these defense systems.

The three essential features of an immune response are pathogen recognition, speed, and potency. Pathogen recognition occurs through cell surface receptors that have been evolutionarily selected to recognize general but essential components in the pathogenic organisms that are not shared by host cells, thereby providing the critical distinction between self and non-self (Janeway 1992; see also Ezekowitz 1997 [in this issue]). These receptors are constitutively expressed by the cells on the front line of defense, so recognition is immediate. The subsequent cellular responses of macrophages and natural killer cells, as well as the secretion of antimicrobial compounds by the epidermis, gut, and airways, are all effective in the elimination of pathogens.

In vertebrates this rapid general response to pathogens has been overlaid with an acquired response. Acquired immunity offers two advantages: exquisite specificity, driven by clonal selection of antibodies and T-cell receptors; and memory, the ability to respond rapidly on reexposure to antigen. However, clonal selection takes time, and thus it is not an effective initial defense against infection. As has been pointed out in a recent review, the clonally selected specificity of the acquired response is complemented by the evolutionarily selected efficacy of the innate response. The clonal response retains the immune memory of the individual, but the innate response retains the evolutionary memory of the species (Fearon 1997). Equally important, the innate response plays an essential role in the regulation of the clonal response (Fearon 1996).

## Insect Immunity

As in vertebrates, the response of insects to microbial infection has both a cellular and a humoral component. Although little is known about the cellular response at the molecular level, it is mediated by hemocytes, cells that circulate in the hemolymph, the arthropod equivalent of blood. Three classes of hemocytes have been defined in *Drosophila*: plasmatocytes, lamellocytes, and crystal cells. Plasmatocytes are the major cell type in larval hemolymph. They are a proliferative population of phagocytic cells. They also secrete antimicrobial peptides into the hemolymph and are hypothesized to send a signal to the primary source of antimicrobial peptides, the fat body. This behavior is much like that of tissue macrophages, neutrophils, and monocytes, the phago-

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cytic vertebrate immune cells that engulf pathogenic microbes and secrete stimulatory cytokines. Lamellocytes are responsible for encapsulating pathogenic organisms such as bacteria, fungi, and parasitoid eggs or larvae. Crystal cells contain the components of the prophenol oxidase cascade that are responsible for melanizing encapsulated pathogens and cuticular wounds. P-element–enhancer detection, a powerful genetic technique for the identification of gene expression in situ, recently has been applied to immune tissues in *Drosophila* embryos (Rodriguez et al. 1996) and larvae (Braun et al. 1997). These studies confirm that hemocytes are not homogeneous and that different genes are expressed among and within different classes of hemocytes.

The humoral response has been far more amenable to molecular analysis; for a general review, see the work of Faye and Hultmark (1993) and Hoffmann and Reichhart (1997). In response to infection, a battery of antimicrobial peptides are secreted into the hemolymph. Antibacterial peptides (e.g., cecropin, diptericin, defensin, and drosocin), bacteriostatic peptides (e.g., attacin), and antifungal peptides (e.g., drosomycin) have been identified in a variety of insects (Cociancich et al. 1994). These peptides are synthesized by hemocytes and the fat body, which has been compared to the vertebrate liver. The regulation of antimicrobial gene expression is currently an area of intense study. Expression is regulated primarily at the level of mRNA transcription: prior to infection, most mRNA levels are at or below the limit of detection, but transcripts accumulate rapidly, $\leqslant 30$ min after infection.

In their studies of insect immune responses, Sun and Faye (1992) characterized the promoter of a *cecropin* gene and recognized a Rel/NF$\kappa$B-binding ($\kappa$B) site. They further demonstrated that protein-factor binding to this $\kappa$B site correlates with immune induction of the *cecropin* gene. $\kappa$B sites have since been found in the promoters of all of the insect antimicrobial peptide genes studied to date, and the integrity of these sites is necessary for inducible immune expression. Rel proteins are therefore critical for immune responsiveness.

Rel proteins function as dimers and form a family of transcription factors whose activity is regulated at the level of nuclear entry. The mammalian transcription factor NF$\kappa$B is the best known of these. NF$\kappa$B is a central regulator of inflammation and immunity, also acting in apoptosis, oncogenesis, growth, differentiation, and HIV infection. Rel dimers are held inactive in the cytoplasm, in association with inhibitory proteins, I$\kappa$Bs. When an extracellular signal is received, I$\kappa$B is phosphorylated and proteolytically degraded, releasing NF$\kappa$B to move into the nucleus and to activate target-gene transcription (reviewed by Baeuerle and Baltimore 1996). Because no protein synthesis is required to activate transcription, this signaling cascade can generate a rapid response.

## Rel Cascades in *Drosophila*

Evidence of a Rel protein signaling cascade in *Drosophila* was first discovered in screens for maternally expressed genes involved in embryonic dorsal/ventral polarity. The *dorsal* gene encodes a Rel protein that is found in the cytoplasm of all the cells of the early embryo. It moves to the nucleus in a signal dependent manner. An extracellular signal, encoded by the *spätzle* gene, binds to a membrane-bound receptor, encoded by the *Toll* gene. The extracellular domain of Toll consists of leucine-rich repeats (LRRs), flanked by cysteine-rich regions. LRRs are found on a wide variety of proteins involved in some form of protein-protein interactions, such as receptors and adhesion molecules. The cytoplasmic domain of Toll shows striking similarity to the cytoplasmic domain of the mammalian interleukin 1 type I receptor (IL-1RI). Spätzle binding to Toll causes activation of a signal-transduction cascade involving a novel protein, Tube, and a serine/threonine kinase, Pelle. This leads to the phosphorylation and subsequent degradation of Cactus, an I$\kappa$B homologue. In the absence of Cactus, Dorsal is able to move into the nucleus, where it acts as a transcription factor to initiate the expression of zygotic genes required for ventral-cell fates, such as mesoderm. The Dorsal signaling pathway recently has been reviewed by (Belvin and Anderson 1996). Lemaitre et al. (1996) have shown that components of the Dorsal-Rel protein-signaling cascade—*spätzle, Toll, tube, pelle,* and *cactus*—also act in the immune response, preferentially activating the antifungal defense.

Because Rel factors function as dimers and, frequently, as heterodimers, Ip et al. (1993) searched for potential partners for Dorsal. They identified Dif, a second *Drosophila* Rel protein, and found that it was expressed not in the embryo but in the larval fat body, where Dorsal is also expressed. A third Rel factor, Relish, was isolated in a molecular screen for genes whose expression is altered after infection. Like the two mammalian regulatory proteins p100 and p105, but unlike Dorsal and Dif, Relish is a compound protein that contains both Rel (activating) and I$\kappa$B (inhibitory) domains (Dushay et al. 1996). We hypothesize that, as with p100 and p105, the Relish inhibitory domain is proteolytically degraded to release an active Rel protein.

There is now evidence that a second distinct Rel cascade is acting in immunity. We have demonstrated that 18-Wheeler, a receptor whose structure is similar to that of Toll, regulates Dif nuclear entry and that this is necessary for the full induction of *attacin* expression (Williams et al. 1997). These striking results suggest a far more complex response than previously had been anticipated. Although septic injury stimulates responses to both bacteria and fungi, mutants in the Toll cascade are unable to mount a robust antifungal response, whereas

mutants in 18-Wheeler are unable to mount a robust antibacterial response. Indeed, as has been shown by Ferrandon et al. (1997), flies subjected to natural infection with fungi, rather than to septic injury, mount a surprisingly specific antifungal response. Thus, contrary to what was previously thought, insects do not mount a simple all-or-nothing response to infection. Different elicitors can lead to specific responses, and different signaling cascades are responsible for the regulation of distinct but overlapping sets of antimicrobial peptides. Components of these known pathways and perhaps of additional, unknown signaling pathways remain to be discovered in the fly.

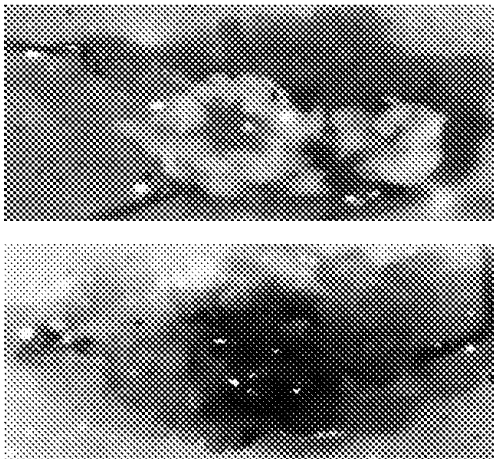
## Why *Drosophila?*

The strength of the *Drosophila* model comes from the combination of molecular, genetic, and, increasingly, genomic techniques that this system provides. It should be possible to isolate mutant flies with specific defects in the immune response and rapidly to identify the affected genes (see sidebar). It is also possible to identify new genes by features of either their sequence or their expression patterns and then to characterize their function. PCR-based differential display, for example, a technique originally developed to identify differentially expressed genes in oncogenically transformed cells (Liang and Pardee 1992), has proved to be a powerful means of isolating both antimicrobial peptide genes (Åsling et al. 1995) and regulatory genes (Dushay et al. 1996). Discovering the function of a cloned gene can be challenging, but in *Drosophila* the methods for moving from gene to mutation are well established. As in the case of human genes, one determines the chromosomal location of the gene of interest and scans the ever-expanding database for previously identified mutations. If none of these affect the gene of interest, P-elements known to have inserted nearby can be mobilized to hop locally into the gene itself—or close enough to generate mutation through imprecise excision. Proof that mutant phenotypes result specifically from changes in the gene of interest can be obtained by restoring the wild-type phenotype, by means of P-element–mediated transformation, to reintroduce a wild-type copy of the gene into the genome of the mutant fly.

Once a gene is identified and cloned in *Drosophila*, it is frequently possible to identify vertebrate homologues to test for immune function. Using the strategy of looking for *Drosophila* motifs conserved in humans, Medzhitov et al. (1997) reported the cloning of a human homologue of *Toll*, *hToll*. Activation of hToll on human monocyte THP-I cells is sufficient to induce the expression of cytokines IL-1 and IL-8 and of B7.1, a surface receptor known to act as an essential costimulator for

### Studying immune responses in the fly

Hemolymph, the equivalent of blood in the fly, would serve as a rich medium to support the growth of pathogenic bacteria or fungi, were it not for the many antimicrobial peptides that are rapidly induced in infected flies. This inductive process is profoundly similar to human humoral immune responses. Many insect antimicrobial peptides have been identified and purified biochemically—for instance, by overlaying bacterial lawns on gels with electrophoretically separated hemolymph proteins. Immunoregulatory genes cannot be identified in this way, but they are amenable to genetic analysis through screens for flies with aberrant antimicrobial gene expression. A critical first step for such a project is the generation of transgenic flies bearing a reporter gene under transcriptional control of a promoter from an immune-response gene.



These micrographs show the fat bodies (organs likened to the mammalian liver) of larvae bearing the *lacZ* reporter gene under control of the inducible *cecropin* promoter (Engström et al. 1993). Septic injury to these larvae causes massive induction of *lacZ* in $\leqslant 2$ h, indicated by the dark-blue staining in the lower panel of this figure. The uninfected larva, in the upper panel, shows only a low level of background staining. Other reporter genes have been used for the same purpose, each with unique advantages. The green fluorescent protein, GFP, is a "vital stain" that allows detection of reporter-gene expression in whole animals. Alcohol dehydrogenase (ADH) can be used for screening by histological staining, as with *lacZ*, but it can also be the basis of a selection for immune mutants. Flies grown in the presence of ethanol will survive only if the reporter gene is expressed. Negative selection is also possible: 1-pentyne-3-ol is harmless to flies by itself, but it is converted by ADH into a toxin, killing only those flies whose immune systems are activated. Tools available from the *Drosophila* genome project now make it easier to clone the genes identified by such screens. As described in the main text of this article, identified *Drosophila* immunoregulatory genes define a pathway that is conserved in the human immune system. Some of the components of this pathway, notably the recently cloned human *Toll*-homologue, had been identified in flies before their importance in human immunity was suspected.

activation of resting T-cells. This finding places the innate immune response squarely in the center of vertebrate immune stimulation and argues that further study of innate immunity is essential if vertebrate immunity is to be understood.

### The Paradigm

The similarities between human and *Drosophila* signaling cascades are outlined in figure 1. Both are initiated by the stimulation of a membrane-bound receptor, through the binding of an extracellular ligand. The identity of the ligands for hToll and 18-Wheeler are unknown. The extracellular domains of these proteins consist of multiple copies of LRRs, 24-amino-acid motifs found on many proteins known to be involved in protein-protein interactions, such as receptors and adhesion molecules. Their involvement in immunity is widespread, as is shown by the fact that plants employ LLR-containing proteins as part of their defense against pathogens (reviewed recently by Yang et al. 1997). Cysteine-rich regions flank the LRR domains, and, although their function is not known, mutations of conserved cysteine residues lead to constitutive signaling by the receptor (Schneider et al. 1991; Medzhitov et al. 1997).

The cytoplasmic domains bear striking similarity to that of IL-1RI, and IL-1 is a potent activator of NF$\kappa$B. Receptor stimulation results in the activation of a cascade of protein phosphorylation, through interleukin-1 receptor–associated kinase, in the case of humans, and through Pelle, in the case of flies. Other components of the pathway remain to be identified, but the result is that I$\kappa$B (human) or Cactus (fly) is phosphorylated and degraded. A transcription-factor dimer—NF$\kappa$B in humans or some combination of Dif, Dorsal, and Relish in the fly— that no longer is held in the cytoplasm moves into the nucleus, where it begins to initiate the expression of downstream genes.

*Drosophila* and *Homo* use this conserved Rel pathway for similar responses in the rapid response to infection, but they also employ the pathway for quite different physiological functions. *Drosophila* uses a Rel cascade to establish the dorsal/ventral axis in the embryo. *Homo* uses Rel cascades in numerous situations, including regulation of apoptosis and oncogenesis, and HIV subverts Rel proteins in order to regulate the expression of its own genes (Swingler et al. 1994). Given the complexity and diversity of mammalian Rel cascades, as well as their conservation in the experimentally more tractable fly, it is likely that *Drosophila* will continue to provide important insights into the regulation of these critical pathways and their immunoregulatory functions.

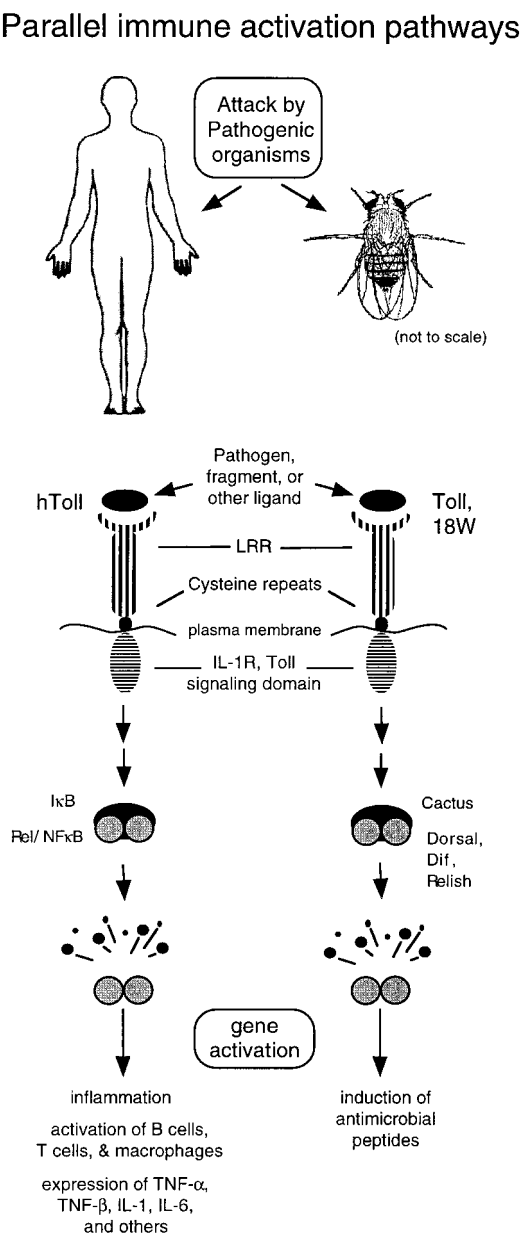



**Figure 1** Comparison of signaling in *Homo* and *Drosophila* immune response, illustrating the striking similarity in immune signaling pathways in *Homo* and *Drosophila*. hToll = recently identified human homologue of the *Drosophila* protein (Medzhitov et al. 1997); 18W = *Drosophila* gene *18-wheeler,* which is similar to Toll; LRR = multiple copies of the LRR, a conserved 24-amino-acid motif; and IL-1R = signaling motif of the cytoplasmic domain of IL-1RI. Other components of the pathways are described in the text. Multiple arrows indicate where components remain to be identified.

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