mechanism of caspase 1 activation by TLRs is not known, one possible scenario would be the activation of a TLR3–Trif–RIP1–RAIDD–caspase 1 or TLR4–Trif–RIP1–RAIDD–caspase 1 pathway. Several other proteins, including TRAF2 and FADD, interact with RIP1 in the TNF receptor pathway, and their possible involvement in Trif-dependent signaling will need to be tested.

The unexpected involvement of RIP1 in TLR signaling is reminiscent of the imd pathway in drosophila. Imd is a death domain adaptor that is most similar to mammalian RIP1, although it lacks the kinase domain. The imd pathway controls the induction of antibacterial responses through the drosophila NF-κB protein relish. In addition, imd mediates caspase-dependent apoptotic signaling in flies. The coupling of the inflammatory and apoptotic signaling pathways is therefore of ancient origin, and RIP-like proteins seem to be critically involved in their regulation. Defining these pathways and their importance in terms of the immune response to infection will be an important challenge.

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Nature's fortress against infection

Steven A Wasserman

Correlative analyses of polymorphism and disease resistance in *Drosophila melanogaster* refine our understanding of the forces that maintain variation in innate immunity loci.

"... this other Eden, demi-paradise, this fortress built by Nature for herself against infection..."
—William Shakespeare, King Richard II. Act ii. Sc. 1.

Innate immunity is a primary defense against microorganisms, coordinating rapid, targeted responses that eradicate invading pathogens and neutralize virulence factors. The fact that naturally occurring microbes threaten both reproduction and survival leads logically to the suggestion that the genes encoding molecules of the innate immune response are subject to considerable directional selection pressure. Verification of this idea has awaited a demonstration that standing variation in such genes has phenotypic consequences for host-pathogen interactions. In a recent article in *Science*, Clark and colleagues provide just such evidence, establishing that natural populations of the fruit fly *Drosophila melanogaster* show allelic variation that is linked to their ability to fight off bacterial infection[1].

Over the last 10 years, *D. melanogaster* has emerged as a favorite model organism for the study of innate immunity[2]. Indeed, work in flies has helped catalyze much of the progress of research in vertebrate innate immunity, beginning with the identification of the Toll receptor and the discovery of its conservation in humans[3]. In mammals, Toll-like receptor (TLR) proteins function in both pathogen recognition and signal transduction[4]. TLR proteins bind specifically to 'pathogen-encoded' macromolecules such as components of the bacterial cell wall that are not present in eukaryotes but are highly conserved among classes of microorganisms. After interacting with these foreign molecules, TLR proteins signal via the NF-κB family of transcription factors to trigger an antimicrobial effector response and to instruct the adaptive immune response via cytokines and costimulatory molecules[5]. There are substantial similarities in the fly innate immune pathways, although recognition and signaling are encoded separately (**Fig. 1**). Dedicated binding proteins interact with microbial cell wall components, initiating extracellular cascades that mainly activate the Toll pathway, for infections with fungi or Gram-positive bacteria, or a second system, the immune deficiency (Imd) pathway, when the

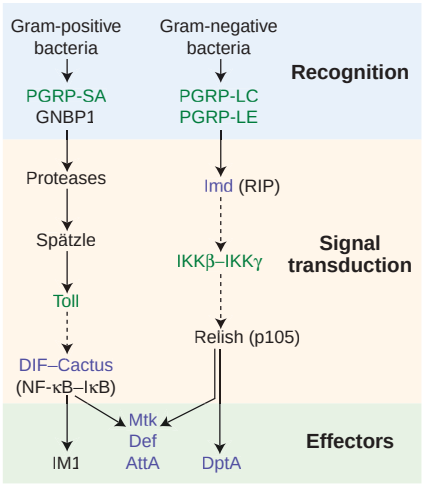

Figure 1 The Toll and Imd pathways of *D. melanogaster* innate immunity. Cell wall components of infecting bacteria bind to and activate recognition factors in *D. melanogaster* belonging to the peptidoglycan recognition protein (PGRP) and Gram-negative binding protein (GNBP) families. For Gram-positive pathogens (left), the result is proteolytic activation of spätzle, the ligand for the transmembrane receptor Toll. Signaling (dotted line) by Toll directs destruction of cactus, an IκB-related inhibitor, and activation of the drosophila immunity factor (DIF), a transcription factor closely related to the mammalian Rel and NF-κB proteins. Gram-negative bacteria (right) engage distinct factors to activate the Imd protein, a counterpart of the mammalian RIP kinase. Acting through a pathway that includes the fly IKK complex, Imd effects cleavage and activation of relish, the *D. melanogaster* counterpart of human p105. The N-terminal, NF-κB-like domain of relish then directs 'downstream' gene expression. Under the control of the Toll and Imd pathways, DIF and relish induce expression of a broad array of innate immune effectors, including the antimicrobial peptide genes shown here. Genes such as those encoding metchnikowin (Mtk), defensin (Def) and attacin A (AttA) respond to both pathways, whereas others, such as those encoding immune-induced molecule 1 (IM1) and diptericin A (DptA), are pathway specific. Lazzaro *et al.*[1] assayed sequence polymorphisms in and around the genes encoding factors shown in blue, as well as the genomic regions encoding homologs of factors shown in green.

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infecting agent is a Gram-negative microbe[2]. Both the Toll and Imd pathways use *D. melanogaster* NF-κB family members to activate expression of arrays of antimicrobial peptides. These peptides are synthesized in a dedicated organ, the adult fat body, and are released throughout the animal in the hemolymph, the fluid constituent of the open insect circulatory system.

Loss-of-function mutations in genes encoding either microbial pattern recognition proteins or signal transducers lead in general to severely immunocompromised individuals. Such mutations, although essential to the successful dissection of the mechanisms underlying innate immunity, are ill suited for investigating how the immune response evolves. For that purpose, researchers have instead examined naturally occurring phenotypic variation at immunity loci within and across drosophila species. Based on detailed studies of polymorphism and divergence, it has been argued that the observed variation in genes of the innate immune system can only be readily explained as the product of natural selection[6]. However, it had not been directly demonstrated that existing polymorphism reflects differences in the ability of individuals to combat infection.

To determine whether detectable polymorphism is in fact functional, Lazzaro *et al.* set out to correlate genotypic variation with resistance to infection. They began by generating a set of 107 lines, each homozygous for chromosome 2 derived from a distinct isolate collected in the wild. As all other chromosomes were isogenic across the set, any differences among the lines could be attributed to chromosome 2 (representing approximately 40% of the genome). Flies from each line were stabbed with a culture of the Gram-negative bacterium *Serratia marcescens*. Infected flies were later collected and the internal pathogen load was assayed by plating of fly homogenates and then counting of colonies. In parallel, DNA was prepared from each line and used to derive sequence for the genomic regions of chromosome 2 that encode 21 genes of interest. Many of these genes are known to function in innate immunity; the remainder have sequence similarity to known immune factors (but no demonstrated immune function). The extent of polymorphism at each site was assayed and statistical methods were used to calculate the correlation between each polymorphic site and the extent of the bacterial load sustained after infection.

The results of the correlation studies were notable. Isolates of chromosome 2 drawn from flies in the wild differed in their effect on the ability of individuals to resist *S. marcescens* infection. At the extremes of the distribution, bacterial counts were on average an order of magnitude higher or lower than the population mean. Moreover, the determinants of susceptibility to infection were not spread uniformly among the genomic regions examined. Several polymorphic regions associated with the level of resistance to infection were linked to loci encoding pattern recognition or signal transduction factors, whereas no such linkage was found for the genes encoding antimicrobial peptides.

These studies provide strong support for the idea that standing variation in innate immune response genes can be the object of selection related to disease resistance. Although this result seems reasonable in the context of current knowledge, well considered models predicted very different scenarios. For example, it has been suggested that one possible consequence of an 'arms race' between predators and prey, or in this case pathogens and hosts, would be that selection would cause resistance alleles to sweep through the population under attack[7]. If this occurred, the steady-state level of polymorphism among resistance loci in the periods between such sweeps would be expected to be low. The findings of Lazzaro *et al.* thus establish important constraints to model building for evolution of the innate immune system and provide evidence against any schemes that cannot accommodate the ongoing interaction of selective forces and extant polymorphism.

While notable, these studies leave many unanswered questions, of which three are considered here. First, are the immune loci associated with variation in resistance in fact responsible for that variation? Experiments to address this issue might involve measurements of polymorphism at other loci distributed across chromosome 2 or, more directly, phenotypic assays of transgenic flies differing only in the observed alleles of a single locus. It will also be essential to exploit a natural route of infection, because wounding of flies, even in sterile conditions, elicits a distinct defensive response. Second, is there any importance in the finding of functional variation among only a subset of the classes of loci tested? The antibacterial peptide genes were the sole effector loci examined in these studies, a limitation that may have skewed the outcome for this class. An elegant series of transgenic experiments have demonstrated that whole groups of antimicrobial peptide loci are functionally redundant when flies are infected with a single bacterial species[8]. Third, what processes maintain this variation? More specifically, how much of the variation results from various forms of positive selection? To answer such a question it will be important to assay multiple natural drosophila pathogens, as particular infectious agents evoke both shared and unique components of the recognition and response network underlying innate immunity[9,10].

Although pathogens are important in innate immune evolution, other forces are also at work. In particular, the close interrelationship between defensive and developmental pathways imposes considerable bounds on the nature and extent of variation in immune factors. For example, one of the immune factors linked to significant phenotypic variation in the paper by Lazzaro *et al.* is cactus, the fly counterpart to the NF-κB inhibitor IκB. As cactus is dispensable for the humoral response to Gram-negative bacteria, this result is at first unexpected. However, cactus has many developmental functions, including an essential function in larval hematopoiesis[11]. It may be, therefore, that the significant variation at this locus modulates the cellular arm of the innate immune system, that is, its function in the lineage of cells responsible for the phagocytic response to infection. Similarly, the involvement of many innate immune genes in embryonic axis formation and patterning may act to limit variation at these loci.

Two other areas of ongoing investigation are likely to have a considerable effect on inquiries into evolution of innate immune function. The first is the genomic sequencing of one half-dozen or more drosophila species. The second is the generation and consolidation of large amounts of microarray data from organisms infected with a broad range of pathogens. Together, these two lines of experimentation should provide a much more detailed picture of the invertebrate immune system and should greatly facilitate further exploration of the mechanism and evolution of host-pathogen interaction. The understanding growing out of such work is in turn likely to inform attempts to generate an effective arsenal against the ever-changing collection of microbes that seem at times 'hell-bent' on our demise.

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