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meeting report

Sensing microbes by diverse hosts

Workshop on Pattern Recognition Proteins and Receptors

Stephen E. Girardin[1], Dana J. Philpott[2+] & Bruno Lemaitre[3]

[1]Pathogénie Microbienne Moléculaire and [2]Immunité Innée et Signalisation, Institut Pasteur, Paris, France, and [3]Centre de Génétique Moléculaire du CNRS, Gif-sur-Yvette, France

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Introduction

Innate immunity has a key role in combating microbial infections in all animals. The innate immune response is activated by receptors known as pattern recognition receptors (PRRs), which recognize surface determinants that are conserved among microbes but are absent in the host, including components such as lipopolysaccharides (LPSs), peptidoglycans (PGs) and mannans (Medzhitov & Janeway, 1998). These pathogen-associated molecular patterns (PAMPs) therefore represent an ideal signature for the presence of infectious agents. After a PAMP is recognized, PRRs activate several complex signalling cascades, which ultimately regulate the transcription of target genes that encode effectors and regulators of the immune response. A transcription profile specific to one class of pathogens can be achieved through the linkage of recognition receptors to distinct signalling pathways.

The concept of 'pattern recognition' has been essential in unifying the work that has accumulated on the innate immune responses in a large variety of invertebrate and vertebrate species. During the past few years, significant progress has been made towards our understanding of pathogen detection. Recent insights into the function and signalling of PRRs were presented at an EMBO workshop, and here we review the most important issues and focus on the new concepts that emerged during this excellent symposium.

The *Drosophila* antimicrobial response

The *Drosophila* antimicrobial response has been the focus of intense studies during recent years. The Toll and immune deficiency (Imd) pathways have emerged as simple paradigms of innate immune-response regulation in animals, and have shown how two distinct signalling pathways can modulate the expression of a complex transcriptional programme in response to different pathogens. In general, the Toll pathway controls the response to fungal and Gram-positive bacterial infections, whereas the Imd pathway is specific for controlling Gram-negative bacterial infections (Fig. 1). Recently, it was shown that microbial recognition acting upstream of the Toll and Imd pathways is achieved, at least in part, through peptidoglycan recognition proteins (PGRPs; Michel *et al*., 2001; Choe *et al*., 2002; Gottar *et al*., 2002; Ramet *et al*., 2002). PGRPs bind to PG, which is a component of the bacterial cell wall, and are found in many species, including insects and mammals. In *Drosophila*, 13 PGRP genes have been identified and the analysis of their function is the focus of intense studies. J. Royet (Strasbourg, France) discussed his work on PGRP-SA and PGRP-LC, which function upstream of the Toll and Imd pathways, respectively. He showed results that indicate that these two receptors are pathway-specific and not microbe-specific, as double-mutant *PGRP-SA:PGRP-LC* flies die rapidly after all types of bacterial challenge. The absence of crosstalk indicates that the recognition specificity of these two PRRs determines the precise activation of the downstream signalling cascades.

[1]Pathogénie Microbienne Moléculaire, and [2]Immunité Innée et Signalisation, Institut Pasteur, 28 rue du Dr Roux, 75724 Paris, France
[3]Centre de Génétique Moléculaire du CNRS, F-91198 Gif-sur-Yvette, France
[+]Corresponding author. Tel: +33 1 45 688993; Fax: +33 1 40 613902;
E-mail: philpott@pasteur.fr

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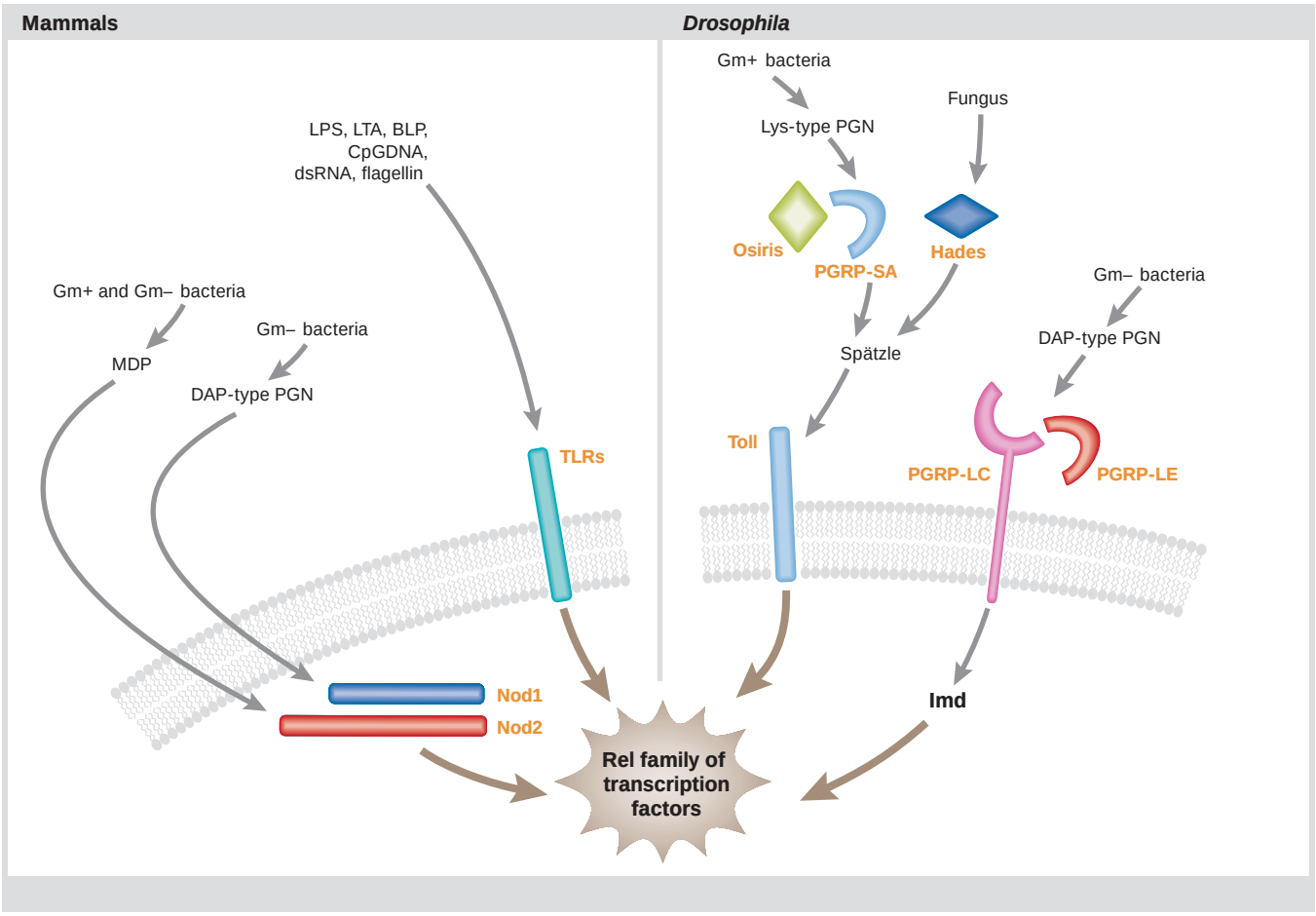

Fig. 1 | Innate immune sensing of microbes by mammals and *Drosophila*. This diagram highlights the pattern recognition receptors (PRRs) in the *Drosophila* and mammalian systems and the pathogen-associated molecular patterns (PAMPs) recognized by these receptors. BLP, bacterial lipoprotein; DAP, diaminopimelic acid; Gm+, Gram positive; Gm–, Gram negative; Imd, immune deficiency; LPS, lipopolysaccharide; LTA, lipoteichoic acid; MDP, muramyl dipeptide; PG, peptidoglycan; PGRP, peptidoglycan recognition protein; TLR, toll-like receptor.

A key issue is the identification of the bacterial determinants that are recognized by these pathways. Royet and D. Ferrandon (Strasbourg, France) both showed preliminary results indicating that lipoteichoic acid (LTA), a component of the Gram-positive cell wall, activates the Toll pathway. In mammals, LTA has been shown previously to be a ligand for the Toll-like receptor 2 (TLR2). B. Lemaitre (Gif-sur-Yvette, France) described his recent studies indicating that diaminopimelic-acid (DAP)-type PG, which is found mainly in the walls of Gram-negative bacteria, is the most potent inducer of the Imd pathway, whereas the Toll pathway is predominantly activated by Gram-positive lysine-type PG. The ability of *Drosophila* to discriminate between Gram-positive and Gram-negative bacteria therefore relies on the recognition of specific forms of PGs (Leulier *et al.*, 2003). However, the structural basis of this recognition and the minimum PG motif required has not been determined. The role of LPS in the insect response to Gram-negative bacterial infections was debated at the meeting, because many of the studies that link it to recognition used commercial preparations of LPS, which are usually contaminated with PG. The data from Lemaitre and colleagues now suggest that LPS is probably not the main determinant of Gram-negative bacteria that is recognized by *Drosophila* adults, but a role for this compound at earlier developmental stages, or in distinct tissues, is still possible. For example, T. Werner (Umeå, Sweden) showed that LPS stimulates antimicrobial peptide gene expression in mbn-2 cells, which are cultured cells derived from *Drosophila* haemocytes. He showed that this effect requires two isoforms of the receptor PGRP-LC (LCa and LCx), whereas another isoform, PGRP-LCx with a different PGRP motif, activates antimicrobial peptide gene expression in response to PG. However, it is not known whether, in this cell system, LPS is recognized through its lipid A core, as it is in mammals, or through its sugar moieties.

Two contributions to the meeting suggested that microbial detection by *Drosophila* involves a multiprotein complex. S. Kurata (Sendai, Japan) has identified a loss-of-function mutation in *PGRP-LE*, which encodes a secreted PGRP protein. Double-mutant *PGRP-LE*:*PGRP-LC* flies are more susceptible to Gram-negative bacterial infections, indicating that both PGRP-LE and PGRP-LC function together to detect this class of bacteria. Consistent with these findings, Ferrandon reported that the phenotype of *Drosophila* with a mutation in the *Osiris* gene is identical to that of those with a mutation in *PGRP-SA*: the *Osiris* mutation blocks the activation of the Toll pathway in response to Gram-positive bacteria. Constitutive activation of the Toll pathway is observed only when PGRP-SA and Osiris are co-expressed,

which indicates that both proteins are required for signalling through the Toll pathway in response to Gram-positive bacterial infection. Surprisingly, Ferrandon reported that *Osiris* encodes Gram-negative binding protein 1 (GNBP1). GNBPs are PRRs that contain an inactive glucanase domain and bind to β-glucan and LPS *in vitro*. He also reported that a mutation in another GNBP encoded by a gene known as *Hades* blocks the activation of the Toll pathway after fungal infection. The implication of a GNBP protein in fungal sensing fits with the capacity of GNBP to bind to β-glucan, a component of the fungal cell wall.

PGRPs contain a conserved PGRP domain that has similarities with the T7 bacteriophage lysosyme, although most receptors are not enzymatically active due to amino-acid changes in this domain. However, recent studies have shown that 6 of the 13 *Drosophila* PGRPs may have retained muramidase activity, which enables them to cleave the peptide chain from the sugar moieties of PG. J. Karlsson (Stockholm, Sweden) showed that, in fact, PGRP-SC1B is an efficient muramidase and also that PG digested by PGRP-SC1B loses its stimulatory effect on the immune response (Mellroth *et al.,* 2003). Interestingly, she also reported that PGRP-L, a mouse PGRP produced by the liver and secreted into the serum, has muramidase activity. The *in vivo* role of these activities awaits further genetic analysis, but her studies suggest that PGRPs could modulate the immune response by digesting PG into non-stimulatory fragments.

The intracellular signalling proteins downstream of the Toll and Imd pathways share striking similarities with the cascades of the Toll-like receptor (TLR) and the tumour necrosis factor-α receptor (TNFα-R) that regulate NF-κB activation in vertebrates (Khush *et al*., 2001). During this process, NF-κB precursors such as p105 are cleaved by the proteasome to release the transcriptionally active Rel protein from the IκB inhibitor. D. Hultmark (Umeå, Sweden) showed that the p105 *Drosophila* homologue Relish is also cleaved, but by a caspase rather than the proteasome. The Relish domains required for efficient processing have been mapped and the caspase Dredd seems most likely to carry out this cleavage event (Stoven *et al.,* 2003). However, the direct, *in vitro* cleavage of Relish by Dredd remains to be shown. In the Toll pathway, the IκB homologue Cactus is released from the transcription factors Dif and Dorsal by the action of the proteasome. T. Tingall (Stockholm, Sweden) has analysed in further detail the role of the two Cactus isoforms, Cact261 and Cact2618 in *Drosophila*. Cact2618, in contrast to Cact261, strongly inhibits the ability of Dif to activate a reporter gene construct. The fact that Cact261 but not Cact2618 is found in the nucleus of mbn-2 cells supports the hypothesis that there are functional differences between these two isoforms. Although previous genetic analyses have identified several components of the Toll and Imd pathways, these studies highlight how little we know about the biochemical steps involved.

Pattern recognition in other arthropods

Even though the importance of LPS sensing in insects has not been clarified, it has been clearly established that this bacterial compound is a potent activator of the clotting reaction in the horseshoe crab *Limulus polyphemus*. In this species, LPS binds to the serine protease factor C and activates a two-step proteolytic cascade leading to the transformation of coagulogen into an insoluble protein, coagulin. S.-I. Kawabata (Fukuoka, Japan) has shifted his interest to a less-characterized aspect of horseshoe crab host defence, the degranulation of granular haemocytes. This reaction is LPS-specific as it is not observed in the presence of either β-glucan or PG. His experiments suggest the existence of an LPS receptor at the haemocyte cell surface that has similarities to factor C and that activates a G-coupled protein that then signals degranulation.

Microbial determinants such as β-glucan and PG also trigger a two-step proteolytic cascade that leads to the activation of the prophenoloxidase enzyme in arthropods. This enzyme cascade is important for the melanization reaction, which is an invertebrate response to microbial entry. B.L. Lee (Jangjeon Dong, Korea) has purified several recognition receptors and serine proteases from the haemolymph of two coleopteran insects, *Tenebrio molitor* and *Holotrichia diomphalia*, and his long-term goal is to reconstitute all the steps of this cascade *in vitro* using purified compounds. He also reported that PGRP purified from *H. diomphalia* binds to both β-glucan and PG *in vitro*. These data and others shown during the meeting suggest that, as already described in the case of TLRs in mammals, most PRRs recognize different PAMPs and that recognition may involve multiple cofactors.

M. Kanost (Manhattan, KS, USA) has identified five classes of PRRs in *Manduca sexta*: haemolin, PGRP, β1-3 glucan recognition proteins, immulectin and leureptin. He is now identifying the ligands of these PRRs and investigating how they interact with components of the phenoloxidase activation system. Interestingly, one of these PRRs, leureptin, has a leucine-rich repeat domain similar to TLRs and Nod proteins in mammals. More information on immulectin 2 (IML2) was provided by X.-Q. Yu (Kansas City, KS, USA). IML2 is a C-type lectin that is induced after the injection of bacteria and aggregates *Escherichia coli* in a Ca^{2+}-dependent manner. It also binds to LPS and enhances prophenoloxidase activity. The injection of antibodies directed against IML2 lowers the survival rate of *M. sexta* after it is challenged with *Serratia marcescens,* which suggests an important role for this protein in fighting bacteria. IML2 binds to a clip domain serine protease *in vitro*, which might link this PRR to the phenoloxidase enzyme (Yu & Kanost, 2003).

Functional studies in other insect models

Functional studies of the proteins involved in pattern recognition in insects have been hampered by the lack of genetic tools to test for their *in vivo* relevance. These types of study are necessary in the PRR field because of the presence of large gene families. Several approaches have now been developed to test the function of a particular protein in a non-*Drosophila* model. For example, the injection of antibody was used to analyse the function of IML2 as described above. Microarrays and genome annotation have revealed the presence of several genes that might be involved in the innate immune response of the malaria vector *Anopheles gambiae*. G. Christophides (Heidelberg, Germany) inactivated several candidate genes in the mosquito by RNA interference (RNAi). Working in the laboratory of F. Kafatos, he has identified several leucine-rich repeat proteins (LRRP) that are upregulated after malaria infection. Interestingly, the targeted inactivation of LRRP1 markedly increases the number of malaria parasites that develop in the mosquito. J. Ishibashi (Kansas City, KS, USA) has identified several serine proteases with clip domains acting downstream of PRRs in *M. sexta*. By injecting mutated, inactivated proteases, he was able to analyse the order of action of the enzymes and suggested a role for one of them in the regulation of antimicrobial peptides in this insect.

Although many parallels can be seen in microbial detection among different arthropods, distinct strategies are also prevalent, which is likely to reflect the adaptation of individual animals to different types of habitat. For this reason, caution must be taken not to over-emphasize the parallels between, for example, *Drosophila* and mammals, without being aware that these similarities may not necessarily be conserved in other arthropods. With this in mind, the RNAi approach developed in *A. gambiae*, a dipteran living in an environmental niche distinct from that of *Drosophila*, should provide important insights into the diversification of innate immune system components. Nevertheless, as will be highlighted in the next section, conserved innate immune strategies do exist between vertebrates and invertebrates, especially at the level of the PAMPs that are detected by these animals and the proteins involved in cell signalling.

Detection of fungi by mammals

Although the inflammatory effects of the fungal product β-glucan have been known for many years, the receptors that mediate these responses in human cells have remained obscure. G. Brown (Oxford, UK) presented his recent findings showing that dectin 1 is the β-glucan receptor present on macrophages. This receptor mediates the cellular responses to β-glucan and live yeast through its interaction with TLR2 (Brown *et al*., 2003; Gantner *et al*., 2003). Although complement receptor 3 has also been identified as a receptor for β-glucan, this is the first indication of a connection between this class of PRRs and the TLRs, and therefore provides an explanation for how the signal is transmitted inside the cell.

Detection of bacteria by mammals

In mammals, it is now clear that bacteria are recognized extracellularly and intracellularly by TLRs and Nod proteins. The membrane-bound TLRs, which are involved in sensing a variety of bacterial, viral and fungal components, detect these products either in the extracellular milieu or possibly within specialized compartments of the cell. Ten TLRs are present in mammals and these receptors individually, jointly or with the aid of cofactors sense different PAMPs. One cofactor that is required for LPS sensing by TLR4 is MD2, and K. Miyake (Tokyo, Japan) showed that mice that are deficient in this molecule can no longer respond to LPS. MD2 seems to be required for the cell-surface expression of TLR4, as in MD2-deficient cells, TLR4 predominantly resides in the Golgi apparatus. Furthermore, Miyake presented evidence that the TLR4–MD2 complex seems to change conformation when LPS is bound, and this might have important consequences for cell signalling.

TLR9 recognizes bacterial DNA and this can be mimicked experimentally using unmethylated CpG oligonucleotides. S. Bauer (Munich, Germany) discussed his work to show that mouse and human TLR9 are able to distinguish between different oligonucleotide sequences, which strongly indicates that there is a direct interaction between TLR9 and stimulatory DNA. Although their natural ligands are unknown, TLR7 and TLR8 are activated by anti-viral compounds such as R848 and loxoribine, which are purine and guanosine analogues, respectively. Bauer suggested that these immunostimulatory compounds interact directly with these TLRs, because he detected differences between the capacity of mouse and human TLR8 to respond to these ligands.

Differences in the ligand specificity in mice and human TLRs was also highlighted in the talk by C. Werts (Paris, France). Her previous work has shown that LPS from the spirochaete *Leptospira interrogans* activates TLR2 rather than TLR4 in human cells. This LPS has an unusual lipid A structure and weak endotoxicity. Her most recent findings indicate that leptospiral lipid A does not activate human cells, whereas it is proficient at stimulating murine cells.

Cell signalling downstream of Toll-like receptors

TLRs connect to intracellular signalling cascades through their cytoplasmic Toll/interleukin-1 receptor (TIR) domains by interacting with TIR-domain-containing adaptor molecules, including MyD88 and TIRAP (also known as Mal). The production of inflammatory cytokines in response to all TLR ligands tested is strictly dependent on MyD88, and TLR2 and TLR4 additionally require TIRAP. However, as shown in LPS- or double-stranded-RNA-treated cells, there is also a MyD88-independent pathway that leads to the activation of interferon regulatory protein 3 (IFR3) and the subsequent expression of interferon-β (IFN-β). K. Takeda (Osaka, Japan) discussed his findings that this MyD88-independent pathway depends on another TIR-containing adaptor protein called TRIF. TRIF interacts with both TLR3 and IFR3 and thus links the TLR to this signal-transduction pathway (Yamamoto *et al*., 2003).

The regulation of signalling downstream of the TLRs was also highlighted in several talks at this meeting. R. Beyaert (Ghent, Belgium) showed that a splice variant of MyD88, called MyD88s, which is an isoform that lacks the intermediate domain of the molecule, is upregulated in macrophages after LPS treatment. This isoform acts as a dominant-negative inhibitor to downregulate NF-κB activation. These findings may have an impact on the phenomenon of endotoxin tolerance, which is characterized by the hyporeactivity of cells after a second stimulation with LPS. The initial stimulation with LPS might increase expression of molecules such as MyD88s, which then act to downregulate the activation pathway.

Hyporeactivity after stimulation with bacterial products was also discussed by M. Adib-Conquy (Paris, France), who investigates the consequences of sepsis, haemorrhagic shock and trauma. Her work has shown that leukocytes isolated from patients who suffer from any one of these conditions produce less inflammatory cytokines when stimulated with bacterial products, but produce high levels of anti-inflammatory cytokines such as IL-10. The observed hyporeactivity may be explained by several mechanisms, including the increased expression of the MyD88s isoform. The reason for the increased capacity to produce anti-inflammatory cytokines, however, remains to be investigated.

The recent findings of R. Ulevitch (La Jolla, CA, USA) have shown that ubiquitination controls TLR expression and this might be a way for the cell to increase or decrease its TLR sensitivity. A ubiquitin-conjugating enzyme interacts with the cytoplasmic domain of TLR4 and TLR9, and overexpression of this enzyme results in TLR degradation and loss of signalling through these receptors. Therefore, the ubiquitination of TLRs might be a new mechanism by which the cell can regulate signalling.

Nod proteins recognize bacterial products inside the cell

The intracellular detection of bacteria relies on cytosolic proteins called Nods, which are structurally similar to the R-protein family in plants that are involved in resistance against pathogen infection. D. Philpott (Paris, France) showed that one of these proteins, Nod1,

detects PG motifs found mainly in Gram-negative bacteria (Girardin *et al*., 2003a). The reason for this discriminatory detection is similar to what has been described for *Drosophila*; Nod1 is specifically activated by DAP-type PG, which is found mainly in the PG from Gram-negative bacteria. G. Nuñez (Ann Arbor, MI, USA) described that the minimal structure recognized by Nod1 is in fact a dipeptide fragment of PG, γ-D-glutamyl-mesoDAP (Chamaillard *et al*., 2003). Nod2, conversely, was shown by S. Girardin (Paris, France) and also Nuñez to sense the minimal component of PG, muramyl dipeptide, which makes it a general sensor of bacteria (Girardin *et al*., 2003b; Inohara *et al*., 2003). Mutations in the gene encoding Nod2 were recently shown to be associated with the inflammatory bowel disease, Crohn's disease, indicating that defects in sensing bacterial PG in the cytoplasmic compartment contributes to the aetiology of this disease.

Perspectives

This symposium highlighted the important role of both PG and β-glucan sensing in vertebrates and invertebrates. The findings that both insect and mammalian cells are able to distinguish microbes on the basis of these structures show that the similarities we see in these recognition systems reflects their specificity for detecting conserved molecular patterns present in microbes. The concept that germ-line-encoded receptors of the innate immune system can recognize a limited but conserved set of molecular motifs from different microbes was a hypothesis first proposed by C. Janeway in 1989 at a Cold Spring Harbor meeting on quantitative biology. In the proceedings, he stated, "the immune system has evolved specifically to recognize and respond to infectious microorganisms, and that this involves recognition not only of specific antigenic determinants, but also of certain characteristics or patterns common on infectious agents but absent from the host" (Cold Spring Harbor Symposia, 1989). Janeway's insightful comments back in 1989 have spawned intensive research in this area, such that today there are entire meetings dedicated to this topic.

Another important concept that emerged from this meeting is that although both vertebrate and invertebrate systems recognize similar PAMPs, the PRRs that have evolved seem to be substantially different (Fig. 1). For example, mammalian TLRs seem to be, at least in some instances, *bona fide* PRRs, whereas in *Drosophila*, the TLR homologue Toll recognizes a host protein (Spätzle) rather than a PAMP. Also, there are no Nod homologues in *Drosophilia*. Conversely, the PGRP proteins seem to be highly homologous in both vertebrates and invertebrates and are able to bind PG. However, even though a role for PGRPs in *Drosophila* innate immune defence has been clearly shown through genetic evidence, their role in mammalian defence is entirely unknown. The systems of signal transduction can also be conserved; many homologous proteins are found downstream of Toll in *Drosophila* and the TLRs in mammals, and also in the Imd and TNF-α pathways (Khush *et al.,* 2001). In addition, these pathways all lead to the activation of members of the Rel family of transcription factors: Dorsal, Dif or Relish in *Drosophila* and NF-κB in mammals.

Finally, this meeting highlighted how little we know about the interaction of PRRs with their putative PAMP ligands. We only know for a few cases the sub-structures of the PAMPs that are recognized by PRRs. For Nod1 and Nod2, for example, the minimal components of PG that are recognized have been defined but a direct interaction between these molecules and these PG fragments has not yet been shown. What is clearly needed in this field are more in-depth evaluations of PRR–PAMP interactions in terms of their direct binding, specificity and affinity.

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