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Institut Pasteur, Viruses and RNA Interference Unit, Centre National de la Recherche Scientifique UMR 3569, 75724 Paris cedex 15, France

The *Drosophila* immune system distinguishes live – and potentially harmful bacteria – from harmless dead bacteria using a novel splice variant of the receptor PGRP-LC.

Distinguishing self from non-self is critical for mounting an appropriate immune response. This historical concept by Janeway[1] was the framework for immune discrimination between host and microorganism. However, it was insufficient to explain the finely-tuned discrimination between harmful *vs.* non-harmful microorganisms or between infection and colonization. Similarly, it could not explain how the immune system could discriminate between living and dead microorganisms. The fact that the same microbial-associated molecular patterns (MAMPs) are shared among microorganisms regardless of pathogenicity or viability is still an unsolved quandary in innate immunity. In this issue of *Nature Immunology* a paper by Neyen *et al*.[2] suggests a resolution of this quandary.

The innate immune response is a tightly regulated process that consists of different phases. Firstly, there's recognition of ligands e.g. MAMPs. This is followed by activation of genes involved in host defense (antimicrobial peptides, inflammatory cytokines, chemokines). Finally the response ends with the resolution phase. One of the principles that governs the immune response in all organisms is that once the threat has passed, the immune system must downregulate activation (resolution) to avoid over reaction that can lead to the death of the host[3].

In insects, the Immune Deficiency pathway (IMD) has a principal role in responses to Gram negative ($Gram^-$) bacterial infection by activating NF-kB-like genes controlling the expression of antimicrobial peptides among others[4, 5]. In contrast to vertebrates, *Drosophila*'s sensing of $Gram^-$ bacteria does not rely on recognition of lipopolysaccharide (LPS) but rather in the

recognition of specific forms of diaminopimelic acid (DAP)-type peptidoglycans (DAP-type PGN) by peptidoglycan recognition proteins (PGRPs)[6, 7, 8].

Drosophila employs PGRP-LC to sense extracellular peptidoglycan (PGN) and activate the IMD pathway, which can discriminate between monomeric PGN (also called tracheal cytotoxin TCT) and polymeric PGN[8]. TCT, the minimal PGN motif, is released during cell wall remodeling after bacterial proliferation and is highly immunogenic- signalling the presence of live bacteria. Polymeric PGN are macromolecules released as a result of cell wall destruction due to bacterial death and is sensed as innocuous by the immune system. Both TCT and polymeric PGN activate the same signaling complex by the recruitment of ligand-induced clustering of PGRP receptor tails. PGRP-LC gene encodes 3 isoforms (LCx, LCa and LCy) with different ectodomains that determine the differential binding capacity to PGN. Recognition of polymeric PGN relies on homotypic clusters (LCx) while TCT is dependent on LCx-LCa clusters. Interestingly, upon TCT or PGN activation, the immune system manages to perform a fast resolution only during PGN activation, suggesting an ability to discriminate between living and dead bacteria[8, 9].

Neyen *et al*. identify and characterize the mechanism by which *Drosophila* discriminates between live and dead Gram$^-$ bacteria. They discover a novel isoform of the PGRP-LC, which specifically recruits polymeric PGN from dead bacteria, therefore contributing to the resolution phase of the immune response. This newly identified sensor controls IMD kinetics and so prevents lethality stemming from an unresolved immune response to dead – and therefore innocuous bacteria. By this discovery, the authors shed light onto one of the most striking concepts in innate immunity, the discrimination between live and dead microorganisms.

Neyen *et al*. evaluated the differential expression of the antimicrobial peptide Diptericin in flies injected with live or dead Gram$^-$ bacteria and observed that dead bacteria had a faster resolution rate. This was also observed when mimicking infection by TCT (live bacteria) or polymeric PGN (dead bacteria) injection in a dose-independent manner. Since both polymeric PGN and TCT activate the IMD pathway through PGRP-LC the authors searched for the determinant of this differential response in PGRP-LC itself. They focused their attention on the PGRP-LC locus and found the presence of an alternative first exon that encodes a different cytosolic tail variant that they named regulatory PGRP-LC (rLC). This new isoform is also alternatively spliced much like the regular PGRP receptor with its isoforms a, x and y. As a result, the number of isoforms increases from 3 (PGRP-LCa, PGRP-LCx and PGRP-LCy) to 6 (including the 3 rPGRP isoforms) with similar expression, tissue distribution and kinetics after immune challenge.

rLC by its unique difference in the cytosolic tail, was capable of dampening the immune response only in the presence of polymeric PGN. Indeed, mutant flies expressing solely rLC isoforms or flies completely depleted for the PGRP-LC locus, did not survive to Gram$^-$ bacteria infection nor express antibacterial peptides, meaning that rLC *per se* was not able to activate the IMD pathway. More precisely, the authors showed that the rLCx isoform was necessary to resolve IMD activation in the presence of polymeric PGN regardless of the presence of rLCa, LCx or LCa. As overexpression of rLCx potently reduced the immune response to polymeric PGN but did not suppress the TCT induced response, they proposed that rLCx is a negative regulator of PGRP-LC and precisely discriminates between polymeric and monomeric PGN contributing to the resolution of the immune activation by sensing the presence of dead bacteria. Interestingly, even if in the absence of rLC isoforms flies can still kill the bacteria, they die because of an inability to resolve autoinflammation triggered by the presence of MAMPs[2]. On the other hand overexpression of rLCx resulted in a poor IMD activation.

But how does rLC downregulate IMD signaling? *In silico* analysis of rLC, demonstrated the presence of a PHD-type zinc finger domain in the cytosolic N-terminal tail which is involved in lipid interaction[10, 11]. Overexpressed GFP-tagged versions of rLCx showed that although full-length GFP-rLCx localized to plasma membrane microdomains, mutant variants lacking the PHD region were regularly distributed, suggesting that the PHD domain might retain rLCx in specific membrane domains. As expected, rLC regulated the levels of surface-exposed LC receptors with endogenous LC receptors accumulated in rLC-deficient flies after challenge with dead bacteria, therefore downregulating the availability of 'activating' receptors (PGRP-LC) in the presence of post-mortem MAMPs. The authors went further and demonstrated a role for the endocytic pathway machinery in the termination of IMD signaling. Accordingly, depletion of endocytic (Rab5, Fab1) and ESCRT (Vps28, Tsg101) components resulted in accumulation of rLCx. It is known that receptor ubiquitynilation can act in the signaling for the endocytic pathway. Indeed, Neyen *et al*. confirmed that rLC could interact with the ubiquitin ligase DIAP2 in *Drosophila* cells via the PHD domain of rLC.

Since LC protein accumulates in rLC-deficient flies, the authors next evaluated the correlation between rLC and the ESCRT machinery in regulating LC. When dead bacteria were injected in rLC and/or Vps28 deficient flies, endogenous GFP-LC accumulated in the fat body and the IMD pathway was not affected in the early steps of activation. rLC and Vps28 acted together to remove LC from the plasma membrane. Thus altering endosome maturation and formation of

multivesicular bodies enhance immune activation and prevent immune resolution. They propose rLC to be considered as an adaptor molecule that targets PGRP-LC and PGRP-rLC to microdomains at the plasma membrane to promote degradation of activating and regulatory receptors via ESCRT trafficking. This mechanism interacts with another negative regulator of the IMD pathway, Pirk, and the ubiquitin ligase DIAP2.

PGRP-LC is well known as a major sensor of Gram$^-$ bacteria in flies but how this receptor contributed to response resolution was unclear. The data by Neyen *et al*. suggest rLC downregulates IMD signaling in response to polymeric PGN via rLC-mediated endocytosis and ESCRT-dependent degradation of PGRP-LC. The outcome of this process is a readjustment of the response according to the threat-level. The model presented by the authors suggests an efficient way to shut down PGRP-LC receptors once the infection is cleared by sensing the presence of dead bacteria ligands (polymeric PGN) (figure 1).

The work of Neyen *et al.* highlights what seems to be a fundamental difference in the function of innate immune receptors in invertebrates *vs.* vertebrates. Namely, if their observation is generalized to other immune receptors and other microorganisms in invertebrates, it is possible that one gene or locus controls not only the discrimination but also functions as a receptor, adaptor and signaling protein. How can regulation of these different functions be achieved in such a '3- in-1' model? In insects, a hint to this appears when studying *Drosophila* MyD88, that has a double function as an adaptor and signalling[12]. Mammals solved this conundrum by extreme subcellular compartmentalization of receptors and downstream adaptors and signalling components[13]. To better dissect this 3-in-1 model and understand why it might be employed in all invertebrates, it would be interesting to understand how does the localization of one protein relates to the sites of signalling complex assembly, if cell-type-specific differences in the subcellular positioning of receptor/signalling protein can explain the cell-type-specific responses to ligands (for example bacteria sensing in gut *vs*. rest of the organism). Further insights could be gleaned by looking at how harmful microorganisms influence the activity of this 3-in-1 protein, and if there's a common strategy that microorganisms use to interfere with these processes?. The answer to these and other questions will move our concept of immunity to new and unexpected places.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Figure Legend

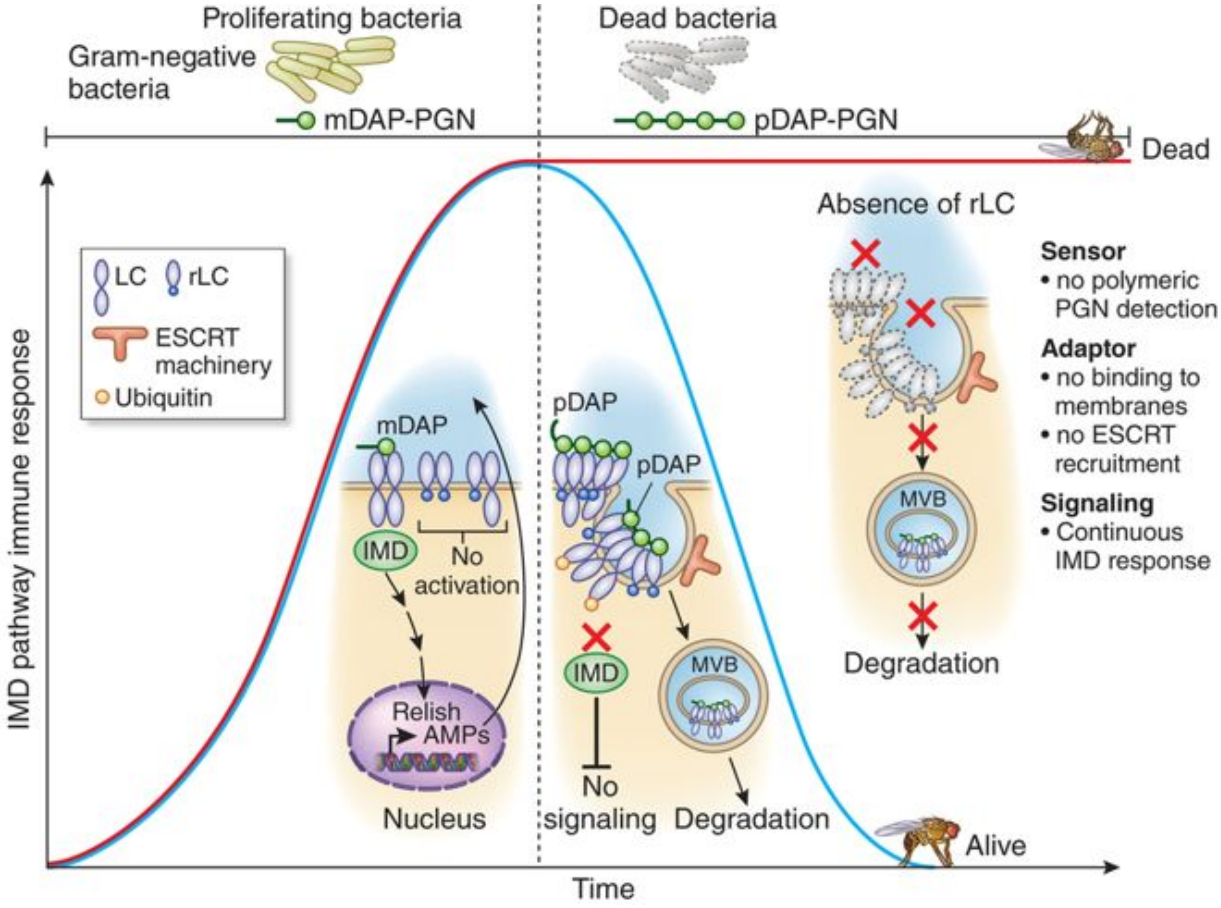

Figure 1: Role of rPGRP-LC in immune response resolution upon Gram– bacteria killing.

Following bacterial infection, *Drosophila* PGRP-LC receptor sense extracellular monomeric peptidoglycan (DAP) and activate the IMD pathway that culminates in the production of antimicrobial peptides (AMP) and the killing of the bacteria. Upon bacterial death the immune system has to move onto resolution phase. Two scenarios can be distinguished. Top panel: green line shows rapid resolution of the IMD immune response in the presence of polymeric DAP-PGN, a dead bacteria-associated MAMP. rLC mediates endosomal recruitment and ESCRT clearance of activating PGRP-LC receptors, promoting its removal from the membrane surface and trafficking multi-vesicular bodies (MVB). Together, this inhibits microbe sensing and the IMD signaling cascade. Bottom panel: red line shows that in the absence of rPGRP-LC, IMD activation is not resolved. As polymeric DAP-PGN is not sensed and activating receptors are not removed from

the membrane surface by ESCRT recruitment there is an over-reactive immune response that can kill the host.

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