Figure 1 PKA negatively regulates the NLRP3 inflammasome. NLRP3 can sense a variety of PAMPs or DAMPs that induce assembly of the inflammasome. Activated caspase-1 cleaves pro-IL-1β and pro-IL-18 to their active forms (IL-1β and IL-18, respectively), which induces their secretion. Caspase-1 induces pyroptosis. Signaling via prostaglandin E_2 (PGE_2) induces cAMP formation and activates PKA, which directly phosphorylates NLRP3 in already formed inflammasomes. Phosphorylated NLRP3 induces disassembly of the inflammasome and loss of caspase-1 activity. LPS, lipopolysaccharide; TLR, Toll-like receptor.

that one or both mechanisms are activated and thus control the NLRP3 inflammasome differentially via the same tool: cAMP.

The study by Mortimer *et al.* also explores how the phosphorylation of NLRP3 by PKA might be important in autoinflammatory diseases mediated by gain of NLRP3 function[3]. Uncontrolled inflammation is linked to a multitude of diseases, while chronic inflammasome activation is known to contribute specifically to Alzheimer's disease, Parkinson's disease, type 2 diabetes, atherosclerosis and the CAPS family of autoimmune disorders[1,13]. The CAPS family of related disorders is caused mainly by gain-of-function single-nucleotide polymorphisms in *NLRP3*. The most serious of these is NOMID, which causes chronic production of IL-1β with potentially life-threatening outcomes, including severe arthritis, kidney damage, splenomegaly, chronic meningitis, uveitis, hearing loss, macrocephaly and other neurological problems. Mortimer *et al.* show that unlike wild-type NLRP3, which is phosphorylated on Ser295 by PKA to inactivate inflammasomes, a cluster of amino acid alterations near Ser295 encoded by CAPS-related single-nucleotide polymorphisms are resistant to negative regulation by PKA[3]. Thus, CAPS mutants might escape negative regulation and lose a molecular break, which results in disease manifestation.

This study highlights the importance of negative regulation of the NLRP3 inflammasome[3]. Previous work has indicated that CAPS arise as a result of gain-of-function single-nucleotide polymorphisms of *NLRP3*. However, this new work suggests that at least some of these single-nucleotide polymorphisms give rise to CAPS because the resulting NLRP3 mutants are unresponsive to negative regulation once activated[3]. It will be interesting to see how many more of the more than 90 identified mutations that induce CAPS are due to lack of responsiveness to negative regulation. CAPS flare-ups can appear spontaneously or can be induced by known stressors such as cold, heat, fatigue or others. The new findings suggest that CAPS-related mutations might be more adept at escaping negative regulation of already activated inflammasomes and thus lead to inflammatory flares associated with the disorder.

1. Guo, H., Callaway, J.B. & Ting, J.P. *Nat. Med.* **21**, 677–687 (2015).
2. Lamkanfi, M. & Dixit, V.M. *Cell* **157**, 1013–1022 (2014).
3. Mortimer, L., Moreau, F., MacDonald, J.A. & Chadee, K. *Nat. Immunol.* **17**, 1176–1186 (2106).
4. Takeuchi, O. & Akira, S. *Cell* **140**, 805–820 (2010).
5. Kayagaki, N. *et al. Nature* **479**, 117–121 (2011).
6. Yang, J., Liu, Z. & Xiao, T.S. *Cell. Mol. Immunol.* http://dx.doi.org/10.1038/cmi.2016.29 (27 June 2016).
7. Latz, E., Xiao, T.S. & Stutz, A. *Nat. Rev. Immunol.* **13**, 397–411 (2013).
8. Brandwein, S.R. *J. Biol. Chem.* **261**, 8624–8632 (1986).
9. Lee, G.S. *et al. Nature* **492**, 123–127 (2012).
10. Sokolowska, M. *et al. J. Immunol.* **194**, 5472–5487 (2015).
11. Yan, Y. *et al. Cell* **160**, 62–73 (2015).
12. Duncan, J.A. *et al. Proc. Natl. Acad. Sci. USA* **104**, 8041–8046 (2007).
13. Broderick, L., De Nardo, D., Franklin, B.S., Hoffman, H.M. & Latz, E. *Annu. Rev. Pathol.* **10**, 395–424 (2015).

R.I.P. dead bacteria, you will not be attacked

Lorena Tomé-Poderti and Maria-Carla Saleh

The Drosophila immune system distinguishes live, and potentially harmful, bacteria from harmless dead bacteria through the use of a splice variant of the receptor PGRP-LC.

Distinguishing self from non-self is critical for mounting an appropriate immune response. This historical concept[1] was the

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framework for the immune system's discrimination between host and microorganism. However, it was insufficient to explain the finely tuned discrimination of harmful microorganisms versus non-harmful microorganisms or between infection and colonization. Similarly, it could not explain how the immune system is able to discriminate between living microorganisms and dead microorganisms. The fact that the same microbe-associated molecular patterns (MAMPs) are shared among microorganisms regardless of pathogenicity or viability yet can elicit different outcomes is still an unsolved quandary in innate immunity. In this issue of *Nature Immunology*, a paper by Neyen *et al.* suggests a resolution to this quandary[2].

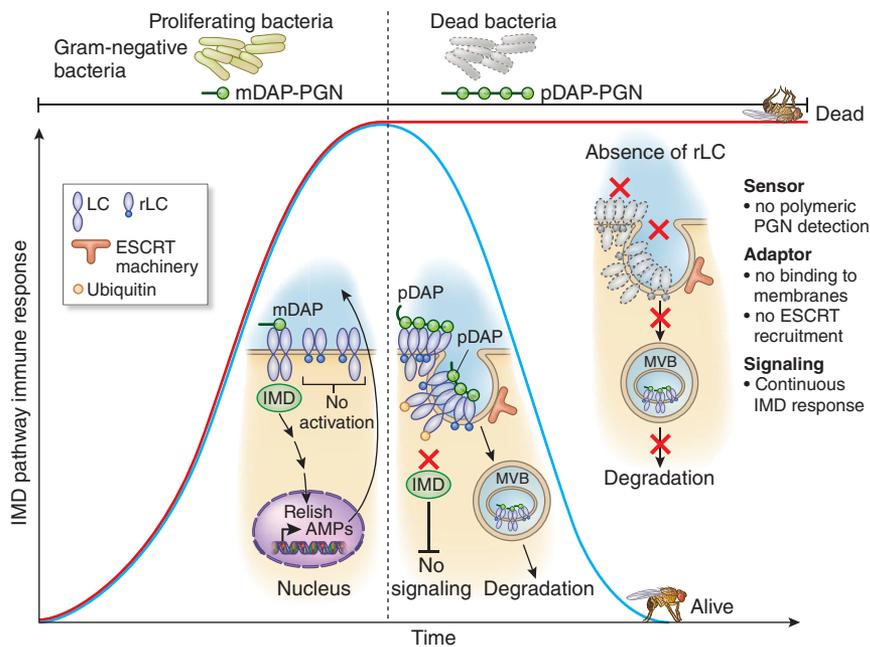The innate immune response is a tightly regulated process that consists of various

Figure 1 Role of rLC in resolution of the immune response after killing of Gram-negative bacteria. After bacterial infection, *Drosophila* PGRP-LC (LC) senses extracellular monomeric DAP-PGN (mDAP-PGN) and activates the IMD pathway, which culminates in the production of antimicrobial peptides (AMPs) and killing of the bacteria. After bacterial death, the immune system must move into a resolution phase. Two scenarios for this can be distinguished. It can comprise rapid resolution of the IMD immune response (blue line) in the presence of polymeric DAP-PGN (pDAP-PGN), a dead-bacteria-associated MAMP. rLC mediates the recruitment of PGRP-LC to the endosome, followed by recruitment of ESCRT, which promotes the removal of PGRP-LC from the membrane surface and its trafficking to multi-vesicular bodies (MVB). This inhibits sensing of microbes and the IMD signaling cascade. In the absence of rLC (red line), IMD activation is not resolved. As polymeric DAP-PGN is not sensed and activating receptors are not removed from the membrane surface by recruitment of ESCRT, there is an unresolved immune response that can kill the host.

phases. First, there is recognition of ligands such as MAMPs. That is followed by the activation of genes encoding products involved in host defense (antimicrobial peptides, inflammatory cytokines and 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 (IMD) pathway serves a principal role in responses to infection with Gram-negative bacteria by activating genes encoding transcription-factor-NF-κB-like products that control the expression of effectors such as antimicrobial peptides[4,5]. In contrast to the sensing of bacteria by vertebrates, the sensing of Gram-negative bacteria by *Drosophila* does not rely on the recognition of lipopolysaccharide but instead relies on the recognition of specific forms of diaminopimelic-acid (DAP)-type peptidoglycans (PGNs) by PGN-recognition proteins (PGRPs)[6–8].

Drosophila employs PGRP-LC to sense extracellular 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, is highly immunogenic and signals the presence of live bacteria. Polymeric PGNs are macromolecules released as a result of cell-wall destruction due to bacterial death and is sensed as innocuous by the fly immune system. Both TCT and polymeric PGNs activate the same signaling complex via the recruitment of ligand-induced clustering of PGRP receptor tails. *PGRP-LC* encodes three isoforms (PGRP-LCx, PGRP-LCa and PGRP-LCy) with different ectodomains that determine the differences in their ability to bind to PGNs. The recognition of polymeric PGNs relies on homotypic clusters (PGRP-LCx), whereas the recognition of TCT is dependent on PGRP-LCx–PGRP-LCa clusters. Interestingly, upon activation of TCT or PGNs, the immune system manages to achieve rapid resolution only during PGN activation, suggestive of an ability to discriminate between living bacteria and dead bacteria[8,9].

Neyen *et al.* identify and characterize the mechanism by which *Drosophila* discriminates between live Gram-negative bacteria and dead Gram-negative bacteria[2]. They discover a previously unknown isoform of the PGRP-LC that specifically recruits polymeric PGNs from dead bacteria and therefore contributes to the resolution phase of the immune response. This newly identified sensor controls IMD kinetics and thus prevents the lethality that stems 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 microorganisms and dead microorganisms.

Neyen *et al.* evaluate differential expression of the antimicrobial peptide Diptericin in flies given injection of live or dead Gram-negative bacteria and observe that dead bacteria generate a faster rate of resolution of the immune response[2]. They also obtain this result, in a dose-dependent manner, when they mimic infection in flies via injection of TCT (live bacteria) or polymeric PGNs (dead bacteria). Since both polymeric PGNs and TCT activate the IMD pathway through PGRP-LC, the authors search for the determinant of this differential response in PGRP-LC itself. They focus their attention on the *PGRP-LC* locus and find an alternative first exon that encodes a different cytosolic-tail variant that they call 'regulatory PGRP-LC' (rLC). This new isoform is also alternatively spliced much like PGRP-LC, with its isoforms PGRP-LCa, PGRP-LCx and PGRP-LCy. As a result, the number of isoforms increases from three (PGRP-LCa, PGRP-LCx and PGRP-LCy) to six (including the three rPGRP isoforms: rLCa, rLCx and rLCy) with similar expression, tissue distribution and kinetics after immunological challenge.

Owing to the unique features of its cytosolic tail, rLC is able to dampen the immune response only in the presence of polymeric PGNs. Indeed, mutant flies expressing solely rLC isoforms and flies that completely lack the *PGRP-LC* locus do not survive infection with Gram-negative bacteria or express antibacterial peptides, which means that rLC itself is not able to activate the IMD pathway. More precisely, the authors show that the rLCx isoform is necessary for the resolution of IMD activation in the presence of polymeric PGNs regardless of the presence of rLCa, PGRP-LCx or PGRP-LCa. As overexpression of rLCx potently reduces the immune response to polymeric PGNs but does not suppress the TCT-induced response, they propose that rLCx is a negative regulator of PGRP-LC that precisely discriminates between polymeric PGNs and monomeric PGN and contributes to the resolution of the immune-system activation by sensing the

presence of dead bacteria. Interestingly, even in the absence of rLC isoforms, flies are still able to kill the bacteria, but they die because of an inability to resolve autoinflammation triggered by the presence of MAMPs[2]. On the other hand, overexpression of rLCx results in poor activation of IMD.

So how does rLC downregulate IMD signaling? *In silico* analysis of rLC demonstrates the presence of a plant homeodomain (PHD)-type zinc-finger domain in the cytosolic amino-terminal tail that is involved in lipid interaction[10,11]. Analysis of overexpressed green-fluorescent-protein (GFP)-tagged rLCx shows that although full-length GFP-rLCx localizes to plasma membrane microdomains, mutant variants lacking the PHD region are evenly distributed, which suggests that the PHD might retain rLCx in specific membrane domains. As expected, rLC regulates the accumulation of surface-exposed PGRP-LC and therefore 'downregulates' the availability of 'activating' receptors (PGRP-LC) in the presence of dead bacteria. The authors go further and demonstrate a role for the endocytic pathway machinery in the termination of IMD signaling. Accordingly, depletion of endocytic components (Rab5 or Fab1) and components of the ESCRT endosomal sorting complex required for transport (Vps28 or Tsg101) results in accumulation of rLCx. It is known that receptor ubiquitination can act in endocytic signaling pathways. Indeed, Neyen *et al.* confirm that rLC interacts with the ubiquitin ligase DIAP2 in *Drosophila* cells via the PHD domain of rLC[2].

Since PGRP-LC protein accumulates in rLC-deficient flies, the authors next evaluate the correlation between rLC and the ESCRT machinery in regulating PGRP-LC. When rLC- and/or Vps28-deficient flies are given injection of dead bacteria, endogenous GFP–PGRP-LC accumulates in the fat body and the IMD pathway is not affected in the early steps of activation. rLC and Vps28 act together to remove PGRP-LC from the plasma membrane. Thus, altering endosome maturation and the formation of multivesicular bodies enhances immune-system activation and prevents resolution of the immune response. Neyen *et al.* propose rLC should be considered an adaptor that targets PGRP-LC to microdomains at the plasma membrane to promote the degradation of activating and regulatory receptors via ESCRT trafficking[2]. This mechanism interacts with another negative regulator of the IMD pathway (Pirk) and DIAP2.

PGRP-LC is well known as a major sensor of Gram-negative bacteria in flies, but how this receptor contributes to resolution of the immune response has remained unclear. The data by Neyen *et al.* suggest that rLC downregulates IMD signaling in response to polymeric PGNs via rLC-mediated endocytosis and ESCRT-dependent degradation of PGRP-LC[2]. The outcome of this process is a readjustment of the response according to the 'threat level'. The model presented by the authors suggests that an efficient way to shut down PGRP-LC receptors once the infection is cleared is by sensing the presence of dead bacterial ligands (polymeric PGNs) (**Fig. 1**).

The work of Neyen *et al.* highlights what seems to be a fundamental difference in the function of innate immune receptors in invertebrates versus that in vertebrates[2]. That is, if their observation is generalized to other immune-system receptors and other microorganisms in invertebrates, it is possible that the product of one gene or locus not only controls such discrimination but also functions as a receptor, adaptor and signaling protein. How can the regulation of these different functions be achieved in such a 'three in-one' model? In insects, a hint to the solution appears in studies of *Drosophila* MyD88, which has a dual function as both an adaptor and a signaling molecule[12]. Mammals solved this conundrum by extreme subcellular compartmentalization of receptors and downstream adaptors and signaling components[13]. Better delineation of this three-in-one model and understanding of why it might be employed in all invertebrates would be provided by elucidation of how the localization of one protein relates to the sites of signaling-complex assembly, if cell-type-specific differences in the subcellular positioning of receptor or signaling protein can explain the cell-type-specific responses to ligands (for example, sensing of bacteria in the gut versus that in the rest of the organism). Further insights could be gleaned by research into how harmful microorganisms influence the activity of this three-in-one protein and if there is a common strategy that microorganisms use to interfere with these processes. The answer to these and other issues will move the concept of immunity to new and unexpected places.

1. Janeway, C.A. Jr. *Cold Spring Harb. Symp. Quant. Biol.* **54**, 1–13 (1989).
2. Neyen, C., Runchel, C., Schüpfer, F., Meier, P. & Lemaitre, B. *Nat. Immunol.* **17**, 1150–1158 (2016).
3. Akira, S., Uematsu, S. & Takeuchi, O. *Cell* **124**, 783–801 (2006).
4. Lemaitre, B. *et al. Proc. Natl. Acad. Sci. USA* **92**, 9465–9469 (1995).
5. Hoffmann, J.A. & Reichhart, J.M. *Nat. Immunol.* **3**, 121–126 (2002).
6. Kaneko, T. *et al. Nat. Immunol.* **7**, 715–723 (2006).
7. Leulier, F. *et al. Nat. Immunol.* **4**, 478–484 (2003).
8. Kaneko, T. *et al. Immunity* **20**, 637–649 (2004).
9. Neyen, C., Poidevin, M., Roussel, A. & Lemaitre, B. *J. Immunol.* **189**, 1886–1897 (2012).
10. DiNitto, J.P., Cronin, T.C. & Lambright, D.G. *Sci. STKE* **2003**, re16 (2003).
11. Gozani, O. *et al. Cell* **114**, 99–111 (2003).
12. Marek, L.R. & Kagan, J.C. *Immunity* **36**, 612–622 (2012).
13. Kagan, J.C. & Medzhitov, R. *Cell* **125**, 943–955 (2006).