this dissociation came from the observations that addition of divalent cations, including Zn^{2+}, blocked the LAG3-induced Lck displacement and that the E-rich C-terminal region of LAG3 can bind Zn^{2+}, although weakly. Because the association of Lck with CD4 and CD8 depends on Zn^{2+} (ref. [9]), it seems likely that the LAG3 C-terminal region competes for the Zn^{2+} when in vicinity of the co-receptors, leading to Lck dissociation and thus to T cell inhibition. The displacement of Lck also relies on local acidification of the membrane at the immune synapse, which depends on the E-rich C-terminal region of LAG3 (Fig. 1). These E-rich intracellular domains control the sorting of proteins from endosomes to lysosomes. Hence, LAG3 may depend on its E-rich domain for its transport to endocytic lysosomal compartments, where it has been described[10]. Recruitment of the low-pH lysosomes containing LAG3 may contribute to the acidification of synaptic membranes and disruption of the association of p56Lck with its co-receptors. In the absence of the E-rich domain, these LAG3-dependent low-pH lysosomes would not be recruited and the pH in the vicinity of the TCR and CD4 or CD8 would not be altered. It is worth noting that LAG3-dependent local membrane acidification might also modify the reported association of Lck with CD3ε[8] and LAT[11]. Furthermore, evaluating whether proteins that contain comparable domains have inhibitory effects similar to those of LAG3 may lead to the characterization of new players in the regulation of T cell activation.

Finally, these results are particularly relevant because, despite the lack of functional understanding, LAG3 is a 'hot' target for anti-cancer immunotherapy. It is currently the subject of over 100 ongoing clinical trials, most of them using antagonistic monoclonal antibodies that block the interaction of LAG3 with MHCII[12]. Novel strategies that rely on the discovery reported here may increase the efficiency of LAG3-based immunotherapy. ❐

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Competing interests

The author declares no competing interests.

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INFLAMMASOME

Dual ligand engagement for noncanonical inflammasome activation

The activation of the noncanonical NLRP3 inflammasome can be elicited by the interaction and interdependent activation of caspase-11 and NLRP3 that follows coincident cytosolic detection of lipopolysaccharide and bacterial mRNA from live Gram-negative bacteria.

Zhang-Hua Yang and Jiahuai Han

Lipopolysaccharide (LPS), also known as endotoxin, is a component of the cell wall and the most studied molecular pattern in Gram-negative bacteria. Murine caspase-11 and human caspase-4 and caspase-5 are cytosolic receptors that detect intracellular LPS and selectively mediate the activation of the noncanonical NLRP3 inflammasome[1,2]. A few mechanisms have been proposed for caspase-11-mediated activation of NLRP3, but these models could not fully explain the activation of noncanonical NLRP3 inflammasome by infection with Gram-negative bacteria[3]. In this issue of *Nature Immunology*, Moretti et al. show that at least some noncanonical inflammasome activations induced by Gram-negative bacteria require not only LPS but also messenger RNA (mRNA) from the bacteria — with LPS engaging with caspase-11 and the mRNA concurrently binding to NLRP3, potentiating caspase-11 and NLRP3 interaction and activation of NLRP3 inflammasome[4].

Detection of LPS is central to host defense against infection with Gram-negative bacteria and to the pathogenesis of endotoxemia and sepsis. Extracellular LPS is sensed by Toll-like receptor 4 (TLR4); whereas cytosolic LPS is detected by caspase-11, which controls the activation of noncanonical inflammasome[2,5,6]. Intracellular LPS binds to and activates caspase-11 directly[2]. The activated caspase-11 cleaves the pore-forming protein gasdermin D (GSDMD)[7–9], which can lead to a lytic form of programmed necrosis called pyroptosis. The NLRP3 inflammasome can be activated downstream of the activation of caspase-11, and in that case is named noncanonical[1]. The noncanonical NLRP3 inflammasome is widely believed to be a secondary event following the caspase-11-triggered formation of the GSDMD pore[7]. It should

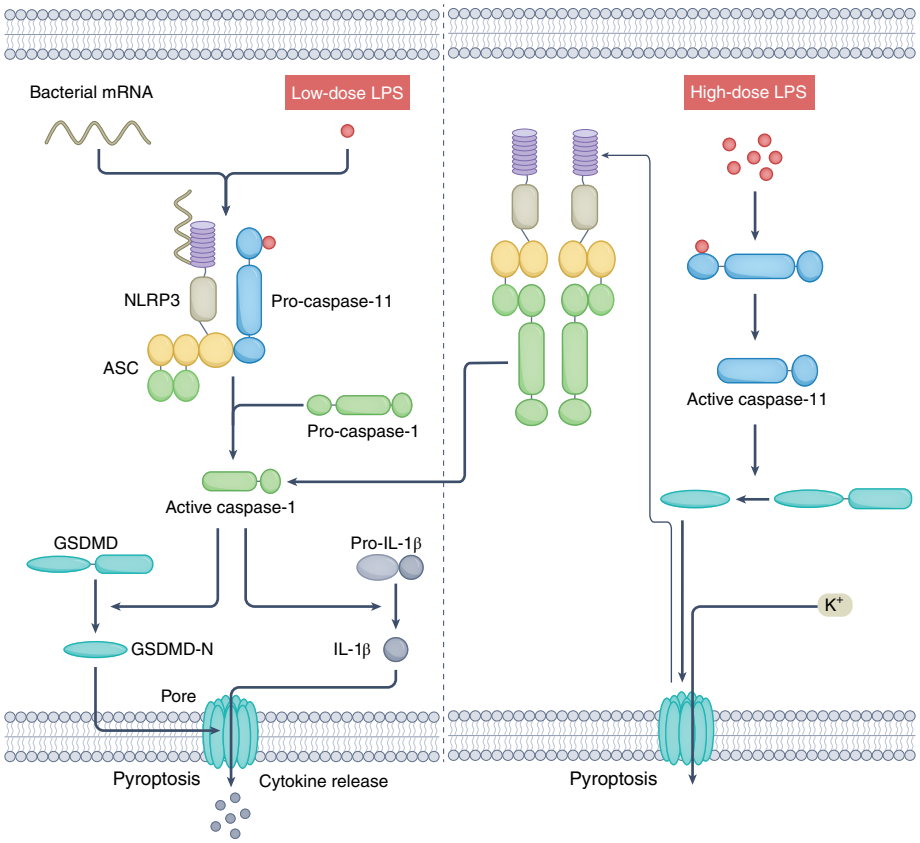

Fig. 1 | Two pathways for activation of the noncanonical NLRP3 inflammasome. If high-dose LPS is delivered into cells by Gram-negative bacterial infection, LPS directly activates caspase-11, resulting in cleavage of GSDMD and the formation of membrane pores. Dissipation of intracellular potassium (K^+) through the pores leads to activation of the NLRP3 inflammasome. If low-dose LPS is delivered to cells during Gram-negative bacterial infection, prokaryotic mRNA is required together with LPS to activate the noncanonical NLRP3 inflammasome. Concurrent detection of bacterial mRNA by NLRP3 and binding of LPS to caspase-11 promotes an interaction between caspase-11 and NLRP3. LPS-bound caspase-11 augments bacterial mRNA-dependent NLRP3 inflammasome assembly, and an assembled NLRP3 inflammasome promotes the activation of LPS-bound procaspase-11. This interdependent activation of caspase-11 and NLRP3 results in cleavage of caspase-1, GSDMD and IL-1β, and leads to pyroptosis and cytokine release. GSDMD-N represents the N-terminal of GSDMD generated by caspase-1/11 cleavage.

be noted that this conclusion is based on the experimental use of high-concentration intracellular LPS, but in many cases bacterial infection cannot deliver such amounts of LPS into cells. There could be other mechanisms.

Live — but not dead — Gram-negative bacteria can elicit noncanonical NLRP3 inflammasomes in macrophages[10]. Moretti et al. observed that, although heat-killed *Escherichia coli* did not induce the activation of caspase-11, addition of prokaryotic mRNA, a pathogen-associated molecular pattern (PAMP) linked to bacteria viability, elicited cleavage of caspase-11, caspase-1, GSDMD and interleukin-1β (IL-1β) to levels comparable to those elicited by live *E. coli*. Using multiple strains of mutant bacteria, Moretti et al. also showed that coincident detection of bacterial LPS and bacterial mRNA triggered the activation of the noncanonical NLRP3 inflammasome during Gram-negative bacterial infection. The role of bacterial mRNA was further confirmed by observations that the NLRP3 inflammasome can be strongly activated by experimental cytoplasmic delivery of bacterial mRNA and LPS into macrophages.

Previous models for the activation of caspase-11 are based on results obtained using cytoplasmic delivery of LPS[2,5,6]. In published studies, 1–50 μg ml^{-1} LPS has been used — this amount of LPS effectively elicits noncanonical inflammasome activation[2,5,6,11]. However, Moretti et al. calculated that the intracellular amount of LPS that resulted from the experimental delivery of 1 μg ml^{-1} LPS was on average 100-fold higher than the amount of cytosolic LPS caused by infection with avirulent *E. coli*, 10-fold higher than that caused by virulent *E. coli* and 6-fold higher than that caused by virulent *Salmonella typhimurium*; while delivery of low-dose LPS (2 ng ml^{-1}) was not sufficient to elicit the activation of the noncanonical inflammasome. This suggested that the amount of LPS delivered into cells by bacterial infection is not sufficient to trigger inflammasome activation on its own, and that other bacterial components could be involved. The authors also showed that delivery of 2 ng ml^{-1} LPS plus 100 ng ml^{-1} of bacterial mRNA, which approximated the amount of endotoxin and bacterial RNA measured in cytosolic fractions of macrophages infected with avirulent *E. coli*, activated the noncanonical NLRP3 inflammasome. These observations indicated the concurrent stimulation of two different PAMPs from infected Gram-negative bacteria might represent the mechanism for the activation of the noncanonical inflammasome.

As a known PAMP, bacterial RNA had been reported to be sufficient to activate NLRP3 inflammasome[10,12]. Because the role of caspase-11 as a receptor for LPS in the activation of the noncanonical NLRP3 inflammasome was not known at that time, contamination of bacterial RNA with LPS might not have been considered. To address this issue, Moretti et al. used RNA from the Gram-positive bacteria *Listeria innocua* (which lacks LPS), in vitro transcribed bacterial RNA or RNA prepared from a mutant *E. coli* that expresses a tetra-acylated, non-stimulatory LPS and observed that bacterial RNA alone could not induce the activation of the noncanonical NLRP3 inflammasome. Thus, LPS alone at high intracellular concentration can elicit noncanonical NLRP3 inflammasome, but bacterial RNA can only synergize with cytosolic LPS to activate the noncanonical NLRP3 inflammasome (Fig. 1).

The activation mechanism of noncanonical NLRP3 inflammasome by the synergized stimulation with LPS and bacterial mRNA appears to be different from that induced by high-dose cytosolic LPS (Fig. 1). A prerequisite for NLRP3 activation is the initial assembly of the NLRP3 inflammasome and subsequent oligomerization of the inflammasomes adaptor ASC into prion-like structures that recruit and activate caspase-1, which applied to both high-dose cytosolic LPS and LPS plus bacterial mRNA-induced activation of the noncanonical NLRP3 inflammasome. However, unlike high-dose cytosolic LPS — which can directly elicit caspase-11 activation, cleavage of GSDMD and pyroptosis — LPS plus bacterial mRNA requires NLRP3 and ASC

to induce these same processes. During high-dose LPS-induced activation of the noncanonical NLRP3 inflammasome, the formation of the NLRP3–ASC complex is downstream of caspase-11-mediated GSDMD pore formation; whereas LPS plus bacterial mRNA-induced activation of the inflammasome results from amplified signaling between the caspase-11 and NLRP3 complex, as NLRP3 inflammasome assembly induced by LPS plus bacterial mRNA requires caspase-11. Of note, the relation between caspase-11 and inflammasome receptors is limited to NLRP3 (Fig. 1). Moretti et al. analyzed caspase-11 activation, GSDMD cleavage and pyroptosis elicited by virulent or avirulent bacteria and found the former triggered faster NLRP3- and ASC-independent pyroptosis, in agreement with the triggering of the caspase-11–GSDMD–pyroptosis pathway, while the latter required assembly of the NLRP3 inflammasome, correlating with the amount of intracellular LPS delivered by infection with virulent or avirulent Gram-negative bacteria. Why virulent and avirulent Gram-negative bacteria deliver different amounts of LPS into infected cells awaits further investigation.

Moretti et al. then asked whether there was a specific interaction between caspase-11 and NLRP3 underlying their interdependent activation by LPS plus bacterial mRNA. They found that bacterial infection could induce disassembly of the trans-Golgi network (TGN) in macrophages, and that caspase-11 colocalized with NLRP3 on the dispersed TGN (dTGN) in an NLRP3-dependent manner (Fig. 1). Of note, disassembly of the TGN and recruitment of NLRP3 to the dTGN have been reported as common upstream events leading to canonical NLRP3 inflammasome activation. Using a coimmunoprecipitation assay, Moretti et al. identified a biochemical interaction between caspase-11 and NLRP3, which was specifically elicited by the combination of LPS and bacterial mRNA or other NLRP3 agonists. Further analysis showed that caspase-11 and NLRP3 interaction remained intact in *Gsdmd*$^{-/-}$, *Pycard*$^{-/-}$ and *Casp1*$^{-/-}$ macrophages, and the interaction did not rely on activation of caspase-11, as caspase-11 interacted with NLRP3 in its pro-form. In addition, the inhibition of caspase-11 with the pan-caspase inhibitor zVAD–FMK had no effect on the interaction between caspase-11 and NLRP3. These findings indicate that coincident cytosolic detection of LPS and bacterial mRNA is sufficient to mediate a specific interaction between caspase-11 and NLRP3, which is upstream of inflammasome assembly and caspase-11 activation.

Collectively, Moretti et al. have elegantly demonstrated a previously undescribed mode of noncanonical NLRP3 inflammasome activation by the coincident cytosolic detection of LPS and bacterial mRNA during live Gram-negative bacterial infection. As simultaneous entry of LPS and bacterial mRNA into cells via Gram-negative bacterial infection should commonly occur, this dual-ligand synergistically triggered noncanonical NLRP3 inflammasome activation is likely to be a primary mechanism, in addition to the existing model of caspase-11–GSDMD pore–NLRP3 assembly. Although more studies are needed to extend these findings to human caspase-4/5 and to determine whether other viability-associated PAMPs can serve similar functions, the characterization of bacterial mRNA and LPS in combination as a trigger for noncanonical inflammasome activation extends our knowledge of innate immune reactions. ❐

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Competing interests

The authors declare no competing interests.

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IMMUNOMETABOLISM

Creating ATP via creatine kinase B for NLRP3 activation

A role for mitochondrial ATP that leads to phosphocreatine and the subsequent generation of cytosolic ATP via creatine kinase B is now proposed in the activation of the NLRP3 inflammasome.

Juliana E. Toller-Kawahisa and Luke A. J. O'Neill

The NLRP3 inflammasome is the primary sensor of phagocytosed material in macrophages[1]. It activates caspase-1 to promote production of the inflammatory cytokines IL-1β and IL-18, and triggers pyroptosis via the cleavage of gasdermins[2]. NLRP3 has been implicated in a number of diseases, including atherosclerosis, asthma, gout and Parkinson's disease[3]. How it is regulated has therefore been the focus of much attention. Reactive oxygen species (ROS) produced by mitochondria, specifically from complex I in the electron-transport chain (ETC), have been implicated in NLRP3 activation.