Galectin-3 promotes noncanonical inflammasome activation through intracellular binding to lipopolysaccharide glycans

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Edited by Hao Wu, Harvard Medical School, Boston, MA, and approved May 23, 2021 (received for review January 10, 2021)

Cytosolic lipopolysaccharides (LPSs) bind directly to caspase-4/5/11 through their lipid A moiety, inducing inflammatory caspase oligomerization and activation, which is identified as the noncanonical inflammasome pathway. Galectins, β-galactoside–binding proteins, bind to various gram-negative bacterial LPS, which display β-galactoside–containing polysaccharide chains. Galectins are mainly present intracellularly, but their interactions with cytosolic microbial glycans have not been investigated. We report that in cell-free systems, galectin-3 augments the LPS-induced assembly of caspase-4/11 oligomers, leading to increased caspase-4/11 activation. Its carboxyl-terminal carbohydrate-recognition domain is essential for this effect, and its N-terminal domain, which contributes to the self-association property of the protein, is also critical, suggesting that this promoting effect is dependent on the functional multivalency of galectin-3. Moreover, galectin-3 enhances intracellular LPS-induced caspase-4/11 oligomerization and activation, as well as gasdermin D cleavage in human embryonic kidney (HEK) 293T cells, and it additionally promotes interleukin-1β production and pyroptotic death in macrophages. Galectin-3 also promotes caspase-11 activation and gasdermin D cleavage in macrophages treated with outer membrane vesicles, which are known to be taken up by cells and release LPSs into the cytosol. Coimmunoprecipitation confirmed that galectin-3 associates with caspase-11 after intracellular delivery of LPSs. Immunofluorescence staining revealed colocalization of LPSs, galectin-3, and caspase-11 independent of host *N*-glycans. Thus, we conclude that galectin-3 amplifies caspase-4/11 oligomerization and activation through LPS glycan binding, resulting in more intense pyroptosis—a critical mechanism of host resistance against bacterial infection that may provide opportunities for new therapeutic interventions.

galectin-3 | lipopolysaccharide | caspase-4/11 | noncanonical inflammasome

Lipopolysaccharides (LPSs) are pathogen-associated molecular patterns that can elicit a host defense response through binding to cell-surface Toll-like receptor 4 (TLR4). Systemic inflammatory response syndrome is induced by overstimulation of the innate immune response via LPSs, resulting in severe multiple organ failure, which is a major cause of death worldwide in intensive care units (1). LPS-induced dimerization of TLR4 initiates signal transduction involving the NF-κB– and MyD88-dependent and -independent pathways, thereby contributing to various inflammatory responses (2). Another set of the immune repertoire, which resides in the cytosol and comprises NLRP1, NLRP3, NAIP/NLRC4, and AIM2, is known as the inflammasome. Inflammasomes can be activated in response to a number of well-defined pathogen-derived ligands and physiological aberrations, which in turn trigger caspase-1–mediated pyroptotic death (3, 4). This process has been associated with strengthening the host defense program to eliminate intracellular bacteria.

Recently, a cytosolic LPS-sensing pathway involving caspase-4/5 in humans and caspase-11 in mice was termed the noncanonical inflammasome pathway, and this pathway is independent of TLR4 (5–8). LPSs from extracellular bacteria can enter the cytoplasm and trigger caspase-4/5/11–dependent responses. LPSs can be delivered into the cytosol when LPS-containing outer membrane vesicles (OMVs) from gram-negative bacteria are taken up by the cells or when intracellular bacteria escape from the phagosomes that are damaged by host resistant factors such as guanylate-binding protein and HMGB1 or microbe-derived hemolysins (9–12). LPSs comprise three regions: lipid A, core oligosaccharide, and O-polysaccharide (also termed O-antigen). The lipid A moiety binds directly to the caspase-4/5/11 caspase activation and recruitment domain (CARD, also known as prodomain), leading to caspase oligomerization and activation (7). This event likely mimics the proximity-induced dimerization model of initiator caspase activation (13). Furthermore, caspase-4/5/11 executes downstream signaling events via gasdermin D. Activated inflammatory caspase proteolytically cleaves gasdermin D to create an N-terminal fragment that self-oligomerizes and then inserts into the cell membrane to form pores, causing lytic cell death (14–17). Various stimuli have been identified in the caspase-1–mediated canonical-inflammasome signaling pathway (3, 4), but

Significance

Cytosolic lipopolysaccharides (LPSs) induce oligomerization of caspase-4/5/11, resulting in pyroptosis, but the involvement of any other host resistance factors in this process is unknown. Galectins bind to components of pathogenic microorganisms, particularly LPSs, in a glycan-dependent manner. However, these proteins are mainly present intracellularly, and little is known regarding their functions associated with binding to components of microorganisms, including LPSs, in the cytosol. Here, we report that galectin-3 recognizes cytosolic LPSs from various bacteria and amplifies LPS-induced caspase-4/11 oligomerization and activation, causing more intense pyroptosis in a carbohydrate-dependent manner. This study defines a unique molecular mechanism based on the carbohydrate-binding and self-association properties of galectin-3, through which this glycan-binding protein enhances cytosolic LPS-induced noncanonical inflammasome activation in macrophages.

Author contributions: T.-H.L. and F.-T.L. designed research; T.-H.L. and C.-I.Y. performed research; H.-L.C., I.-C.W., C.-S.L., C.-C.H., N.-J.C., C.-H.L., and F.-T.L. contributed new reagents/analytic tools; H.-L.C., C.-I.Y., I.-C.W., C.-S.L., and F.-T.L. analyzed data; and T.-H.L. and F.-T.L. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2026246118/-/DCSupplemental.

Published July 23, 2021.

the detailed mechanism underlying noncanonical inflammasome activation mediated by caspase-4/5/11 remains unclear.

Galectins, a family of β-galactoside–binding proteins, can decode host-derived complex glycans and are involved in various biological responses (18–23). Galectins are nucleocytoplasmic proteins synthesized without a classical signal sequence, although they can be secreted through unconventional pathways (19, 21, 23, 24). Recent studies have revealed prominent roles of cytosolic galectins in host defense programs (12, 25, 26). The proposed molecular mechanisms involve the binding of galectins to host glycans exposed to the cytosolic milieu upon endosomal or phagosomal membrane damage. In addition to binding host glycans, galectins also recognize microbial glycans, particularly LPSs (27–30). However, the contribution of galectins to the host response through binding to cytosolic LPSs is unknown.

Galectin-3 is an ∼30-kDa protein that contains a carbohydrate-recognition domain (CRD) connected to N-terminal proline, glycine, and tyrosine-rich tandem repeats. Upon binding to multivalent glycoconjugates through its CRD, the protein forms oligomers, which is attributable to the self-association property of its N-terminal region (31, 32). Galectin-3 binds to LPSs of various gram-negative bacteria by recognizing their carbohydrate residues (33–36).

Although structural information is scarce (37), existing information suggests that ligand-induced oligomerization of caspase CARD is necessary for the activation of inflammatory caspases (7, 38). Therefore, we hypothesized that galectin-3 may be an intracellular LPS sensor that participates in LPS-induced CARD-mediated inflammatory caspase activation. Specifically, highly ordered arrays of LPS–galectin-3 complexes may amplify caspase-4/5/11 oligomerization and activation. Here, we investigated the formation of galectin-3–LPS–caspase-4/11 complexes in cell-based and cell-free systems. Our findings provide evidence regarding a role of galectin-3 as an intracellular mediator in noncanonical inflammasome activation through LPS glycan recognition.

Results

LPSs Form Ternary Complexes with Galectin-3 and Caspase-11. We first confirmed that galectin-3 binds to *Escherichia coli* LPSs in a carbohydrate-dependent manner (*SI Appendix*, Fig. S1), with a dissociation rate constant (K_D) of ∼100 nM, but does not bind to muramyl dipeptide (MDP) (*SI Appendix*, Fig. S2 *A* and *B*). Also, in a cell-free system with various truncated domains of caspase-11 (Fig. 1*A*), we found that both caspase-11 CARD and galectin-3 could be coisolated with LPSs (Fig. 1*B*). Importantly, we found that the caspase-11 CARD was coimmunoprecipitated with galectin-3 in the presence of *E. coli* LPSs (Fig. 1*C*). Here, such coimmunoprecipitation was not observed in the presence of *Helicobacter pylori* LPSs (Fig. 1*C*), which contain tetra-acylated lipid A, unlike *E. coli,* which contains hexa-acylated lipid A, although both strains display sugar moieties recognizable by galectin-3 (33, 36). This is consistent with the fact that *H. pylori* LPSs have been shown to escape caspase-11 surveillance, although the mechanism is unknown (8). Interestingly, the caspase-11 catalytic domain was coimmunoprecipitated with galectin-3 in the presence of *H. pylori* LPSs but not *E. coli* LPSs (Fig. 1*D*). Galectin-3 also formed complexes with the caspase-11 CARD in the presence of *Salmonella minnesota* LPSs (*SI Appendix*, Fig. S3). These findings suggest that LPSs from various bacterial strains interact with different domains of caspase-11 and that galectin-3 is associated with this complex through binding to LPSs. The formation of galectin-3–LPS–caspase-11 complexes was prevented by the presence of lactose, an inhibitor of galectin-3–glycan interaction, but not by sucrose, which does not bind to galectin-3 (Fig. 1*E*). Hence, galectin-3 binds to LPSs and forms ternary complexes with caspase-11 in a carbohydrate-dependent manner.

Galectin-3 Amplifies LPS-Induced Caspase-11 Complex Assembly and Activation in a Carbohydrate-Dependent Manner. Various amounts of galectin-3, as well as LPSs, were added to recombinant caspase-11 CARD to test whether galectin-3 could promote the assembly of caspase-11 complexes upon binding to LPSs. As expected, LPSs induced the formation of caspase-11 oligomeric complexes (>1,000 kDa) in a dose-dependent manner (Fig. 2*A*). Galectin-3 significantly amplified the formation of these complexes at lower but not higher LPS concentrations (Fig. 2*B*). High-molecular-weight molecules containing galectin-3 of similar size to oligomeric caspases were also detected (Fig. 2*B*). Consistent with the fact that formation of caspase-11 oligomeric complexes results in its activation (7), we found galectin-3 enhanced LPS-induced caspase-11 enzymatic activity (Fig. 2*C*). This promoting effect was blocked by lactose but not sucrose (Fig. 2*C*), suggesting that recognition of LPS β-galactoside residues by galectin-3 is involved in this effect. Moreover, while galectin-3 increased LPS-induced caspase-11 enzymatic activity in a dose-dependent manner, the carboxyl-terminal domain fragment of galectin-3 (galectin-3C) was ineffective in this regard (Fig. 2*D*). The N-terminal domain of galectin-3 is known to be essential for the multivalent property of the full-length protein because this domain is engaged in protein self-association. Hence, these results suggest that the functional multivalency of galectin-3 is critical for this promoting effect. Overall, these observations indicate that galectin-3 promotes LPS-induced caspase-11 oligomerization and activation by recognizing LPS glycans.

Intracellular Galectin-3 Enhances Caspase-11 Complex Assembly and Gasdermin D Cleavage. Next, we tested the effect of galectin-3 on caspase-11 oligomerization via LPSs inside the cell. Human HEK 293T cells, which contain only a low amount of galectin-3, were transfected with galectin-3 alone or in combination with caspase-11 followed by transfection with LPSs. Galectin-3 did not cause caspase-11 oligomerization in the absence of LPSs. In the absence of galectin-3, LPSs were insufficient to induce caspase-11 oligomers (Fig. 3*A*). In contrast, a large amount of caspase-11 oligomers were detected in cells cotransfected with galectin-3 and caspase-11 when combined with intracellular delivery of LPSs (Fig. 3*A*). Moreover, caspase-11 oligomerization was not observed in cells transfected with a galectin-3 mutant lacking functional carbohydrate-binding activity (Fig. 3*B*). These data indicate that CRD of galectin-3 is required for caspase-11 oligomerization in the presence of LPSs.

Activated caspase-4/5/11 proteolytically cleaves gasdermin-D (p50) to generate its 30-kDa N-terminal fragment (p30), thereby initiating pyroptotic cell death (15). HEK 293T cells were cotransfected with gasdermin D, galectin-3, and caspase-11, followed by transfection with LPSs, to test whether galectin-3 could facilitate cleavage of gasdermin D. Galectin-3 did not increase cleavage of gasdermin D in the absence of LPSs but amplified cleavage in cells transfected with low but not high doses of LPSs (Fig. 3*C*). It is to be noted that a low level of gasdermin D N-terminal fragments (p30) was detected when cells were cotransfected with caspase-11 and gasdermin D, but not when cells were cotransfected with LPSs (Fig. 3*C*), which might be related to the spontaneous formation of caspase-11 oligomers, which exhibit proteolytical activity to cleave gasdermin D even in the absence of its ligand.

Galectin-3 Promotes Intracellular LPS-Induced Noncanonical Inflammasome Activation in Macrophages. We then investigated whether cytosolic galectin-3 could contribute to noncanonical inflammasome activation in macrophages. Bone marrow–derived macrophages (BMDMs) contain low levels of caspase-11, but a priming step [mediated by substances such as Pam3CSK4, poly(I:C), and LPSs] for TLR-mediated transcriptional activation can significantly enhance its expression (39). We first determined whether galectin-3 regulates the expression of caspase-11. When BMDMs from wild-type (WT) and galectin-3 knockout (KO) mice were primed with Pam3CSK4, two isoforms of caspase-11 (38 and 43 kDa) were highly induced, but both were comparably expressed between two

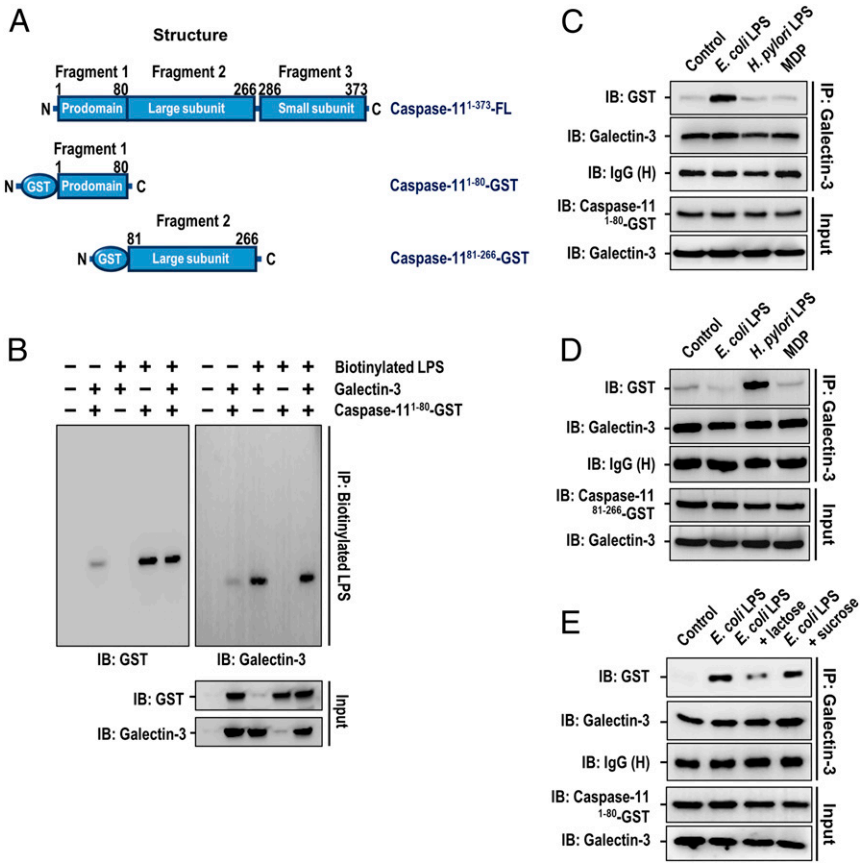

Fig. 1. LPSs form ternary complexes with galectin-3 and caspase-11. (*A*) Schematic illustration of full-length caspase-11 and its truncated domains (CARD and catalytic domain) with a GST tag. (*B*) GST-tagged caspase-11 CARD and/or galectin-3 were incubated with biotinylated *E. coli* LPSs and then mixed with streptavidin beads. The bound protein was eluted from the beads, followed by immunoblotting analysis. (*C* and *D*) Reaction mixtures containing galectin-3 and GST-tagged caspase-11 CARD (*C*) or catalytic domain (*D*) were incubated with *E. coli* or *H. pylori* LPSs. The mixtures were treated with anti–galectin-3 antibodies, and the immunoprecipitates were subjected to immunoblotting with anti-GST antibodies for detection of caspase-11 truncated domains. (*E*) Galectin-3 was incubated with GST-tagged caspase-11 CARD and LPSs in the presence of lactose or sucrose. The mixtures were treated with anti–galectin-3 antibodies, and the immunoprecipitates were subjected to immunoblotting with anti-GST antibodies. In *B* through *E*, the immunoblots of the total input proteins are shown in the lower sections. Data are representative of three independent experiments.

genotypes of BMDMs (*SI Appendix*, Fig. S5). This indicates that galectin-3 does not affect caspase-11 expression through transcriptional regulation.

Next, BMDMs were transfected with LPSs from *E. coli*, *H. pylori*, *Pseudomonas aeruginosa*, *S. minnesota*, or *Klebsiella pneumoniae*. Significantly lower levels of pyroptosis, indicated by lactate dehydrogenase (LDH) release (Fig. 4*A*) and IL-1β secretion (Fig. 4*B*), were observed in galectin-3 KO BMDMs compared to WT cells in response to all bacterial LPSs used except that from *H. pylori*. Compared to WT cells, galectin-3 KO BMDMs also exhibited a lower extent of caspase-11 activation, which was measured by the appearance of caspase-11 in the culture supernatant (Fig. 4*C*) (8, 12). These data indicate that galectin-3 promotes intracellular LPS-induced noncanonical inflammasome activation in macrophages. The negative result for the *H. pylori* LPS is consistent with the fact this LPS poorly triggers noncanonical inflammasome activation (5).

We also generated galectin-3 KO RAW 264.7 mouse macrophage cell lines and noted that these cells exhibited a lower cytosolic LPS-induced pyroptotic response than WT cells after they were transfected with LPSs (Fig. 4 *D* and *E*). This was also confirmed when LPSs from *E. coli* or *S. minnesota* were electroporated into the cells (*SI Appendix*, Fig. S6). Moreover, ectopic expression of galectin-3 in galectin-3 KO RAW 264.7 cells restored the ability of cytosolic *E. coli* and *S. minnesota* LPSs to induce pyroptosis. These findings were not observed with ectopic expression of a galectin-3 mutant lacking functional carbohydrate-binding activity (*SI Appendix*, Figs. S7 and S8 *A* and *B*).

β-galactoside–containing glycans are present in the O-antigen and core sections of LPSs. Sugar-free LPS Re variants (derived from *E. coli* and *S. minnesota* LPSs) were transfected into the RAW 264.7 macrophage cell line, and LPSs were isolated from the cell lysate using beads coated with antibodies against lipid A. In lysates from cells transfected with full-length LPSs, galectin-3 could be coisolated with LPSs but not from those transfected with LPS Re (*SI Appendix*, Fig. S9 *A* and *B*). Moreover, caspase-11 could be coisolated with galectin-3 in the RAW 264.7 macrophage cell line transfected with LPSs but not sugar-free LPS Re (*SI Appendix*, Fig. S9 *A* and *B*). Compared with sugar-free LPS Re, the transfection of cells with full-length LPSs resulted in more intense pyroptotic death (*SI Appendix*, Fig. S9 *A* and *B*). It is to be noted that the ability of sugar-free LPS Re to induce pyroptotic death is not as strong as that reported previously (7), which needs to be addressed in future studies. These data emphasize that LPS glycans are required for mediating galectin-3 interaction with caspase-11, which results in increased pyroptotic death.

Infection with *Listeria monocytogenes* (LM) combined with LPSs reportedly triggers the cytosolic LPS sensing pathway due to phagosome lysis by the pore-forming toxin listeriolysin O (8), resulting in pyroptotic cell death. Under these conditions, galectin-3 KO BMDMs also exhibited less LDH release and IL-1β secretion than WT cells (*SI Appendix*, Fig. S10 *A* and *B*). Finally, bacterial OMVs are known to be taken up by cells and mediate LPS intracellular delivery and subsequent caspase-11–mediated pyroptosis (9). OMVs isolated from *E. coli* triggered lower cell death in galectin-3 KO cells (Fig. 4*F* and *SI Appendix*, Fig. S11). Altogether, these data indicate that galectin-3 promotes noncanonical inflammasome activation in macrophages and that its CRD is essential for this function.

Galectin-3 Forms Complexes with Caspase-11 and Increases Cleavage of Gasdermin D in Macrophages after LPS Intracellular Delivery. We then proceeded to detect the formation of galectin-3–caspase-11 complexes in macrophages induced by LPS intracellular delivery. BMDMs were transfected with *E. coli* LPSs followed by treatment

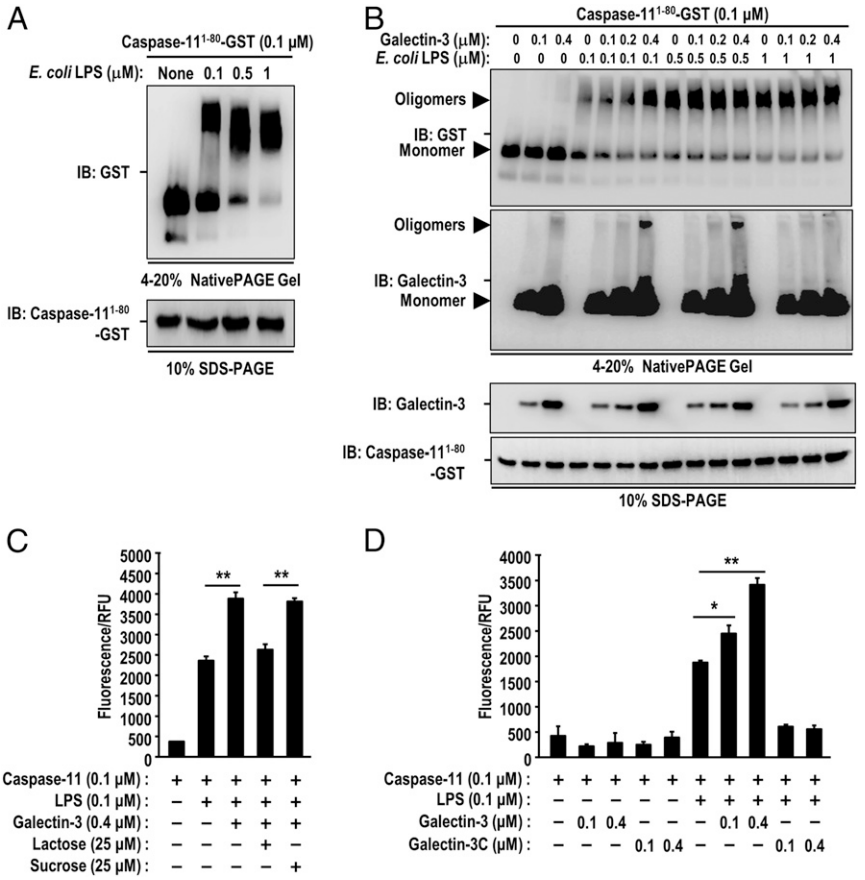

Fig. 2. Galectin-3 amplifies LPS-induced caspase-11 complex assembly and activation in a carbohydrate-dependent manner. (*A*, *Top*) Various amounts of LPSs were added to caspase-11 CARD, and the mixtures were assessed by 4 to 20% native PAGE followed by immunoblotting. High-molecular-weight complexes were noted at the top of the gel. (*Bottom*) The mixtures were assessed by 10% SDS-PAGE followed by immunoblotting. (*B*, *Top*) Various amounts of galectin-3 and LPSs were mixed with caspase-11 CARD, and the mixtures were assessed by 4 to 20% native PAGE followed by immunoblotting. (*Bottom*) The mixtures were assessed by 10% SDS-PAGE followed by immunoblotting. The black arrowheads point to the bands corresponding to monomers and oligomers, respectively, as indicated. (*C*) Galectin-3 and LPSs were added to caspase-11 along with the addition of lactose or sucrose. (*D*) Various amounts of galectin-3 or galectin-3C were added to LPSs and caspase 11. In both *C* and *D*, caspase-11 enzymatic activity was assayed using the fluorogenic substrate Z-VAD-AMC. Error bars indicate the mean ± SD. Data are representative of three independent experiments. Statistical analysis was performed using unpaired Student's *t* tests; $*P < 0.05$, $**P < 0.01$.

with a membrane-permeable chemical crosslinker, dithiobis succinimidyl propionate (DSP). As shown in Fig. 5*A*, galectin-3 could be coimmunoprecipitated with caspase-11 from these cells. Similar results were obtained in RAW 264.7 and J774 mouse macrophage cell lines transfected with LPSs from *E. coli*, *H. pylori*, *P. aeruginosa*, *S. minnesota*, or *K. pneumoniae* (Fig. 5 *B* and *C*). These data indicate that the association between galectin-3 and caspase-11 occurs in macrophages after LPS intracellular delivery. Here, the *H. pylori* LPS also promotes the association between galectin-3 and caspase-11 (Fig. 5 *B* and *C*), because this LPS binds to the catalytic domain of caspase-11 (Fig. 1*D*) and is recognized by galectin-3 even though it does not trigger pyroptotic cell death.

We also addressed whether galectin-3 regulates caspase-11–mediated gasdermin D cleavage in macrophages. Cytosolic LPSs induced gasdermin D cleavage in a caspase-11–dependent manner, which occurred at lower levels in galectin-3 KO RAW 264.7 cells (Fig. 5*D*). Additionally, we found that OMV-induced caspase-11 activation and gasdermin D cleavage were abrogated in galectin-3 KO RAW 264.7 cells (Fig. 5*E*). Gasdermin D (p50) was detected in the culture supernatant after LPS transfection (Fig. 5*D*) but not after OMVs treatment (Fig. 5*E*). This may be due to the smaller pores formed, reflecting a lower extent of pyroptosis (40) and thus more limited lytic release of larger molecules in cells treated with OMV compared to those transfected with LPSs. These data indicate that galectin-3 promotes gasdermin D cleavage after LPS intracellular delivery in macrophages.

Galectin-3 Colocalizes with Caspase-11 in Macrophages after LPS Intracellular Delivery. The J774 mouse macrophage cell line was transfected with the *S. minnesota* LPS and examined at various times by immunofluorescence staining to determine the association between galectin-3 and caspase-11. Galectin-3 began colocalizing with caspase-11 4 h after transfection, and peak colocalization was observed 8 h after transfection (Fig. 6 *A*–*E*) as confirmed by calculating Pearson's correlation coefficient (Fig. 6 *B* and *C*) (41). These results suggest that galectin-3 colocalizes with caspase-11 in macrophages after LPS intracellular delivery.

Exposure of host glycans due to damaged intracellular vesicles (or pathogen-containing vacuoles) can lead to galectin recruitment followed by activation of the autophagy machinery (25). We generated mutant RAW 264.7 cells lacking *N*-acetylglucosaminyltransferase I (encoded by *Mgat1*), which is responsible for the biosynthesis of the complex type-N glycans, the glycan ligands of galectin-3 (42). Galectin-3 still colocalized with both LPSs and caspase-11 in *Mgat1* KO RAW 264.7 cells (Fig. 6*F*), indicating that galectin-3 forms complexes with caspase-11 in the presence of LPSs independently of host *N*-glycans.

Galectin-3 Promotes LPS-Induced Caspase-4 Oligomerization and Activation, Leading to More Severe Pyroptosis in Macrophages. Caspase-4 and -5 are the human orthologs of mouse caspase-11, with caspase-4 sharing 59% homology with mouse caspase-11. The functional similarities between mouse caspase-11 and human caspase-4 and -5 in response to LPSs were recently highlighted (7, 43, 44). Therefore, we examined whether galectin-3 is involved in caspase-4–mediated pyroptosis using the galectin-3 knockdown human THP-1 cell line (Fig. 7*A*). After transfection with *E. coli* or *S. minnesota* LPSs, galectin-3 knockdown THP-1 cells exhibited less LDH release and IL-1β secretion than WT cells (Fig. 7 *B* and *C*). We also performed an oligomerization assay with human HEK 293T cells to test whether galectin-3 causes caspase-4 oligomerization in the presence of LPSs. These cells were transfected with galectin-3 alone or in combination with caspase-4. LPSs alone were insufficient to induce the formation of high-molecular-mass

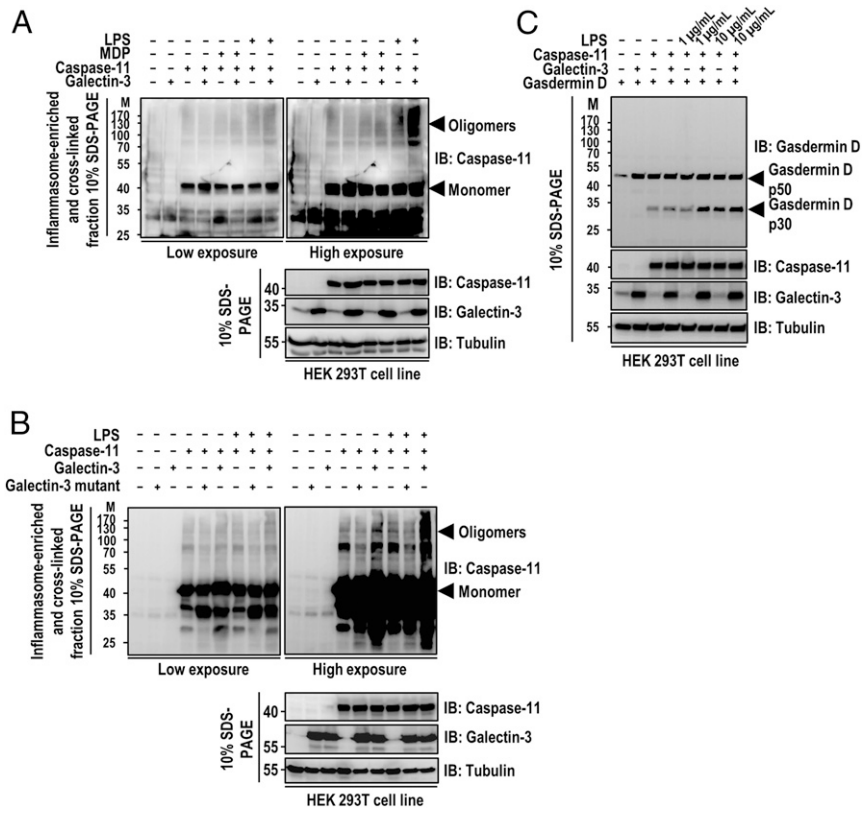

Fig. 3. Intracellular galectin-3 enhances caspase-11 complex assembly and gasdermin D cleavage. (*A*) HEK 293T cells were transfected with a combination of expression vectors containing galectin-3 and caspase-11, respectively, followed by transfection with *E. coli* LPSs or MDP. After cell lysis, inflammasome protein complexes were enriched by serial centrifugation and treated with a noncleavable cross-linker. The cross-linking multiprotein inflammasome complexes were analyzed by SDS-PAGE followed by immunoblotting. The caspase-11 complex was noted at the top of the gel. (*B*) HEK 293T cells were transfected with a combination of expression vectors carrying WT galectin-3 or a galectin-3 CRD mutant (H158A/N160A/R162A) and caspase-11, respectively, followed by transfection with the *E. coli* LPS and then processed as described in *A*. In *A* and *B*, the immunoblots of the total input proteins (not cross-linked) are shown in the lower sections. (*C*) HEK 293T cells were transfected with a combination of expression vectors carrying gasdermin D, caspase-11, and galectin-3, respectively, followed by transfection with the *E. coli* LPS. A total of 8 h after LPS transfection, the cell lysates were subjected to immunoblotting analysis for detection of full-length gasdermin D (p50) and N-terminal gasdermin D (p30). The black arrowheads point to the bands corresponding to monomers, oligomers, and gasdermin D, respectively, as indicated. Data are representative of two independent experiments.

oligomeric caspase-4 complexes (Fig. 7*D*), and galectin-3 did not cause caspase-4 oligomerization in the absence of LPSs (Fig. 7*D*). Caspase-4 oligomerization was only detected in cells cotransfected with galectin-3 and caspase-4 when combined with intracellular delivery of LPSs from *E. coli*, *P. aeruginosa*, or *S. minnesota* but not MDP (Fig. 7*D*). These data indicate that galectin-3 is required for caspase-4 oligomerization in the presence of LPSs.

We also conducted a protein cross-linking and oligomerization assay using a cell-free system to investigate the promoting effect of galectin-3 on caspase-4 oligomerization. Caspase-4 oligomerization was triggered by the *E. coli* LPS (Fig. 7*E*). In the presence of galectin-3, higher-order caspase-4 oligomeric complexes were observed. Coimmunoblotting with anti–galectin-3 antibodies revealed the presence of galectin-3–containing oligomeric complexes (Fig. 7*E*). Caspase-4 monomers and dimers were not detected, which may be due to size exclusion in that recombinant proteins with an N-terminal glutathione S-transferase (GST) tag exhibit a lower propensity to assemble into smaller size complexes (37). By native polyacrylamide gel electrophoresis (PAGE), galectin-3 was noted to significantly amplify the formation of caspase-4 oligomeric complexes in the presence of LPSs. High-molecular-weight molecules containing galectin-3 of similar size to oligomeric caspase-4 were also detected (Fig. 7*F*).

The formation of caspase-4 oligomeric complexes results in its activation (7) as in the case of caspase-11. We thus examined whether enhanced caspase-4 oligomerization by galectin-3 through LPS binding heightens caspase-4 enzymatic activity. Galectin-3 enhanced LPS-induced caspase-4 enzymatic activity in a carbohydrate-dependent manner (Fig. 7*G*). Moreover, galectin-3C was not able to increase LPS-induced caspase-4 enzymatic activity, suggesting that the N-terminal region of galectin-3 is critical for this promoting effect (Fig. 7*H*).

Discussion

Our findings from experiments using cell-based and cell-free systems indicate that galectin-3 is a host resistance factor in the noncanonical inflammasome pathway. It amplifies intracellular LPS-induced inflammatory caspase-4/11 assembly and activation resulting in more severe pyroptosis. The N-terminal region and carboxyl-terminal CRD of galectin-3 are both essential to this function. Moreover, galectin-3 functions in this regard in a carbohydrate-dependent manner, indicating that its recognition of LPS glycans is critical (Fig. 8). Furthermore, the formation of galectin-3–LPS–caspase-11 ternary complexes is independent of host *N*-glycans. Therefore, by intracellularly recognizing LPS glycan moieties, galectin-3 acts as a critical regulator of cytosolic LPS-mediated caspase-4/11 activation and pyroptosis.

Although many extracellular bacteria, such as *E. coli*, *Haemophilus influenzae*, and *Vibrio cholerae*, rarely gain access to the cytosol (45), bacterial OMVs can enter cells and release LPSs intracellularly, which is followed by acute inflammatory responses that lead to sepsis (9). Moreover, intracellular bacteria, including

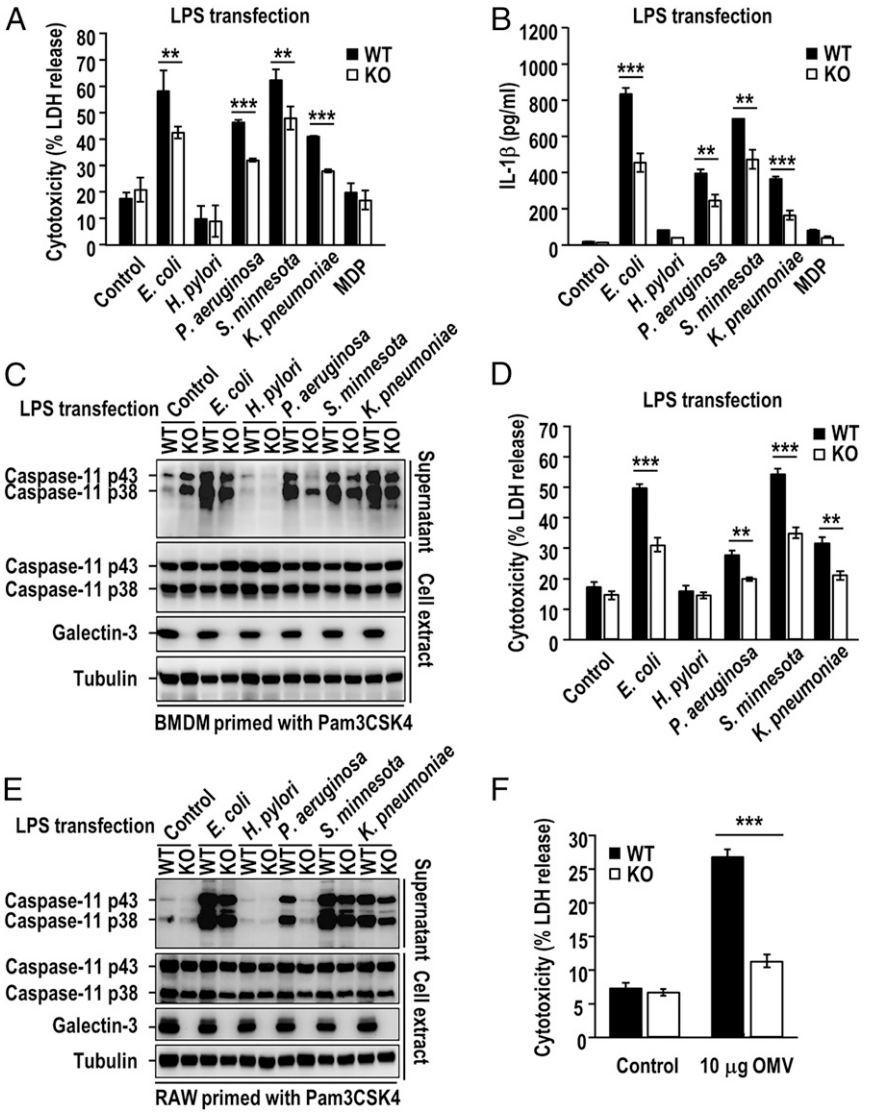

Fig. 4. Galectin-3 promotes intracellular LPS-induced noncanonical inflammasome activation in macrophages. (*A*) LDH release and (*B*) IL-1β secretion were measured 16 h after transfection of Pam3CSK4-primed WT and galectin-3 KO BMDMs with the indicated LPSs or MDP. (*C*) Pam3CSK4-primed WT and galectin-3 KO BMDMs were transfected with the indicated LPSs for 16 h. Supernatants and cell lysates were collected and immunoblotted to detect caspase-11 (38 and 43 kDa), galectin-3, and tubulin levels. (*D*) LDH release was measured 16 h after transfection of Pam3CSK4-primed WT and galectin-3 KO RAW 264.7 cells with the indicated LPSs. (*E*) Supernatants and cell lysates were collected from primed WT and galectin-3 KO RAW 264.7 cells 16 h after LPS transfection, analyzed by immunoblotting with the indicated antibodies. (*F*) LDH release was measured 24 h after treatment of RAW 264.7 cells with bacterial OMVs. Error bars indicate the mean ± SD. Data are representative of three independent experiments. Statistical analysis was performed using unpaired Student's *t* tests; $^{**}P < 0.01$, $^{***}P < 0.001$.

Salmonella typhimurium and *Legionella pneumophila*, are able to survive intracellularly within pathogen-containing vacuoles (46, 47), but the integrity of these vacuoles is lost in the presence of bacterial secretion systems, thereby releasing bacterial virulence components, such as LPSs, into the cytosol (26, 48). The presence of intracellular LPSs is known to activate autonomous cell immunity, thus contributing to host protection against pathogens.

Glycan modification found in eukaryotes is long believed to modulate crucial host cellular processes. Our previous work used LM to dissect the mechanism of host autophagic machinery and suggests that the recruitment of galectin-3 to LM-containing vacuoles was significantly decreased in *Mgat1* KO RAW 264.7 cells compared to WT cells and that lack of galectin-3 results in enhanced autophagic activation (49). Similarly, in *Mgat1* KO HeLa cells treated with L-leucyl-L-leucine methyl ester, the recruitment of galectin-3 to damaged lysosomes was impaired (50). Although these data indicate that host *N*-glycans are critical for autophagic regulation, few studies have examined the role of glycans on the surface of intracellular pathogenic bacteria in the cellular response.

In fact, highly antigenic bacterial surface polysaccharides exhibit considerable intraspecies variability and have a significant impact on immune recognition (51, 52). Here, we report that galectin-3 recognizes the "nonself" glycan structures on LPSs and decodes their glycan information into cellular function through inflammatory caspases. Extracellular galectin-3 was previously reported to regulate LPS-mediated inflammation in macrophages (36). However, this was shown with galectin-3 exogenously added to cells cultured in the presence of LPSs. Also, we have reported that endogenous galectin-3 positively regulates the phagocytic capacity of macrophages (53) and inflammasome activation by forming complexes with NLRP3 (54) and negatively regulates T cell activation (55), but these were not glycan dependent.

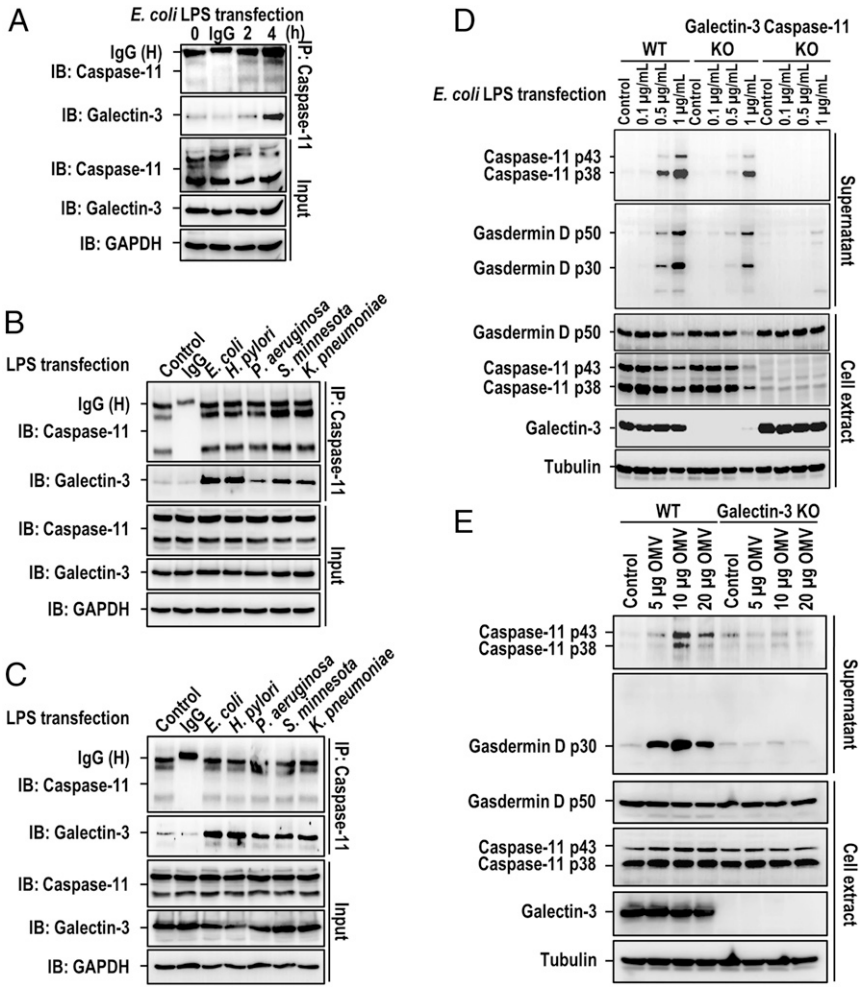

Fig. 5. Galectin-3 forms complexes with caspase-11 and increases cleavage of gasdermin D in macrophages after LPS intracellular delivery. (*A*) Pam3CSK4-primed BMDMs were transfected with the *E. coli* LPS followed by treatment with a cell-permeable cleavable chemical cross-linker. The cell lysates were immunoprecipitated with anti–caspase-11 antibodies, and galectin-3 and caspase-11 in the immunoprecipitates were assessed by immunoblotting. (*B* and *C*) As described in *A*, but Pam3CSK4-primed RAW 264.7 cells (*B*) and J774 cells (*C*) were used and transfected with LPSs from different bacterial strains. In *A* through *C*, immunoblots of the total input proteins are shown in the lower sections. (*D*) Pam3CSK4-primed WT, galectin-3 KO, and caspase-11 KO RAW 264.7 cells were transfected with LPSs. Supernatants and cell lysates were collected and analyzed by immunoblotting with the indicated antibodies. (*E*) The whole-cell lysates and culture supernatants were collected from primed WT and galectin-3 KO RAW 264.7 cells after *E. coli* OMV treatment and analyzed by immunoblotting with the indicated antibodies. Data are representative of three independent experiments.

It is to be noted that galectin-3 deletion in cells did not result in a complete absence of LPS-induced responses. This is because LPSs alone are able to induce caspase-4/5/11 activation (7). Thus, galectin-3 serves as a modulator that cross-links LPSs, leading to a higher-order array of LPS aggregates to amplify caspase activation. Whether other galectins could participate in the noncanonical inflammasome pathway remains to be addressed. However, analysis of RNA expression from RNA-sequencing data (PRJNA547621 and PRJNA594638) revealed that in both BMDMs and RAW 264.7 cells, galectin-3 is the most abundantly expressed galectin, while other galectins are either expressed at very low levels (galectin-1, galectin-8, and galectin-9) or are undetectable. Moreover, our preliminary results from the enzymatic assay indicate that galectin-7 does not have such activity.

We propose that the ability of galectin-3 to form oligomers upon binding to LPSs is highly relevant to its enhancement of LPS-induced caspase oligomerization and activation, which is related to the well-established ability of galectin-3 to form oligomers upon binding to glycoconjugates. We envision that the type-N model (31, 32) is operative here, in which galectin-3 molecules self-oligomerize via their N-terminal region upon binding to LPS glycan epitopes through their carboxyl-terminal CRD. Recently, a type-C self-association model was proposed, in which galectin-3 self-oligomerizes when the carbohydrate-recognition site of one galectin-3 molecule binds to another, with the N-terminal domain remaining unoccupied (56). Additional investigation is required to elucidate how galectin-3 forms oligomers upon binding to LPS–caspase. It is to be noted that galectin-3 can bind to the lipid A moiety of LPSs through its N-terminal domain, which is distinct from the recognition of LPS β-galactoside via its CRD (35). How this interaction affects the formation of galectin-3–LPS–caspase ternary complexes also remains to be determined.

Our findings that the carboxyl-terminal carbohydrate-binding domain fragment (galectin-3C) inhibited LPS-induced caspase-11 enzymatic activity is worth commenting on (Fig. 2*D*). While galectin-3C retains the ability to bind to glycoconjugates, it is unable to mediate their oligomerization, unlike full-length galectin-3. In some biological systems, galectin-3C was reported to act as a negative regulator of responses mediated by endogenous galectin-3 (57–59); thus, its inability to enhance LPS-mediated caspase-11 activation was predictable. However, its ability to inhibit this process is surprising and intriguing (Fig. 2*D*). LPS-sensing molecules

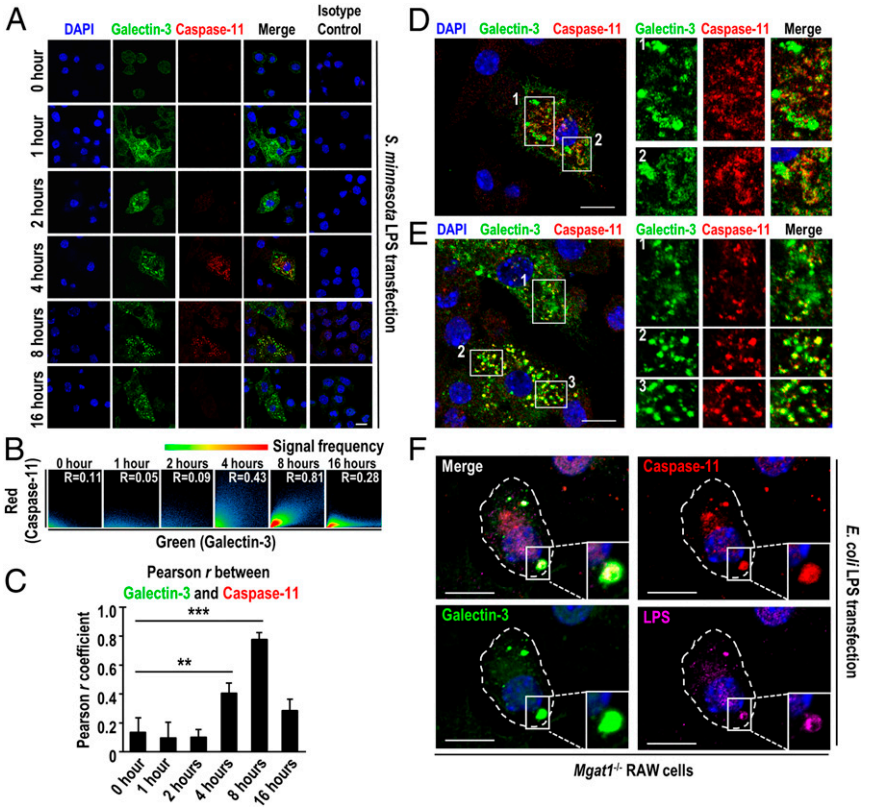

Fig. 6. Galectin-3 colocalizes with caspase-11 in macrophages after LPS intracellular delivery. (*A*) Pam3CSK4-primed J774 cells were transfected with LPSs from *S. minnesota* and immunostained to detect galectin-3 (green) and caspase-11 (red) using corresponding antibodies at the indicated time points by confocal microscopy. (*B* and *C*) Images from *A* were analyzed for colocalization of galectin-3 (green) with caspase-11 (red) at the indicated time points by calculating Pearson's correlation coefficient using ZEN software. Green color intensity was plotted against red color intensity for each pixel. Correlation coefficient values >0.6 indicate colocalization. (*D* and *E*) High-magnification confocal images of primed J774 cells from *A* show merged signals (yellow) of stained caspase-11 (red) with galectin-3 (green) at 4 and 8 h after transfection, respectively. (*F*) Primed *Mgat1* KO RAW 264.7 cells transfected with the *E. coli* LPS were immunostained to detect LPSs (purple), galectin-3 (green), caspase-11 (red), and nuclei (blue). Merged signals of LPSs, galectin-3, and caspase-11 are shown as white. (Scale bars, 10 μm.) Error bars indicate the mean ± SD of 10 to 15 cells per experiment. Data are representative of three independent experiments. Statistical analysis was performed using unpaired Student's *t* tests; $^{**}P < 0.01$, $^{***}P < 0.001$.

are known to either enhance or neutralize LPS-mediated inflammatory responses (60). A previous study demonstrated that two to five molar equivalents of LPSs are required for the formation of complexes with inflammatory caspase, while the molar ratio of 1:1 is not sufficient for this process (61). It has been shown that LPS aggregates were more potent in inducing cytokine production from mononuclear cells, whereas at the same concentration, LPS monomers were ineffective (62). Therefore, LPS self-association may be critical for its mediation of caspase oligomerization and activation, and galectin-3C, through binding to LPS glycans, may inhibit this process, possibly due to steric hindrance. Full-length galectin-3, on the other hand, is able to self-associate, thus mediating the formation of caspase-bound LPS clusters and triggering caspase oligomerization. Regardless of the mechanism by which galectin-3C inhibits LPS-induced caspase oligomerization and activation, this molecular entity has the potential to be developed into a therapeutic agent to suppress cytosolic LPS-induced pyroptosis.

Our study used LPSs from different bacterial strains that display glycans containing β-galactoside in the LPS O-antigen (*E. coli* and *H. pylori*), outer core (*K. pneumoniae* and *P. aeruginosa*), or inner core (*S. minnesota*) for galectin-3 binding. Through binding to these LPSs, galectin-3 enhances pyroptotic death in a caspase-dependent manner. Further research is required to determine whether LPSs presenting β-galactoside from other bacterial species function similarly for noncanonical inflammasome activation.

In addition to LPS glycan diversity, lipid A of LPSs can vary in number and length, resulting in various degrees of pyroptotic responses. Caspase-4/5/11 has been shown to respond to penta- and hexa-acylated lipid A (*E. coli* and *P. aeruginosa*) and mediate cell pyroptosis. Although hepta-acylated lipid A may not be recognized by caspase-11 as efficiently as canonical LPSs (63), *S. minnesota* (hepta-acylated lipid A) used in our study still showed strong immunostimulation. By contrast, tetra-acylated lipid A species (e.g., from *Francisella novicida*) recently have been shown to escape caspase-11 recognition in mice but drive caspase-4 activation in human macrophages, possibly due to the ability of caspase-4 to recognize a wider variety of lipid A variants (64). In addition to having a reduced number of acyl chains, *H. pylori*, which induces a low pyroptotic response, even displays longer acyl chains, C16 to C18, in contrast to the C12 to C14 long fatty acids found in *E. coli* (65). Oxidized phospholipids (oxPAPC), an LPS mimic, from dying cells retain the ability to bind to the caspase-11 catalytic domain (but not the CARD) (66). Liquid chromatography tandem mass spectrometry analysis showed that the lipid peroxidation products of oxPAPC can be C16 or C20 long fatty acids (67). Thus, the *H. pylori* LPS, with a longer acyl chain (C16 or C18), might have diminished endotoxic properties by mimicking host components like oxPAPC and bind to the caspase-11 catalytic domain. Indeed, in a cell-free system, we found that the *H. pylori* LPS binds to the caspase-11 catalytic domain instead of CARD and forms ternary complexes with galectin-3 (Fig. 1*D*). Although

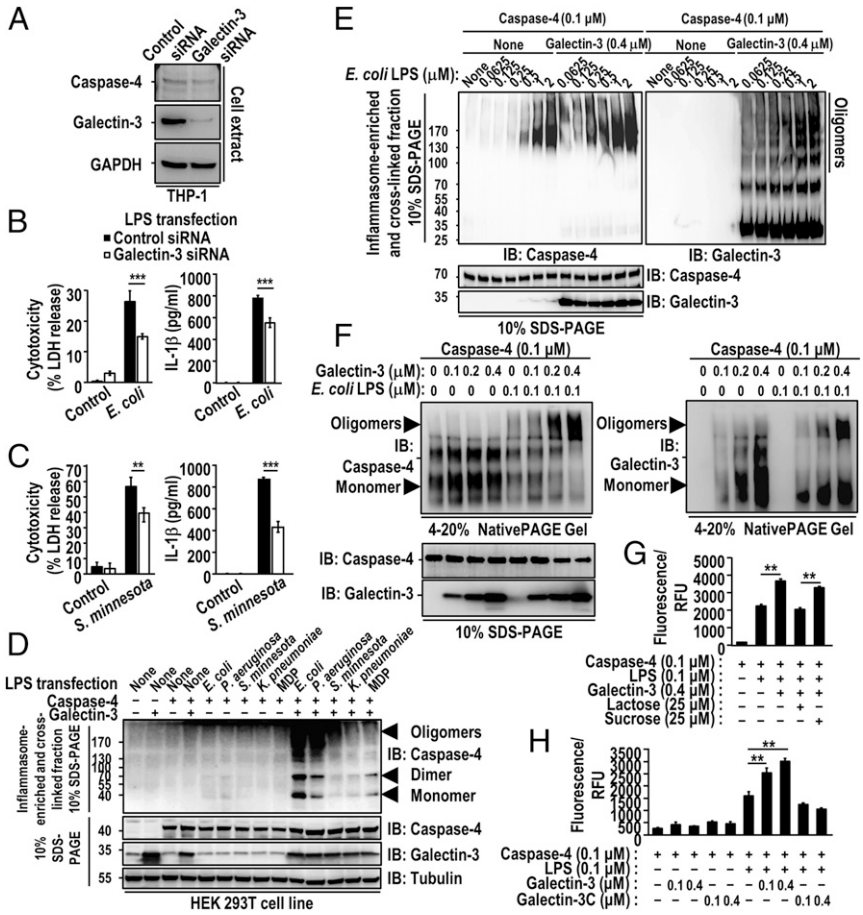

Fig. 7. Galectin-3 promotes LPS-induced caspase-4 oligomerization and activation, leading to more severe pyroptosis in macrophages. (*A*) THP-1 cells were transfected with siRNAs targeting galectin-3, and the levels of galectin-3 and caspase-4 were measured by immunoblotting. (*B* and *C*) LDH release and IL-1β secretion were measured 16 h after transfection of WT and galectin-3 knockdown THP-1 cells with LPSs from *E. coli* (*B*) or *S. minnesota* (*C*). (*D*) HEK 293T cells were transfected with a combination of expression vectors containing galectin-3 and caspase-4, respectively, followed by transfection with LPSs from various bacterial strains, as indicated, or MDP. After cell lysis, inflammasome protein complexes were enriched by serial centrifugation and treated with a non-cleavable cross-linker. The cross-linking multiprotein inflammasome complexes were analyzed by SDS-PAGE followed by immunoblotting. The immunoblots of the cell lysates not treated with the cross-linker are shown below. The black arrowheads point to the bands corresponding to monomers, dimers, and oligomers, respectively, as indicated. (*E*) Human caspase-4 was incubated with increasing concentrations of the *E. coli* LPS and galectin-3. The enriched inflammasome protein complexes were collected and processed as described in *D* and then analyzed by SDS-PAGE followed by immunoblotting. The immunoblots of the total input proteins are shown (*Bottom*). (*F*) Human caspase-4 was incubated with various amounts of galectin-3 and *E. coli* LPSs, and the mixtures were assessed by 4 to 20% native PAGE or 10% SDS-PAGE followed by immunoblotting with anti–caspase-4 and anti–galectin-3 antibodies, respectively. (*G*) Galectin-3 was treated with 25 mM lactose or sucrose for 15 min before being incubated with LPSs and caspase-4. The level of caspase enzymatic activity was measured using the fluorogenic substrate LEVD-AFC. (*H*) Full-length galectin-3 or galectin-3C was added to LPSs and caspase-4, and caspase-4 enzymatic activity was measured using the fluorogenic substrate LEVD-AFC. Error bars indicate the mean ± SD. Data are representative of three independent experiments. Statistical analysis was performed using unpaired Student's *t* tests; $^{**}P < 0.01$, $^{***}P < 0.001$.

galectin-3 could still cross-link the *H. pylori* LPS, why this does not promote caspase-11 oligomerization and activation is unclear. The *H. pylori* LPS binding to the catalytic domain may still trigger the assembly of caspase-11 but possibly in an unfitting orientation to induce its activation. Alternatively, the *H. pylori* LPS may block the caspase-11 active site in the catalytic domain from being accessible to gasdermin D.

The galectin-3 level in HEK 293T cells is similar to that in BMDMs but much higher than macrophage cell lines (*SI Appendix*, Fig. S12). Intriguingly, unlike macrophages, in HEK 293T cells, LPSs were insufficient to induce the formation of caspase-4/11 complexes in the absence of galectin-3, as complexes were detected only in cells cotransfected with galectin-3 and caspase-4/11 combined with intracellular delivery of LPSs (Figs. 3 *A* and *B* and 7*D*). Differential regulation of noncanonical inflammasome activation by galectin-3 may exist between macrophages and HEK 293T cells, and this also implies that galectin-3 may be indispensable for noncanonical inflammasome activation in nonimmune cells. Another point to discuss is that galectin-3 amplified the formation of caspase-11 oligomeric complexes at lower but not higher LPS concentrations (Fig. 2*B*). We believe this is because at high concentrations of LPSs, amphipathic LPSs may self-aggregate into supramolecular micellar structures, resulting in its glycans being inaccessible to galectin-3 (68).

In conclusion, we describe a mechanism by which galectin-3 senses exogenous danger signals, LPSs, intracellularly. Galectin-3 self-association is involved specifically in LPS-mediated caspase-4/11 oligomerization and activation, leading to macrophage cytokine release and pyroptosis. Our findings highlight how cytosolic galectin-3 triggers host innate responses through recognition of LPS glycans. Further elucidation of the role of galectin-3 in the noncanonical inflammasome signaling pathway may provide opportunities for new therapeutic interventions for bacterial infections.

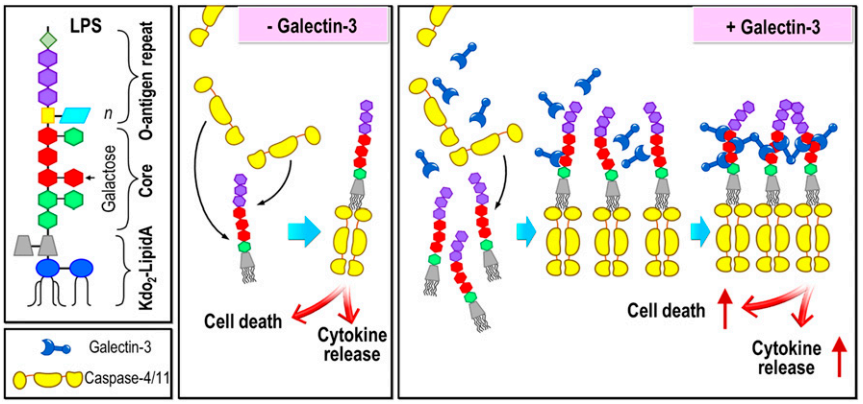

Fig. 8. A model for regulation of the noncanonical inflammasome pathway by galectin-3. Intracellularly delivered lipopolysaccharides (LPSs) are known to bind to caspase-4/11 and induce oligomerization and activation of the latter, resulting in pyroptosis (cell death and IL-1β production). When LPSs are delivered into cells that express galectin-3, cytosolic galectin-3 binds to LPS glycans and enhances LPS-mediated caspase-4/11 oligomerization and activation, resulting in increased pyroptosis—a critical mechanism against bacterial infections.

Materials and Methods

Pull-Down Assay. Recombinant galectin-3 was purified as previously described (31), and endotoxins were removed by phase separation using detergent to avoid endotoxin contamination (*SI Appendix*, Fig. S4) (69). The reaction mixtures containing 1.2 μM recombinant galectin-3, 0.4 μM recombinant caspase-$11^{1\text{-}80}$-GST (Abnova), and/or 0.1 μM biotinylated LPS (O111:B4) (InvivoGen) were incubated for 4 h at 4 °C. Pull-down of LPSs was carried out by treating the mixtures with streptavidin beads (GE Healthcare Life Sciences). The biotinylated LPS-containing protein complexes were analyzed by immunoblotting. For coimmunoprecipitation, the reaction mixtures containing 0.1 μM *E. coli* LPS (O111:B4) (Sigma-Aldrich), *H. pylori* LPS (FUJIFILM Wako Chemicals), or MDP (InvivoGen) were incubated with 1.2 μM recombinant galectin-3 and 0.4 μM recombinant caspase-$11^{1\text{-}80}$-GST (Abnova) or 0.4 μM caspase-$11^{81\text{-}266}$-GST (Abnova) for 4 h at 4 °C. The reaction mixtures were further incubated with anti–galectin-3 antibodies (GeneTex, Inc.) overnight at 4 °C and then mixed with Protein A/G Mix Magnetic Beads (GE Healthcare Life Sciences). The beads containing proteins were washed with lysis buffer (Bio-Rad Laboratories) and then treated with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer at 100 °C. The eluted proteins were analyzed by immunoblotting for GST (Bethyl Laboratories), the heavy chain of immunoglobulin G (IgG) (Bethyl Laboratories), and galectin-3. To analyze the effect of saccharide competition, recombinant galectin-3 was mixed with 25 mM competing lactose (Sigma-Aldrich) or noncompeting sucrose (Sigma-Aldrich) for 15 min at room temperature before being incubated with LPSs and caspase-11 for 4 h at 4 °C.

Native PAGE. This was accomplished by using a commercially available native PAGE gel (Bio-Rad Laboratories) to analyze multiprotein complex assembly, as previously reported (70). Briefly, 100 μL 1× reaction buffer (BioVision Inc.) containing different concentrations of recombinant human galectin-3 [purified as previously described (31)] was incubated with LPSs from *E. coli* (O111:B4) (Sigma-Aldrich) overnight at 4 °C. Caspase-$11^{1\text{-}80}$-GST (Abnova) or human caspase-4-GST (Abnova) was then added, and the mixtures were further incubated for 30 min at 37 °C. Afterward, Native Sample Buffer (Bio-Rad Laboratories) was added, and the reaction mixtures were then separated by 4 to 20% blue native polyacrylamide gel electrophoresis (BN-PAGE) in Tris-Glycine native running buffer followed by immunoblotting using antibodies against galectin-3, GST, or caspase-4 (Cell Signaling Technology).

Caspase Activity. In the in vitro studies, recombinant galectin-3 or galectin-3C purified as described previously (31) was incubated with caspase reaction buffer (BioVision Inc.) containing *E. coli* LPSs overnight at 4 °C followed by incubation with full-length caspase-11-GST (Abnova) or caspase-4-GST (Abnova) and 75 μM fluorogenic substrates Z-VAD-AMC (Cayman Chemical) or LEVD-AFC (BioVision Inc.) for caspase-11 or caspase-4, respectively. In the saccharide competition experiments, competing lactose or noncompeting sucrose was preincubated with galectin-3 for 15 min at room temperature. The reaction mixtures were incubated for 30 min at 37 °C in a 96-well plate, and free AMC or AFC released by hydrolysis was monitored using a fluorescence microplate reader (Tecan Group Ltd.) at 450-nm emission and 365-nm excitation wavelengths for caspase-11 and 505-nm emission and 400-nm excitation wavelengths for caspase-4.

Caspase Inflammasome Complex Cross-Linking Assay and Gasdermin D Cleavage. The caspase-4/11 oligomerization assay was conducted as previously described, with minor modifications (39). Briefly, HEK 293T cells were transfected with the following vectors alone or in combination for 24 to 48 h to establish a cell-reconstitution system: pBK-CMV carrying WT galectin-3 [as previously described (71)], a pEGFP-N1 carrying WT galectin-3 or galectin-3 CRD mutant (H158A/N160A/R162A, which was developed using the QuikChange Site-Directed Mutagenesis Kit [Agilent Technologies]), PCMV-flag-Caspase11 (Addgene plasmid No. 21145; http://n2t.net/addgene:21145; RRID: Addgene_21145) (72), and caspase-4 (Origene). Cells were then transfected using FuGENE HD (Promega) with 1 μg/mL LPS from *E. coli* (Sigma-Aldrich), *P. aeruginosa* (Sigma-Aldrich), *S. minnesota* (Sigma-Aldrich), or *K. pneumoniae* (Sigma-Aldrich) or with MDP for 4 h. Cell lysates were obtained, and inflammasome fractions were enriched using serial centrifugation (39, 73). In a cell-free system, reaction mixtures containing LPSs, caspase-4, and galectin-3 were incubated for 30 min at 37 °C and the complexes were also enriched using serial centrifugation. Enriched protein complexes from both the cell-free and cell-based systems were treated with a noncleavable cross-linker, 2 mM disuccinimidyl suberate (Thermo Fisher Scientific), for 30 min at room temperature. The cross-linked samples were solubilized in a sample buffer (62.5 mM Tris base, 10% glycerol, 2% SDS, and 20 mM 2-mercaptoethanol) and analyzed by immunoblotting for galectin-3, caspase-4/11 (Cell Signaling Technology), β-actin (Abnova), and α-tubulin (BD Biosciences). For gasdermin D cleavage, HEK 293T cells were cotransfected with caspase-11, galectin-3, and flag–gasdermin D (Addgene plasmid No. 80950; http://n2t.net/addgene:80950; RRID: Addgene_80950) (14) followed by transfection with *E. coli* LPSs. A total of 8 h afterward, the total cell lysates were collected and subjected to immunoblotting analysis for gasdermin D (Abcam).

Mice. Experiments were performed using WT and galectin-3 KO littermates with a C57BL/6J background obtained from galectin-3 heterozygous breeders as previously described (74). Mouse strains were maintained under specific pathogen-free conditions at Academia Sinica. The animal protocol was approved by the Academia Sinica Institutional Animal Care and Utilization Committee in accordance with established guidelines.

Cell Cultures and Treatments. Mouse RAW 264.7 cells and J774 cells were cultured in Dulbecco's Modified Eagle Medium containing 2 mM glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin, and 10% fetal bovine serum (FBS). To obtain BMDMs, femur and tibia cells were harvested from WT and galectin-3 KO littermates and then cultured in RPMI containing 10% FBS supplemented with 10 ng/mL granulocyte-macrophage colony-stimulating factor, 20 mmol/L Hepes, and 1% nonessential amino acids. Cells were cultured for 7 to 11 d at 37 °C in a 5% CO_2 humidified incubator and then primed with 200 ng/mL Pam3CSK4 (InvivoGen) for 4 h. Human monocytic THP-1 cells were treated with 1 μM Accell SMARTpool small interfering RNA (siRNA)-targeting galectin-3 or Accell nontargeting control siRNA in Accell delivery media (Thermo Fisher Scientific) for 96 h. Then, cells were differentiated by phorbol

12-myristate 13-acetate (20 ng/mL, Sigma-Aldrich) for 48 h. Cells were transfected with 1 μg/mL LPS from either *E. coli* (O111:B4), *P. aeruginosa*, *S. minnesota*, *K. pneumoniae*, *H. pylori*, or MDP using FuGENE HD (Promega) for different time periods depending on the purpose of the experiment. Electroporation was performed using the ECM 830 Square Wave Electroporation System (BTX Harvard Apparatus). Cells (2×10^6 cells/mL) were harvested, washed, and resuspended in the BTXpress electroporation buffer containing 1 μg/mL LPS from either *E. coli* or *S. minnesota* and then added to an electroporation 4-mm gap cuvette (BTX Harvard Apparatus). The cell suspensions were subjected to electroporation at room temperature with the following parameters: voltage: 260 V; pulse duration: 15 ms; number of pulses: 1. Following electroporation, the cell suspensions were subjected to centrifugation at $300 \times g$ for 5 min at room temperature, and the cell pellets were resuspended in culture medium for further analysis.

Purification of Bacterial OMVs. OMVs were purified from the *E. coli* O86 bacterial strain as previously described (9). Briefly, the *E. coli* O86 bacterial strain was grown in 200 mL Lysogeny Broth until the optical density at 600 nm reached 0.5 to 1.5. The bacteria-free supernatant was obtained by centrifugation at $10{,}000 \times g$ for 10 min at 4 °C. This supernatant was filtered through 0.45-μm filters and then through 0.22-μm filters to remove any bacteria. OMVs were pelleted by ultracentrifugation at ~$400{,}000 \times g$ for 4 h at 4 °C in a Beckman NVT 65 rotor. The purified OMVs were resuspended in 500 μL sterile phosphate buffered saline (PBS), and LPS levels were determined by the limulus amebocyte lysate assay (Thermo Fisher Scientific). The protein content of OMVs was quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Cells were treated with OMV for 24 h in 6-well plates.

Immunoprecipitation. Cells transfected with LPSs for 6 h were washed with PBS and pretreated with 0.4 μg/mL cleavable cross-linker DSP (Sigma-Aldrich) for 10 min at room temperature. To terminate the cross-linking process, cells were incubated with 0.1 M Tris HCl (pH 7.5) for 15 min. Next, cells were washed and lysed in 200 μL Pierce IP Lysis Buffer (Thermo Fisher Scientific) containing protease inhibitor mixture and then incubated on ice for 30 min with consecutive pipetting cycles every 10 min. The lysates were centrifuged at $15{,}000 \times g$ for 10 min, and the supernatants were collected. The collected supernatants were incubated with anti–caspase-11 antibodies (Cell Signaling Technology), anti-lipid A antibodies (Abcam), or IgG control antibodies (Bethyl Labs) overnight at 4 °C and then mixed with Protein A/G Mix Magnetic Beads (GE Healthcare Life Sciences). The beads were washed with lysis buffer containing 25 mM lactose and then treated with sample buffer at 95 °C for 10 min. The eluted proteins were analyzed by immunoblotting with anti–galectin-3 and anti–caspase-11 antibodies. The LPS Re (*E. coli* and *S. minnesota*) was purchased from Enzo Life Sciences. To optimize Western blotting detection of primary antibodies, Clean-Blot IP Reagent (Thermo Fisher Scientific) was used.

Immunofluorescence Assays. Mouse J774 and RAW 264.7 cells were grown on 12-mm glass culture slides overnight. Cells were washed twice with PBS and fixed with 4% paraformaldehyde for 10 min. Cells were then permeabilized using 0.5% Triton X-100 in PBS for 10 min and blocked with 3% bovine serum albumin in PBS for 30 min to prevent nonspecific binding of antibodies. Cells immobilized on glass slides were incubated with primary antibodies against galectin-3, caspase-11 (Cell Signaling Technology), and LPSs (Abcam), respectively, overnight at 4 °C. Cells were washed three times with PBS and stained with the corresponding fluorescent-labeled secondary antibody for 1 h at room temperature. Corresponding isotype control antibodies were purchased from Bethyl Labs. Slides were mounted for fluorescent nuclear staining using water-soluble DAPI Fluoromount-G (SouthernBiotech). The stained slides were analyzed using an LSM 780 Laser Scanning Confocal Microscope (ZEISS), and image processing was performed using the ZEN Image Analysis module. Colocalization was assessed by calculating Pearson's correlation coefficient using ZEN software (ZEISS).

Statistical Analysis. Results are presented as the mean ± SD. The significance of the differences between means was assessed using two-tailed Student's *t* tests. *P* values <0.05 were considered statistically significant.

Data Availability. All study data are included in the article and/or *SI Appendix*.

ACKNOWLEDGMENTS. This work was funded by the Ministry of Science Technology (MOST 104-0210-01-09-02, MOST 105-0210-01-13-01, and MOST 106-0210-01-15-02) and Academia Sinica Thematic Project (AS-105-TP-B08). The pAll-Cas9.pPuro, an all-in-one CRISPR/Cas9 expressing vector, was designed by the National RNAi Core Facility at Academia Sinica, Taiwan. The National RNAi Core Facility is supported by the National Core Facility Program for Biotechnology Grants from the Taiwan National Science Council (NSC 100-2319-B-001-002). We thank the staff of the Common Equipment and Technical Core, Institute of Biomedical Sciences, Academia Sinica, Taiwan, for their technical support and the staff in the Biophysics Core Facility, funded by the Academia Sinica Core Facility and Innovative Instrument Project (AS-CFII108-111), for support with data acquisition and analysis. We also thank Ting-Jui Tu for kindly providing some of the reagents used in the study and Dr. Ming-Zong Lai, Dr. Jong-Young Yen, and Dr. Li-Chung Hsu for valuable discussion.

1. D. C. Angus, T. van der Poll, Severe sepsis and septic shock. *N. Engl. J. Med.* **369**, 2063 (2013).
2. S. Akira, K. Takeda, Toll-like receptor signalling. *Nat. Rev. Immunol.* **4**, 499–511 (2004).
3. V. A. Rathinam, S. K. Vanaja, K. A. Fitzgerald, Regulation of inflammasome signaling. *Nat. Immunol.* **13**, 333–342 (2012).
4. B. K. Davis, H. Wen, J. P. Ting, The inflammasome NLRs in immunity, inflammation, and associated diseases. *Annu. Rev. Immunol.* **29**, 707–735 (2011).
5. N. Kayagaki *et al.*, Noncanonical inflammasome activation by intracellular LPS independent of TLR4. *Science* **341**, 1246–1249 (2013).
6. N. Kayagaki *et al.*, Non-canonical inflammasome activation targets caspase-11. *Nature* **479**, 117–121 (2011).
7. J. Shi *et al.*, Inflammatory caspases are innate immune receptors for intracellular LPS. *Nature* **514**, 187–192 (2014).
8. J. A. Hagar, D. A. Powell, Y. Aachoui, R. K. Ernst, E. A. Miao, Cytoplasmic LPS activates caspase-11: Implications in TLR4-independent endotoxic shock. *Science* **341**, 1250–1253 (2013).
9. S. K. Vanaja *et al.*, Bacterial outer membrane vesicles mediate cytosolic localization of LPS and caspase-11 activation. *Cell* **165**, 1106–1119 (2016).
10. M. Deng *et al.*, The endotoxin delivery protein HMGB1 mediates caspase-11-dependent lethality in sepsis. *Immunity* **49**, 740–753.e7 (2018).
11. S. Chen *et al.*, Dysregulated hemolysin liberates bacterial outer membrane vesicles for cytosolic lipopolysaccharide sensing. *PLoS Pathog.* **14**, e1007240 (2018).
12. E. Meunier *et al.*, Caspase-11 activation requires lysis of pathogen-containing vacuoles by IFN-induced GTPases. *Nature* **509**, 366–370 (2014).
13. Y. Shi, Caspase activation: Revisiting the induced proximity model. *Cell* **117**, 855–858 (2004).
14. X. Liu *et al.*, Inflammasome-activated gasdermin D causes pyroptosis by forming membrane pores. *Nature* **535**, 153–158 (2016).
15. J. Shi *et al.*, Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. *Nature* **526**, 660–665 (2015).
16. N. Kayagaki *et al.*, Caspase-11 cleaves gasdermin D for non-canonical inflammasome signalling. *Nature* **526**, 666–671 (2015).
17. J. Ding *et al.*, Erratum: Pore-forming activity and structural autoinhibition of the gasdermin family. *Nature* **540**, 150 (2016).
18. R. D. Cummings, F. T. Liu, G. R. Vasta, "Chapter 36 Galectins" in *Essentials of Glycobiology*, A. Varki, R. D. Cummings, J. D. Esko, Eds. *et al.* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2017).
19. L. Johannes, R. Jacob, H. Leffler, Galectins at a glance. *J. Cell Sci.* **131**, jcs208884 (2018).
20. F. T. Liu, G. A. Rabinovich, Galectins as modulators of tumour progression. *Nat. Rev. Cancer* **5**, 29–41 (2005).
21. F. T. Liu, R. Y. Yang, D. K. Hsu, Galectins in acute and chronic inflammation. *Ann. N. Y. Acad. Sci.* **1253**, 80–91 (2012).
22. G. A. Rabinovich, M. A. Toscano, Turning 'sweet' on immunity: Galectin-glycan interactions in immune tolerance and inflammation. *Nat. Rev. Immunol.* **9**, 338–352 (2009).
23. S. Thiemann, L. G. Baum, Galectins and immune responses-just how do they do those things they do? *Annu. Rev. Immunol.* **34**, 243–264 (2016).
24. S. Bänfer *et al.*, Molecular mechanism to recruit galectin-3 into multivesicular bodies for polarized exosomal secretion. *Proc. Natl. Acad. Sci. U.S.A.* **115**, E4396–E4405 (2018).
25. T. L. Thurston, M. P. Wandel, N. von Muhlinen, A. Foeglein, F. Randow, Galectin 8 targets damaged vesicles for autophagy to defend cells against bacterial invasion. *Nature* **482**, 414–418 (2012).
26. E. M. Feeley *et al.*, Galectin-3 directs antimicrobial guanylate binding proteins to vacuoles furnished with bacterial secretion systems. *Proc. Natl. Acad. Sci. U.S.A.* **114**, E1698–E1706 (2017).
27. R. E. Mandrell, M. A. Apicella, R. Lindstedt, H. Leffler, Possible interaction between animal lectins and bacterial carbohydrates. *Methods Enzymol.* **236**, 231–254 (1994).
28. G. R. Vasta, Roles of galectins in infection. *Nat. Rev. Microbiol.* **7**, 424–438 (2009).
29. S. R. Stowell *et al.*, Microbial glycan microarrays define key features of host-microbial interactions. *Nat. Chem. Biol.* **10**, 470–476 (2014).
30. S. R. Stowell *et al.*, Innate immune lectins kill bacteria expressing blood group antigen. *Nat. Med.* **16**, 295–301 (2010).
31. D. K. Hsu, R. I. Zuberi, F. T. Liu, Biochemical and biophysical characterization of human recombinant IgE-binding protein, an S-type animal lectin. *J. Biol. Chem.* **267**, 14167–14174 (1992).
32. S. M. Massa, D. N. Cooper, H. Leffler, S. H. Barondes, L-29, an endogenous lectin, binds to glycoconjugate ligands with positive cooperativity. *Biochemistry* **32**, 260–267 (1993).

33. M. Fowler, R. J. Thomas, J. Atherton, I. S. Roberts, N. J. High, Galectin-3 binds to *Helicobacter pylori* O-antigen: It is upregulated and rapidly secreted by gastric epithelial cells in response to *H. pylori* adhesion. *Cell. Microbiol.* **8**, 44–54 (2006).
34. S. K. Gupta, S. Masinick, M. Garrett, L. D. Hazlett, *Pseudomonas aeruginosa* lipopolysaccharide binds galectin-3 and other human corneal epithelial proteins. *Infect. Immun.* **65**, 2747–2753 (1997).
35. A. Mey, H. Leffler, Z. Hmama, G. Normier, J. P. Revillard, The animal lectin galectin-3 interacts with bacterial lipopolysaccharides via two independent sites. *J. Immunol.* **156**, 1572–1577 (1996).
36. Y. Li *et al*., Galectin-3 is a negative regulator of lipopolysaccharide-mediated inflammation. *J. Immunol.* **181**, 2781–2789 (2008).
37. A. Lu *et al*., Molecular basis of caspase-1 polymerization and its inhibition by a new capping mechanism. *Nat. Struct. Mol. Biol.* **23**, 416–425 (2016).
38. S. M. Srinivasula *et al*., The PYRIN-CARD protein ASC is an activating adaptor for caspase-1. *J. Biol. Chem.* **277**, 21119–21122 (2002).
39. V. A. Rathinam *et al*., TRIF licenses caspase-11-dependent NLRP3 inflammasome activation by gram-negative bacteria. *Cell* **150**, 606–619 (2012).
40. Y. Liu *et al*., Visualization of perforin/gasdermin/complement-formed pores in real cell membranes using atomic force microscopy. *Cell. Mol. Immunol.* **16**, 611–620 (2019).
41. J. Adler, I. Parmryd, Quantifying colocalization by correlation: The Pearson correlation coefficient is superior to the Mander's overlap coefficient. *Cytometry A.* **77**, 733–742 (2010).
42. W. Chen, P. Stanley, Five Lec1 CHO cell mutants have distinct Mgat1 gene mutations that encode truncated N-acetylglucosaminyltransferase I. *Glycobiology* **13**, 43–50 (2003).
43. L. A. Knodler *et al*., Noncanonical inflammasome activation of caspase-4/caspase-11 mediates epithelial defenses against enteric bacterial pathogens. *Cell Host Microbe* **16**, 249–256 (2014).
44. C. N. Casson *et al*., Human caspase-4 mediates noncanonical inflammasome activation against gram-negative bacterial pathogens. *Proc. Natl. Acad. Sci. U.S.A.* **112**, 6688–6693 (2015).
45. G. Greub, D. Raoult, Microorganisms resistant to free-living amoebae. *Clin. Microbiol. Rev.* **17**, 413–433 (2004).
46. E. A. Creasey, R. R. Isberg, The protein SdhA maintains the integrity of the Legionella-containing vacuole. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 3481–3486 (2012).
47. J. Ruiz-Albert *et al*., Complementary activities of SseJ and SifA regulate dynamics of the Salmonella typhimurium vacuolar membrane. *Mol. Microbiol.* **44**, 645–661 (2002).
48. C. N. Casson *et al*., Caspase-11 activation in response to bacterial secretion systems that access the host cytosol. *PLoS Pathog.* **9**, e1003400 (2013).
49. I. C. Weng *et al*., Cytosolic galectin-3 and -8 regulate antibacterial autophagy through differential recognition of host glycans on damaged phagosomes. *Glycobiology* **28**, 392–405 (2018).
50. S. E. Stewart *et al*., A genome-wide CRISPR screen reconciles the role of N-linked glycosylation in galectin-3 transport to the cell surface. *J. Cell Sci.* **130**, 3234–3247 (2017).
51. L. E. Comstock, D. L. Kasper, Bacterial glycans: Key mediators of diverse host immune responses. *Cell* **126**, 847–850 (2006).
52. Q. Lu, S. Li, F. Shao, Sweet talk: Protein glycosylation in bacterial interaction with the host. *Trends Microbiol.* **23**, 630–641 (2015).
53. H. Sano *et al*., Critical role of galectin-3 in phagocytosis by macrophages. *J. Clin. Invest.* **112**, 389–397 (2003).
54. Y. J. Chen *et al*., Galectin-3 enhances avian H5N1 Influenza A virus-induced pulmonary inflammation by promoting NLRP3 inflammasome activation. *Am. J. Pathol.* **188**, 1031–1042 (2018).
55. H. Y. Chen *et al*., Galectin-3 negatively regulates TCR-mediated CD4+ T-cell activation at the immunological synapse. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 14496–14501 (2009).
56. A. Lepur, E. Salomonsson, U. J. Nilsson, H. Leffler, Ligand induced galectin-3 protein self-association. *J. Biol. Chem.* **287**, 21751–21756 (2012).
57. C. M. John, H. Leffler, B. Kahl-Knutsson, I. Svensson, G. A. Jarvis, Truncated galectin-3 inhibits tumor growth and metastasis in orthotopic nude mouse model of human breast cancer. *Clin. Cancer Res.* **9**, 2374–2383 (2003).
58. L. Mirandola *et al*., Galectin-3 inhibition suppresses drug resistance, motility, invasion and angiogenic potential in ovarian cancer. *Gynecol. Oncol.* **135**, 573–579 (2014).
59. M. Sundqvist *et al*., Galectin-3 type-C self-association on neutrophil surfaces: The carbohydrate recognition domain regulates cell function. *J. Leukoc. Biol.* **103**, 341–353 (2018).
60. T. Gutsmann *et al*., Dual role of lipopolysaccharide (LPS)-binding protein in neutralization of LPS and enhancement of LPS-induced activation of mononuclear cells. *Infect. Immun.* **69**, 6942–6950 (2001).
61. J. An *et al*., Caspase-4 disaggregates lipopolysaccharide micelles via LPS-CARD interaction. *Sci. Rep.* **9**, 826 (2019).
62. M. Mueller *et al*., Aggregates are the biologically active units of endotoxin. *J. Biol. Chem.* **279**, 26307–26313 (2004).
63. C. A. Lacey, W. J. Mitchell, A. S. Dadelahi, J. A. Skyberg, Caspase-1 and caspase-11 mediate pyroptosis, inflammation, and control of Brucella joint infection. *Infect. Immun.* **86**, e00361-18 (2018).
64. B. Lagrange *et al*., Human caspase-4 detects tetra-acylated LPS and cytosolic Francisella and functions differently from murine caspase-11. *Nat. Commun.* **9**, 242 (2018).
65. T. W. Cullen *et al*., *Helicobacter pylori* versus the host: Remodeling of the bacterial outer membrane is required for survival in the gastric mucosa. *PLoS Pathog.* **7**, e1002454 (2011).
66. I. Zanoni *et al*., An endogenous caspase-11 ligand elicits interleukin-1 release from living dendritic cells. *Science* **352**, 1232–1236 (2016).
67. Z. Ni *et al*., Evaluation of air oxidized PAPC: A multi laboratory study by LC-MS/MS. *Free Radic. Biol. Med.* **144**, 156–166 (2019).
68. N. C. Santos, A. C. Silva, M. A. Castanho, J. Martins-Silva, C. Saldanha, Evaluation of lipopolysaccharide aggregation by light scattering spectroscopy. *ChemBioChem* **4**, 96–100 (2003).
69. Y. Aida, M. J. Pabst, Removal of endotoxin from protein solutions by phase separation using Triton X-114. *J. Immunol. Methods* **132**, 191–195 (1990).
70. E. M. Kofoed, R. E. Vance, Innate immune recognition of bacterial ligands by NAIPs determines inflammasome specificity. *Nature* **477**, 592–595 (2011).
71. R. Y. Yang, P. N. Hill, D. K. Hsu, F. T. Liu, Role of the carboxyl-terminal lectin domain in self-association of galectin-3. *Biochemistry* **37**, 4086–4092 (1998).
72. J. Li *et al*., Caspase-11 regulates cell migration by promoting Aip1-Cofilin-mediated actin depolymerization. *Nat. Cell Biol.* **9**, 276–286 (2007).
73. F. Martín-Sánchez, A. I. Gómez, P. Pelegrín, Isolation of particles of recombinant ASC and NLRP3. *Bio Protoc.* **5**, e1480 (2015).
74. D. K. Hsu *et al*., Targeted disruption of the galectin-3 gene results in attenuated peritoneal inflammatory responses. *Am. J. Pathol.* **156**, 1073–1083 (2000).