



Phosphatidic acid production on the vacuole harboring *Legionella pneumophila* is a signal for recognition of interferon-induced GTPases

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Edited by Yasuko Rikihisa, The Ohio State University, Columbus, OH; received September 30, 2024; accepted July 10, 2025

The interferon (IFN)-inducible GTPases play a crucial role in cell autonomous immunity against intracellular pathogens. Particularly, these GTPases directly recognize the host membrane-derived vacuole containing pathogens and subsequently destroy it. Although it has been revealed that these GTPases target the membrane of *Legionella pneumophila* (*L. pneumophila*)-containing vacuole (LCV), molecular mechanism has been totally unclear. Here, we show that mouse guanylate-binding protein 2 (mGBP2) is specifically recruited to the LCV and subsequently ruptures it. Furthermore, we also show that mGBP2 recognizes phosphatidic acid (PA) produced by the *Legionella* effector Lpg2552 and fails to target the vacuole harboring Lpg2552-depleted *L. pneumophila*. Consistently, this strain successfully grows in cells expressing mGBP2. We additionally identified lysine 585 (K585) of mGBP2 is required for the binding to PA and K585-mutated mGBP2 fails to recognize LCV. Interestingly, this lysine residue is only conserved in GBP1 among human GBPs and the conserved lysine residue is important for PA recognition and subsequent LCV distribution. Importantly, *L. pneumophila* lacking Lpg2552 exhibits high resistance against IFN stimulation in THP-1-derived human macrophage.

cell autonomous immunity | GBP | *Legionella pneumophila* | Lpg2552 | phosphatidic acid

The interferon gamma (IFN- γ) is extremely important cytokine for mammalian host defense against intracellular pathogens, and it promotes translation of antipathogenic proteins including indole 2,3-deoxygenase (IDO), inducible nitric oxide synthase (iNOS), and IFN-inducible GTPases (1).

Functions of IDO or iNOS for engagement of intracellular pathogens are depletion of tryptophane that is essential for proliferation of microorganisms or production of nitric oxidant and subsequently reactive oxygen species that damages microorganisms, respectively (1). However, IFN-inducible guanosine triphosphate (GTP)ases including p47 immunity-related GTPases (IRGs) and p65 guanylate-binding proteins (GBPs) directly associate with the vacuolar membrane in which pathogens reside in and then promote the sequestration of pathogens by autophagosome or disruption of the vacuolar membrane to kill them (1, 2). In the IRGs or GBPs-mediated killing of microorganisms, specific recognition of membranous compartment harboring pathogens is essential. To this end, these GTPases detect slight but significant molecular alterations such as changing phospholipid composition (3). For instance, Irgb6, a member of IRGs, detects the abnormal enrichment of phosphatidylinositol 5 phosphate (PI5P) or phosphatidylserine (PS) on the vacuolar membrane containing human and animal parasitic protozoan *Toxoplasma gondii* (*T. gondii*) and destroys it (4, 5). Given that the level of PI5P is generally suppressed low in the host cells (6), Irgb6 senses this pathogen-derived molecular alteration as a “nonself” signal.

Intracellular pathogen, *Legionella pneumophila* (*L. pneumophila*) is a gram-negative bacterium and is known for a causative microorganism of legionnaires' disease exhibiting severe pneumonia symptom (7, 8). Initiation of human infection of *L. pneumophila* is caused by uptake of this pathogen through respiration, and then it accesses to a lung. Thereafter, *L. pneumophila* infects alveolar macrophage via phagocytosis and forms membrane-decorated compartment termed *Legionella*-containing vacuole (LCV) for creating specialized infectious niche (9, 10). Notably, this pathogen secretes bacterial proteins, “*Legionella* effectors” into the host cytosol through the Dot/Icm type IV secretion system (T4SS) to hijack the several host physiological functions, i.e., membrane trafficking, autophagy, apoptosis, etc. (11–15).

Significance

To engage with intracellular pathogens, cell autonomous immunity should discriminate between pathogen-occupied vacuolar membrane and host-derived organelle membrane. Therefore, revelation of how cell autonomous immunity-related molecules specifically recognize pathogen-containing vacuolar membrane is quite important for understanding of boundary between “self” and “nonself” inside the cell. Even though vacuolar pathogen, *Legionella pneumophila* (*L. pneumophila*), is known to be targeted by cell autonomous immunity-related molecules, it has been unknown how these proteins specifically recognize the *L. pneumophila*-occupied vacuole. In here, we demonstrate that one of the molecules, mouse guanylate-binding protein 2 (mGBP2) and hGBP1 recognizes *Legionella*-produced phosphatidic acid (PA) on the *L. pneumophila*-containing vacuole (LCV), suggesting that accumulation of PA is one of the nonself signals inside the cell.

The authors declare no competing interest.

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This article contains supporting information online at <https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2420096122/-/DCSupplemental>.

Published August 8, 2025.

On the other hand, mammalian host is also known to combat with *L. pneumophila* infection by inflammasome formation (16–18). In this process, noncanonical inflammasome formed by caspase-11 (human caspase-4 and -5) inhibits the intracellular growth of this pathogen (19, 20). In addition, several GBPs target the LCV and subsequently rupture the membrane for restriction of *L. pneumophila* proliferation (21–23), indicating that GBPs recognize LCV membrane as a compartment to be destroyed. However, molecular machinery in which GBPs recognize LCV membrane has been obscured.

We here show that mouse GBP2 (mGBP2) is specifically recruited to the LCV in several mouse GBPs and it binds to phosphatidic acid (PA) that is produced by a *Legionella* effector Lpg2552. We also show that mGBP2 facilitates the rupture of LCV and restricts intracellular growth of *L. pneumophila*. Furthermore, we identified lysine 585 (K585) of mGBP2 as an important residue for binding to PA, and K585-mutated mGBP2 abolishes the localization of LCV as well as restriction of intracellular growth of *L. pneumophila*. Notably, key lysine residue (K585 of mGBP2) is only conserved in GBP1 in human GBPs, and the residue is also important for both PA recognition and LCV distribution. Finally, we demonstrate that mutant *L. pneumophila* lacking Lpg2552 avoids the recognition by hGBP1 and subsequently replicates in IFN- γ stimulated macrophages.

Results

The *Legionella* Effector Lpg2552 Is Required for Recruitment of mGBP2 to the LCV. Given the previous reports showing several GBPs are recruited to the LCV (21, 22), we first explored the specificity of recruitment of GBPs. To do this, we infected *L. pneumophila* with HeLa-Fc γ RII cells ectopically expressing FLAG-tagged individual mouse GBPs (GBP1, 2, 3, 5, and 7 that are known as an antimicrobial GBPs). As shown in *SI Appendix, Fig. S1A*, only mGBP2 is detected on the LCV. Additionally, a function of some *Legionella* effector(s) should be required for mGBP2 recruitment to the LCV because the signal of mGBP2 was negative for the vacuole harboring T4SS-deficient strain Δ dotA (*SI Appendix, Fig. S1B*). Therefore, we tried to identify the effector(s) using five genomic islands-depleted *Legionella* strains (24, Fig. 1A). In cells infected with Δ 7a (Fig. 1B, Bottom row) as well as Δ pentuple (Fig. 1B, second row), recruitment of mGBP2 to the LCV was not detected, implying that island 7a-encoded *Legionella* effector(s) has responsible for the recruitment of mGBP2 to the LCV.

If mGBP2 recognizes some alteration in which it is induced by *Legionella* effector, mGBP2 might coexist with it. To test this, we evaluated the correlation of intracellular distribution between mGBP2 and island7a-encoded 25 *Legionella* effectors. As a result, specific co-localization was observed in cells expressing mGBP2 and Lpg2552 (*SI Appendix, Fig. S2A*). Furthermore, mGBP2-positive vacuoles were significantly reduced in mutant *L. pneumophila* strain lacking Lpg2552 (Fig. 1C). Of note, mGBP2 does not interact with Lpg2552 (*SI Appendix, Fig. S2B*), suggesting that mGBP2 recognizes Lpg2552-derived some alteration on the LCV.

mGBP2 Recognizes Lpg2552-Derived PA in a Concentration-Dependent Manner. Because previous study demonstrated that Lpg2552 produces diacylglycerol (DAG) on the LCV (25), DAG might be a signal for recruitment of mGBP2 to the LCV. To address this, we incubated glutathione S-transferase (GST) or GST-fused mGBP2 with a 15 different lipids-spotted nitrocellulose membrane (PIP-Strip membrane) and analyzed the specificity of mGBP2-lipids binding. Contrary to the expectation, however, specific binding was observed only in PA spotted area (Fig. 2A).

Because DAG is known to be immediately converted into PA in the cell (26, 27), it is possible that Lpg2552-produced DAG is also converted into PA. Of note, previous study also suggests that Lpg2552-produced DAG might be converted into PA via activity of host DAG kinase (25). Consistent with the idea, Lpg2552-positive structures are positive for not only DAG but also PA sensor [PA-binding domain of Nir2; (28)] (*SI Appendix, Fig. S2C*). Moreover, both sensors were detected on the vacuole containing wild type but not Δ lpg2552 strain (Fig. 2B and C), indicating that mGBP2 recognizes Lpg2552-derived PA on the LCV. To show a more physiological evidence that mGBP2 recognizes to PA, we performed liposome floatation assay as described in Fig. 2D. In a liposome containing phosphatidylcholine (PC) and phosphatidylethanolamine (PE), the signal of both GST and GST-mGBP2 was not detected in the top two fractions enriched floating liposomes (Fig. 2E and F, Left). However, significant signal of GST-mGBP2 but not GST was detected in the top two fractions when the liposomes are made by PC/PE plus PA (Fig. 2E and F, Middle and Right). Interestingly, mGBP2 preferentially binds to unsaturated acyl chain-conjugated PA (DOPA) rather than saturated one (DPPA) (Fig. 2F, compare to Middle and Right panels). These results suggest that mGBP2 directly binds to PA for recognition of LCV.

Given that PA is a component of several organelle membranes, mGBP2 ought to have specificity for recognition of PA to avoid targeting organelle membrane. To consider this, PA concentration seems to be a factor for specificity. To test this, we examined whether threshold of PA-binding is different between mGBP2 and canonical PA-binding protein such as PA sensor that specifically binds to PA (*SI Appendix, Fig. S3A*). As shown in Fig. 2G, GST-PA sensor was detected in the liposomal fractions containing 5% or 25% of PA. In contrast, GST-mGBP2 was not detected in the liposomal fractions when it was incubated with the 5% PA-containing liposome (Fig. 2H). In addition, our data indicated that over 10% of PA might be required for the association of mGBP2 with membrane-embedded PA (*SI Appendix, Fig. S3B, Right*).

Lysine 585 of mGBP2 Is Required for LCV Recognition as Well as PA Binding. Because previous studies suggest that the effector domain of mGBP2 has responsible for its membrane interaction [Fig. 3A, (29, 30)], we infected *L. pneumophila* with cells expressing several mGBP2 truncation mutants. Consistent with these studies, recruitment of mutant protein lacking effector domain to the LCV was not detected (Fig. 3B). Of note, one of these studies also shows that lysine residue at 585 (K585) of mGBP2 requires for the binding to a cardiolipin which consists of two PA molecules linked through another glycerol (30). Therefore, we next examined whether the lysine residue is required for the association with LCV as well as the binding to PA. As shown in Fig. 3C, Middle row, efficiency of mGBP2 recruitment to the LCV was significantly reduced upon mutation of K585. Furthermore, we confirmed previous study showing that C-terminus isoprenylation CaaX motif of mGBP2 was necessary for the distribution of vacuolar membrane (Fig. 3C, Bottom row, 29). Importantly, liposome floatation assay showed that affinity of mGBP2 with liposome containing PA is drastically decreased by mutation of K585 (Fig. 3D), suggesting that two activities such as PA recognition by K585 and membrane insertion by CaaX motif were required for the recruitment of mGBP2 to the LCV.

Accumulation of PA-Mediated mGBP2 to the LCV Causes Rupture of LCV Membrane and Restricts Intracellular Growth of *L. pneumophila*. Given that GBPs have an ability to destroy the LCV membrane by themselves (20, 21), LCV accumulated mGBP2 seems to be able to rupture it. To test this, we performed

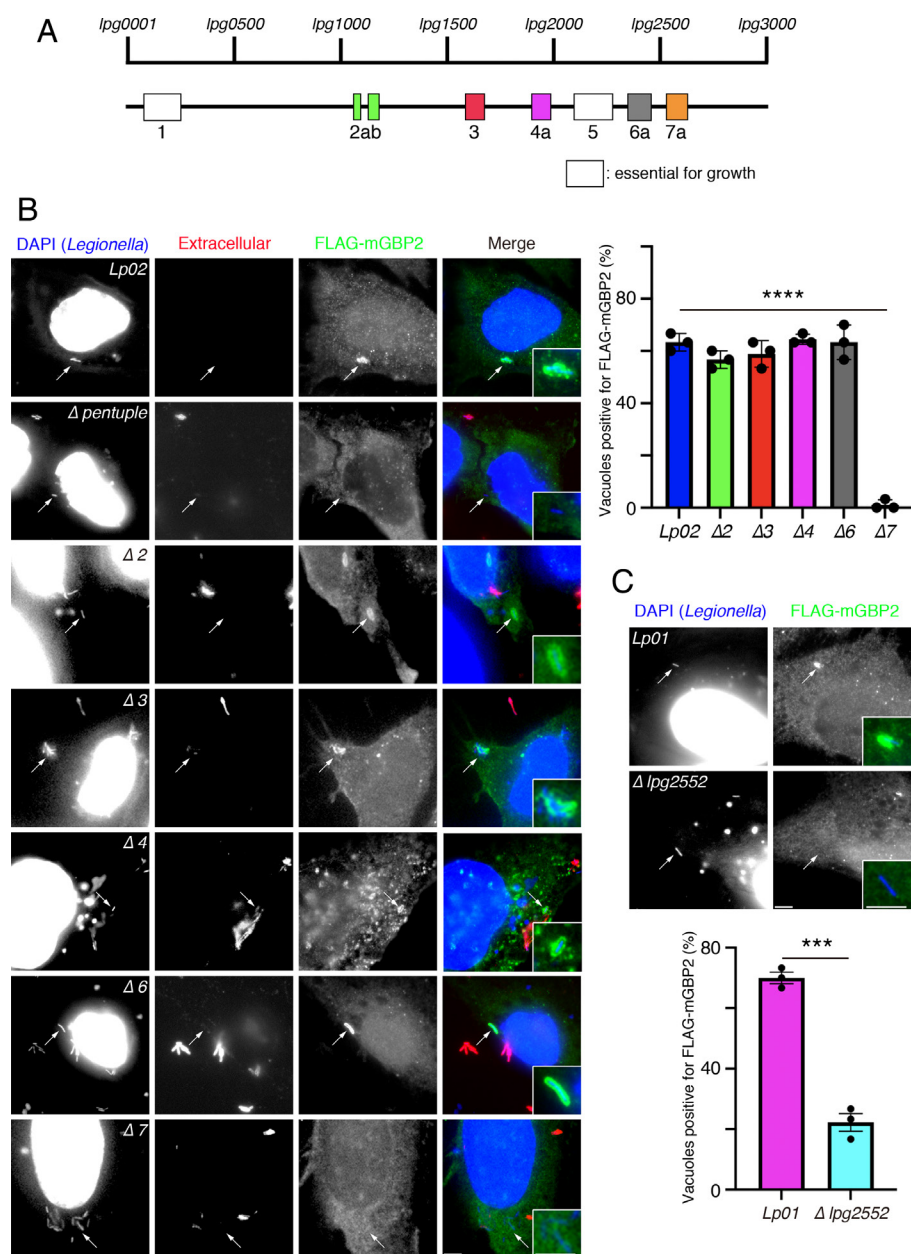


Fig. 1. Identification of *Legionella* effector that facilitates recruitment of mGBP2 to the LCV. (A) Schematic representation of the Δ pentuple and single island-depleted strains of *L. pneumophila* (Lp02). (B) HeLa-Fc γ RII cells expressing FLAG-mGBP2 were infected with the indicated *L. pneumophila* strains for 1 h at MOI of 10. After infection, cells were fixed and stained with anti-*Legionella* antiserum for detection of extracellular bacteria, then permeabilized and further stained with anti-FLAG antibody and DAPI for detection of FLAG-mGBP2 and intracellular bacteria, respectively. Bar, 2 μ m. FLAG-mGBP2 positive LCVs were quantified, and representative data of three independent experiments were presented (30 vacuoles were scored in each experiment). Results are shown as the mean $\pm$ SD. **** P < 0.0001. (C) HeLa-Fc γ RII cells expressing FLAG-mGBP2 were infected with Lp01 or Δ lpg2552 strains for 1 h at MOI of 10. After infection, cells were fixed and stained with anti-*Legionella* antiserum for detection of extracellular bacteria, then permeabilized and further stained with anti-FLAG antibody and DAPI for detection of FLAG-mGBP2 and intracellular bacteria, respectively. Bar, 2 μ m. FLAG-mGBP2 positive LCVs were quantified, and representative data of three independent experiments were presented (30 vacuoles were scored in each experiment). Results are shown as the mean $\pm$ SD. *** P < 0.001.

LCV integrity assay (Fig. 4A, 21). In this assay, *L. pneumophila*-infected cells were permeabilized by mild detergent digitonin for only permeabilization of plasma membrane, allowing small compounds such as an antibody to access into the cell. If the LCV membrane is ruptured, therefore, *L. pneumophila* opsonizing primary antibody that exists luminal side of LCV is recognized by secondary antibody. Even though the signal of secondary antibody was not observed in the vacuole positive for Rab1B that is recruited to the LCV for subversion of early secretory pathway (31–33), significant signal of secondary antibody was detected in mGBP2 positive vacuole (Fig. 4B), indicating that mGBP2 has an ability to make a pore in LCV by itself. Because integrity of LCV membrane is important for the intracellular growth of *L. pneumophila* (34), we next examined whether mGBP2 restricts the intracellular growth of *L. pneumophila*. As shown in Fig. 4C, intracellular growth of *L. pneumophila* was strikingly restricted in cells expressing mGBP2. In addition, K585-mutated mGBP2 failed to restrict *L. pneumophila* growth (Fig. 4D). Furthermore, Δ lpg2552 strain successfully replicated in cells expressing wild-type mGBP2

(Fig. 4E). Taken together, mGBP2 destroys LCV membrane and subsequent restricts intracellular growth of *L. pneumophila* to recognize Lpg2552-mediated PA accumulation.

GBP1 Specifically Targets LCV in Human GBPs through Lysine Residue That Is Corresponding with K585 of mGBP2. In previous study, the efficacy of *L. pneumophila* restriction by GBP1 is higher than GBP2 in human-derived macrophage (21), suggesting that human guanylate binding protein 1 (hGBP1) has high affinity to LCV. However, specificity of LCV distribution among hGBPs has been obscured. Therefore, we observed the LCV distribution of hGBPs. And as a result, we could detect the specific recruitment of hGBP1 to the LCV (SI Appendix, Fig. S4A), and Lpg2552 is required for this recruitment (Fig. 5A). Of note, sequence alignment of C-terminus membrane association region between mGBP2 and hGBPs shows that only hGBP1 includes both lysine and CaaX motif of mGBP2 (SI Appendix, Fig. S4B), indicating that hGBP1 recognizes Lpg2552-derived PA on the LCV through its lysine residue at 587 (K587). Meanwhile, triple arginine cluster (R584/585/586; 3R) of hGBP1 is reported as a key region for

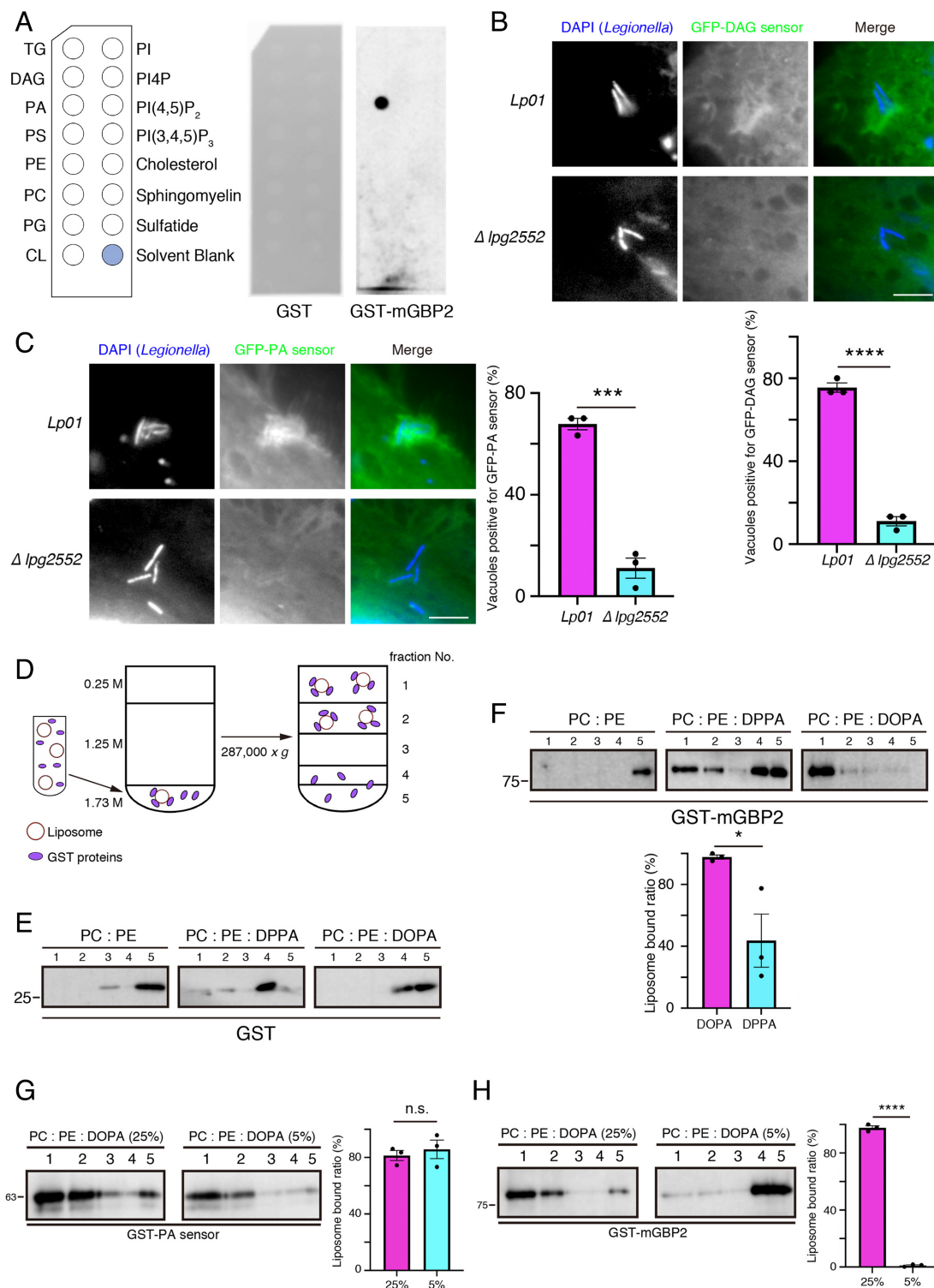


Fig. 2. mGBP2 binds and recognizes to Lpg2552-derived PA in a concentration-dependent manner. (A) GST or GST-mGBP2 (1 μ g/mL each) was incubated with a PIP strip membrane, and lipid binding proteins were detected using anti-GST antibody. *Template* shows the position of each lipid spotted. TAG, triacylglycerol; DAG, diacylglycerol; PA, phosphatidic acid; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PG, phosphatidylglycerol; CL, cardiolipin; PI, phosphatidylinositol; PI(4)P, phosphatidylinositol 4-phosphate; PI(4, 5)P₂, phosphatidylinositol 4,5-bisphosphate; PI(3, 4, 5)P₃, phosphatidylinositol 3,4,5-trisphosphate. (B and C) HeLa-Fc γ RII cells expressing GFP-DAG sensor (B) or -PA sensor (C) were infected with *Lp01* or $\Delta lpg2552$ strains for 1 h at MOI of 10. After infection, cells were fixed and stained with anti-*Legionella* antiserum for the detection of extracellular bacteria, then permeabilized and further stained with DAPI for the detection of intracellular bacteria, respectively. Bar, 2 μ m. GFP-PA sensor-positive LCVs were quantified, and representative data of three independent experiments were presented (30 vacuoles were scored in each experiment). Results are shown as the mean $\pm$ SD. *** P < 0.001, **** P < 0.0001. (D) Schematic representation of liposome floatation assay. (E and F) GST (E) or GST-mGBP2 (F) (each 0.5 μ g) was incubated with liposomes (DOPC/DOPE, DOPC/DOPE/DPPA or DOPC/DOPE/DOPA) and subjected to the floating assay. After centrifugation, fractions were collected from top to bottom, and the collected fractions were analyzed using anti-GST antibody. (F) The graph shows the average percentage of GST-mGBP2 band intensity in fractions 1 and 2 (liposomes containing DPPA or DOPA). Values are the mean $\pm$ SD (n = 3). ** P < 0.01. (G and H) GST-PA sensor (G) or GST-mGBP2 (H) was incubated with liposomes [DOPC/DOPE/DOPA (25%) or DOPC/DOPE/DOPA (5%)] and subjected to the floating assay. After centrifugation, fractions were collected from top to bottom, and the collected fractions were analyzed using anti-GST antibody. The graph shows the average percentage of GST-PA sensor (G) or GST-mGBP2 (H) band intensity in fractions 1 and 2 (liposomes containing 25% DOPA or 5% DOPA). Values are the mean $\pm$ SD (n = 3). n.s., not significance. **** P < 0.0001.

recognition of cytosolic *Shigella flexneri* (35). Then, we examined whether 3R or K587 of hGBP1 is necessary for targeting of LCV. Unlike previous observation (35), 3R/A-mutated hGBP1 was successfully recruited to the LCV (Fig. 5 B, Middle row). On the other hand, recruitment of hGBP1 was significantly reduced

upon mutation of K587 (Fig. 5 B, Bottom row). Furthermore, liposome floatation assay showed that hGBP1 also binds to PA through its K587 (Fig. 5C), suggesting that binding to PA through the lysine residue plays a crucial role in targeting of mGBP2/hGBP1 to the LCV.

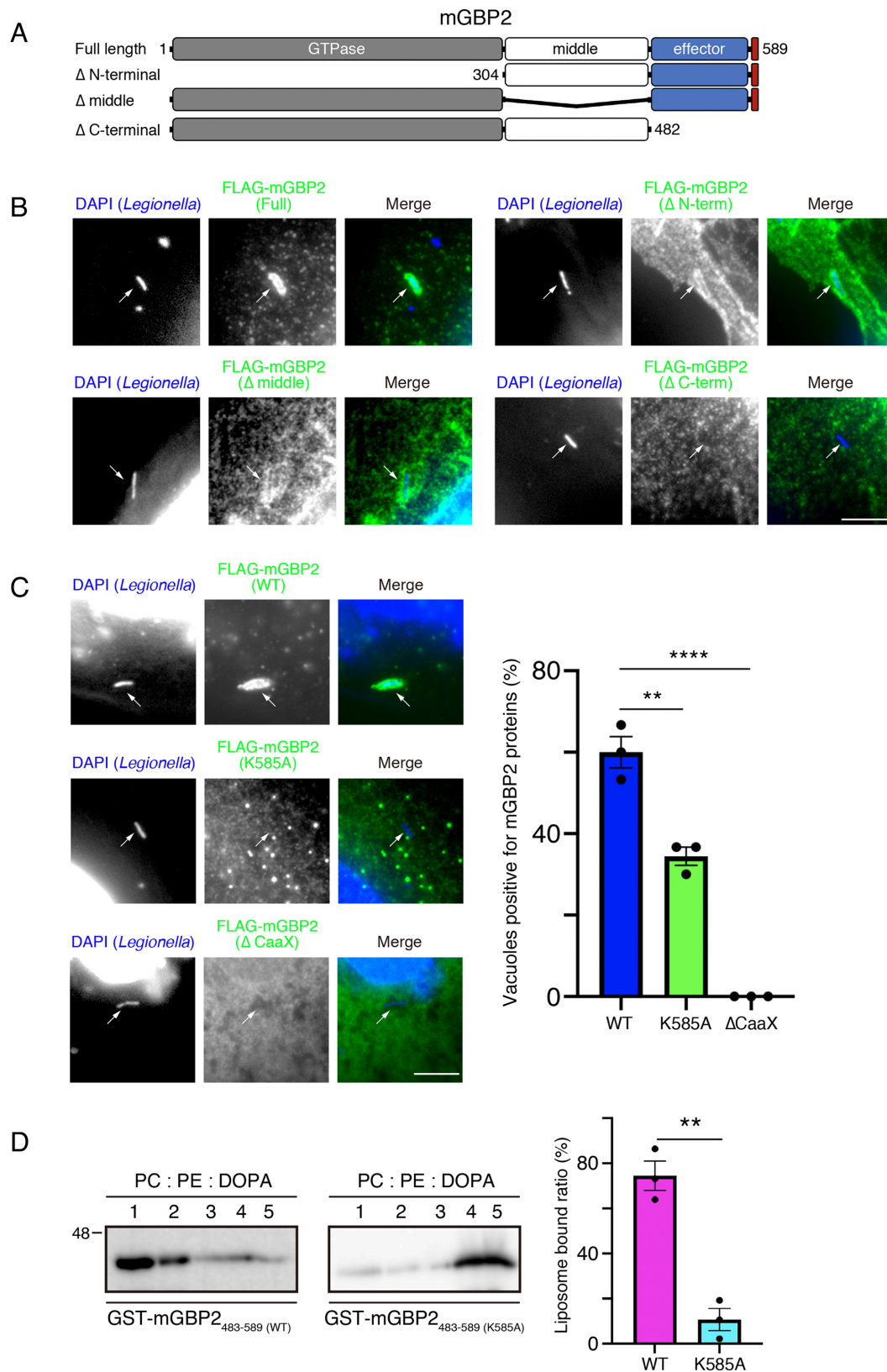


Fig. 3. Lys585 is required for both LCV distribution and PA binding of mGBP2. (A) Schematic representation of mGBP2 domains. (B) HeLa-FcyRII cells expressing FLAG-mGBP2 or its derivatives were infected with *Lp01* for 1 h at MOI of 10. After infection, cells were fixed and stained with anti-*Legionella* antiserum for detection of extracellular bacteria, then permeabilized and further stained with anti-FLAG antibody and DAPI for the detection of FLAG-mGBP2 and intracellular bacteria, respectively. Bar, 2 μ m. (C) HeLa-FcyRII cells expressing FLAG-mGBP2 (WT), -mGBP2 (K585A) or -mGBP2 (Δ CaaX) were infected with *Lp01* for 1 h at MOI of 10. After infection, cells were fixed and stained with anti-*Legionella* antiserum for detection of extracellular bacteria, then permeabilized and further stained with anti-FLAG antibody and DAPI for detection of FLAG-mGBP2 and intracellular bacteria, respectively. Bar, 2 μ m. (D) GST-mGBP2₄₈₃₋₅₈₉ (WT) or -mGBP2₄₈₃₋₅₈₉ (K585A) was incubated with liposomes (DOPC/DOPE/DOPA) and subjected to the floating assay. After centrifugation, fractions were collected from top to bottom, and the collected fractions were analyzed using anti-GST antibody. The graph shows the average percentage of GST-mGBP2 proteins positive LCVs were quantified, and representative data of three independent experiments were presented (30 vacuoles were scored in each experiment). Results are shown as the mean $\pm$ SD. ** P < 0.01. **** P < 0.0001.

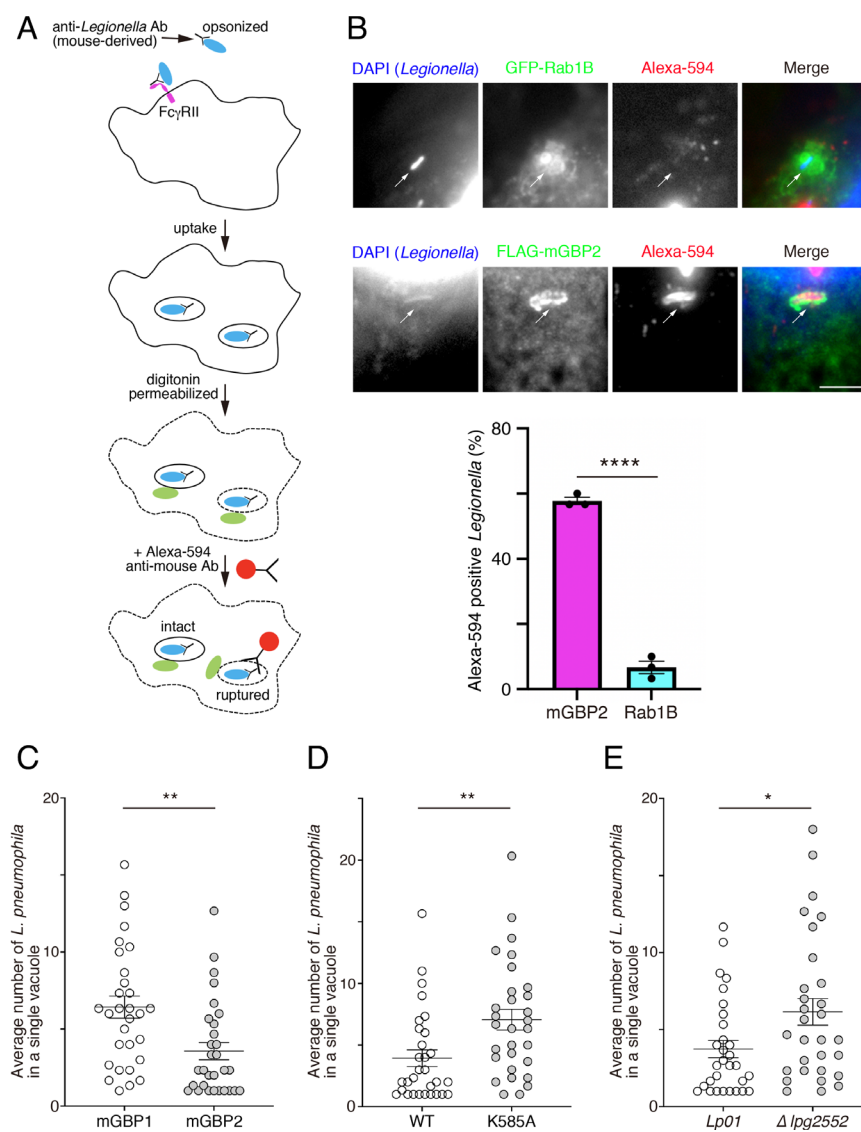


Fig. 4. PA-mediated LCV association of mGBP2 restricts intracellular *Legionella* growth by rupturing LCV membrane. (A) Schematic representation of LCV integrity assay. (B) HeLa-FcγRII cells expressing GFP-Rab1B or FLAG-mGBP2 were infected with *Lp01* for 1 h at MOI of 10, fixed and permeabilized by digitonin. After permeabilization, cells were stained with DAPI and Alexa-594-conjugated anti-mouse IgG antibody for detection of *L. pneumophila* and opsonized antibody, respectively. Bar, 2 μm. Alexa-594 positive *L. pneumophila* decorated with GFP-Rab1B or FLAG-mGBP2 were quantified, and representative data of three independent experiments were presented (30 vacuoles were scored in each experiment). Results are shown as the mean ± SD. *****P* < 0.0001. (C–E) HeLa-FcγRII cells expressing indicated proteins [FLAG-mGBP1/-mGBP2 (C), FLAG-mGBP2 (WT) or -mGBP2 (K585A) (D), FLAG-mGBP2 (E)] were infected with *L. pneumophila* strains [*Lp01* (C and D), *Lp01* and Δ*lpg2552* (E)] for 8 h at MOI of 1. After infection, cells were fixed and stained with anti-*Legionella* antiserum for the detection of extracellular bacteria, then permeabilized and further stained with anti-FLAG antibody and DAPI for detection of FLAG-tagged proteins and intracellular bacteria, respectively. The graph shows the scatter plot of average number of *L. pneumophila* in a single vacuole. Data are representative of three independent experiments (30 vacuoles were scored in each experiment). Results are shown as means ± SEM. **P* < 0.05. ***P* < 0.01.

Mutant *L. pneumophila* Lacking Lpg2552 Shows Resistance to IFN-γ Stimulus. Because macrophage is a bona fide host of *L. pneumophila* and expression of endogenous GBPs is induced by IFN-γ, it is quite important for the evaluation of our findings using IFN-γ stimulated macrophage. To do this, we employed infection experiment using Tohoku Hospital Pediatrics-1 (THP-1) derived human macrophage, and we first confirmed that expression level of hGBP1 and hGBP2 is elevated upon IFN-γ treatment (SI Appendix, Fig. S5A). In addition, silencing experiment revealed that loss of hGBP1 but not hGBP2 permits *L. pneumophila* to survive in the IFN-γ stimulated THP-1-derived macrophages (SI Appendix, Fig. S5B and Fig. 6A), implying that hGBP1 plays a crucial role in a clearance of *L. pneumophila* which is consistent with previous study (21). We next examined the distribution of IFN-γ induced endogenous hGBP1. As shown in Fig. 6B, Top row, endogenous hGBP1 is recruited to the vacuole containing *Lp01*. However, efficiency of hGBP1 recruitment was significantly reduced in vacuole harboring Δ*lpg2552* strain (Fig. 6B, Bottom row). Importantly, Δ*lpg2552* strain shows high survival rate, compared to *Lp01* in IFN-γ-stimulated THP-1-derived macrophages (Fig. 6C). Furthermore, mostly the same results were obtained in HeLa-FcγRII cell, in which expression of GBPs is induced by IFN-γ stimulus (36, SI Appendix, Fig. S5C–E).

Discussion

Based on our present results, we propose a working model by which mGBP2/hGBP1 recognizes *L. pneumophila*-occupied vacuolar membrane (SI Appendix, Fig. S6). After uptake, *L. pneumophila* creates LCV and secretes *Legionella* effectors through T4SS. After that, one of the *Legionella* effectors Lpg2552 associates with LCV membrane (I) and it promotes DAG synthesis on the pathogen-occupied vacuole (II). Synthesized DAG might be metabolically converted into PA (III), and then accumulated PA is recognized by mGBP2/hGBP1 through its K585/K587 residue (IV). Finally, LCV-associated mGBP2/hGBP1 promotes the rupture of LCV membrane and restricts intracellular growth of *L. pneumophila* (V).

This study sheds light on the molecular machinery of mGBP2/hGBP1 for discrimination of boundary between “self” and nonself inside the cell: one of the important determinant factors of this is a concentration of PA. Because PA is activator of cell signaling or several ion channels, it is known that concentration of PA is regulated in a lower level (37, 38).

Therefore, it is quite reasonable that mGBP2 (hGBP1) senses abnormal PA accumulation as a nonself pattern.

However, several important questions still exist for understanding the whole aspect of this machinery. Even though our liposome

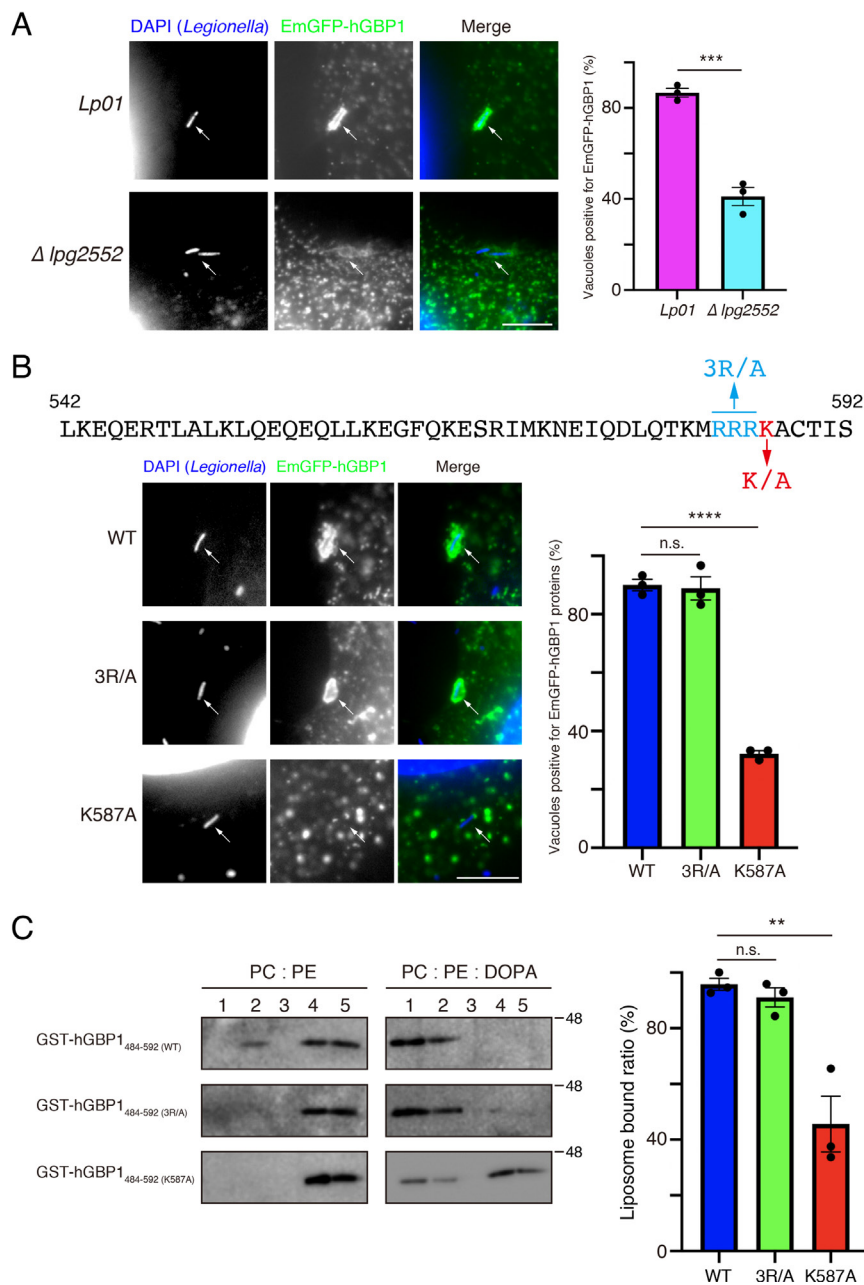


Fig. 5. hGBP1 recognizes Lpg2552-derived PA through its lysine residue corresponding lysine 585 of mGBP2. (A) HeLa-FcyRII cells expressing EmGFP-hGBP1 were infected with Lp01 or $\Delta lpg2552$ strains for 1 h at MOI of 10. After infection, cells were fixed and stained with anti-*Legionella* antiserum for the detection of extracellular bacteria and DAPI for detection intracellular bacteria, respectively. Bar, 2 μ m. EmGFP-hGBP1 positive LCVs were quantified, and representative data of three independent experiments were presented (30 vacuoles were scored in each experiment). Results are shown as the mean $\pm$ SD. *** P < 0.001. (B) Schematic representation of 3R/A or K587A mutation. Right blues indicate triple arginine cluster. Red indicates lysine residue corresponding with K585 of mGBP2. HeLa-FcyRII cells expressing indicated proteins were infected with Lp01 strain for 1 h at MOI of 10. After infection, cells were fixed and stained with anti-*Legionella* antiserum for detection of extracellular bacteria and DAPI for detection intracellular bacteria, respectively. Bar, 2 μ m. EmGFP-hGBP1 proteins positive LCVs were quantified, and representative data of three independent experiments were presented (30 vacuoles were scored in each experiment). Results are shown as the mean $\pm$ SD. n.s., not significance. **** P < 0.0001. (C) GST-hGBP1₄₈₄₋₅₉₂ (WT) (Top), GST-hGBP1₄₈₄₋₅₉₂ (3R/A) (Middle) or GST-hGBP1₄₈₄₋₅₉₂ (K587A) (Bottom) was incubated with liposomes (DOPC/DOPE) or [DOPC/DOPE/DOPA (25%)] and subjected to the floating assay. After centrifugation, fractions were collected from top to bottom, and the collected fractions were analyzed using anti-GST antibody. The graph shows the average percentage of GST-hGBP1₄₈₄₋₅₉₂ (WT) (Top), GST-hGBP1₄₈₄₋₅₉₂ (3R/A) (Middle), or GST-hGBP1₄₈₄₋₅₉₂ (K587A) (Bottom) band intensity in fractions 1 and 2 (liposomes containing 25% DOPA or 5% DOPA). Values are the mean $\pm$ SD (n = 3). n.s., not significance. ** P < 0.01.

floatation assay demonstrates that mGBP2/hGBP1 preferentially binds to unsaturated rather than saturated acyl-chain conjugated PA (Fig. 2F), it has been unclear that which type of acyl-chain conjugated PA is accumulated on the LCV. In order to address this, comprehensive lipidomic analysis using purified vacuole containing wild-type or $\Delta lpg2552$ strain of *L. pneumophila* is necessary in the future. In addition, structural analysis of mGBP2/hGBP1 in PA coupling condition might be necessary for elucidating how mGBP2/hGBP1 binds to PA in a concentration-dependent manner. Additionally, investigation of another *Legionella* effector(s) encoded in island 7a for recruitment of mGBP2/hGBP1 to the LCV might also be needed. Based on our data using island-truncated mutant strains, almost all vacuoles harboring $\Delta 7a$ strain were negative for mGBP2 (Fig. 1B). Meanwhile, some vacuoles containing $\Delta lpg2552$ were detected in mGBP2/hGBP1 association (Figs. 1C, 5A, and 6B), suggesting that some *Legionella* effector(s) assists the recruitment of mGBP2/hGBP1 to the LCV. Because it is known that vacuolar ubiquitination is implicated in recruitment of mGBP2 (39) and several ubiquitin-related effectors are encoded

in island 7a, examination of mGBP2/hGBP1 localization using *L. pneumophila* strains lacking Lpg2552 and these effectors is necessary in the future.

It is also interesting question why *L. pneumophila* has the *Legionella* effector Lpg2552. To consider this, previous studies related with the *Legionella* effector LegC4 might be informative. Roy and colleagues demonstrate that the function of LegC4 is necessary for the intracellular growth of *L. pneumophila* in a protozoan host such as amoeba but it attenuates *L. pneumophila* replication in a mouse lung (40). Moreover, Shames and colleagues show that high susceptible *L. pneumophila* infection is observed in $\Delta legC4$ strain in tumor necrosis factor or IFN- γ primed macrophage (41). Given these findings, therefore, Lpg2552 might play an important role in proliferation of *L. pneumophila* in a protozoan host. Even though the previous study could not detect the significant growth defect of $\Delta lpg2552$ strain in amoeba (25), more precise analyses such as intracellular localization or integrity of vacuole harboring $\Delta lpg2552$ strain in a protozoan host will be needed.

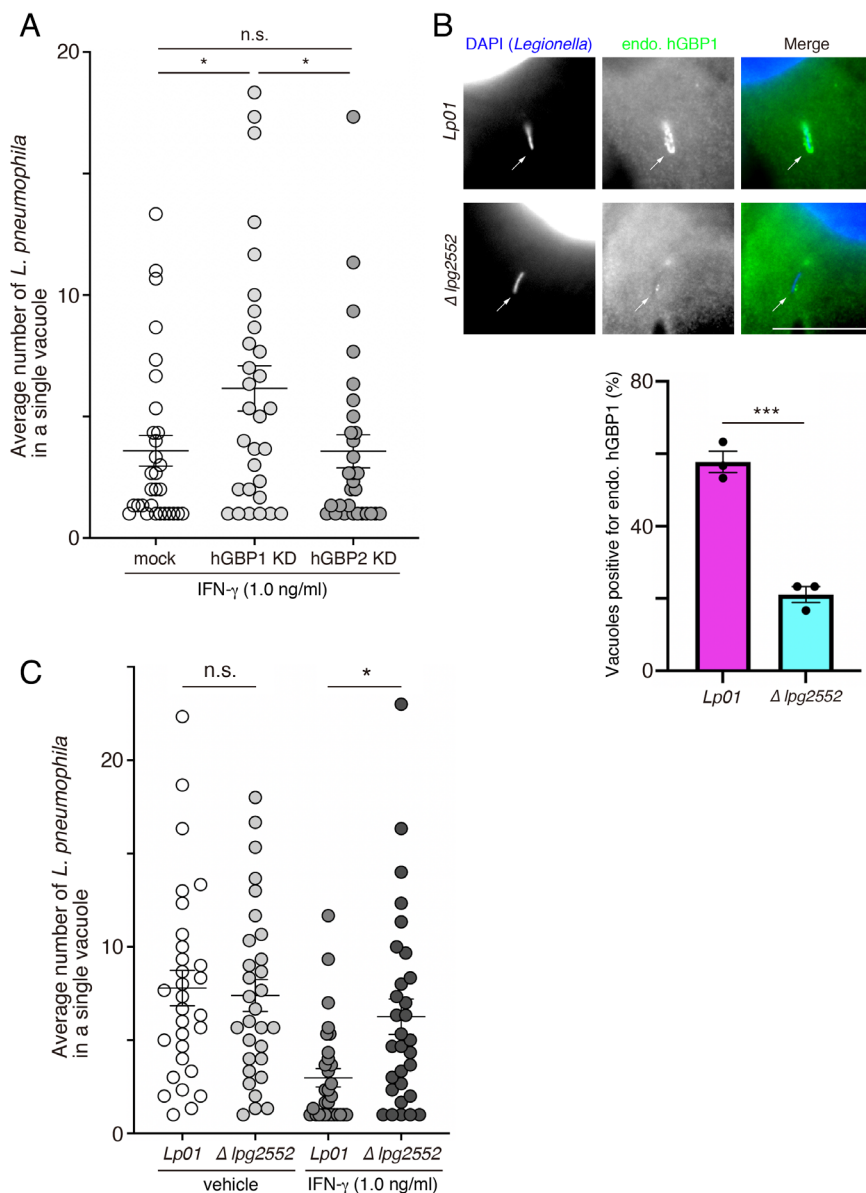


Fig. 6. Lpg2552 is required for the targeting of IFN- γ induced endogenous hGBP1 and clearance of *L. pneumophila* in human macrophage. (A) THP-1-derived macrophages were transfected without (Mock) or with small interfering RNA (siRNA) targeting for hGBP1 or hGBP2. At 48 h after transfection, cells were additionally treated with IFN- γ (1 ng/mL) for 24 h and infected with *Lp01* strain for 8 h at MOI of 50, fixed and stained with anti-*Legionella* antiserum for detection of extracellular bacteria, then permeabilized and further stained with DAPI for detection of intracellular bacteria. The graph shows the scatter plot of average number of *L. pneumophila* in a single vacuole. Data are representative of three independent experiments (30 vacuoles were scored in each experiment). Results are shown as means $\pm$ SEM. n.s., not significance. * P < 0.05. (B) THP-1-derived macrophages treated with IFN- γ (1 ng/mL) for 24 h were infected with *Lp01* or Δ lpg2552 strains for 1 h at MOI of 50, fixed and stained with anti-*Legionella* antiserum for detection of extracellular bacteria, then permeabilized and further stained with DAPI for detection of intracellular bacteria, respectively. Bar, 2 μ m. endogenous hGBP1 positive LCVs were quantified, and representative data of three independent experiments were presented (30 vacuoles were scored in each experiment). Results are shown as the mean $\pm$ SD. *** P < 0.001. (C) THP-1-derived macrophages were treated without (vehicle) or treated with IFN- γ (1 ng/mL) for 24 h. After treatment, cells were infected with *Lp01* or Δ lpg2552 strains for 8 h at MOI of 50, fixed and stained with anti-*Legionella* antiserum for detection of extracellular bacteria, then permeabilized and further stained with DAPI for detection of intracellular bacteria. The graph shows the scatter plot of average number of *L. pneumophila* in a single vacuole. Data are representative of three independent experiments (30 vacuoles were scored in each experiment). Results are shown as means $\pm$ SEM. n.s., not significance. * P < 0.05.

In conclusion, our study demonstrates that “molecular alteration” such as an accumulation of PA is one of the determinant factors of boundary between “self” and “nonself” for mGBP2/hGBP1. Considering that, together with the accumulation of PI5P or PS is trigger for recognition of Irgb6 (4, 5) to the vacuole containing *T. gondii*, alteration of composition or concentration of phospholipid on the pathogen-occupied vacuole might behave as a nonself signature. Since IRGs or GBPs recognize the several vacuolar pathogens other than *L. pneumophila* or *T. gondii*, phospholipid analysis of membrane harboring these pathogens provides a novel pattern of phospholipid alteration for the intracellular nonself signal.

Because IRGs or GBPs are related to the several autoimmune diseases (42), regulation of these protein is a putative therapeutic strategy for these diseases. Therefore, analyses of activity of phospholipid-synthesizing or -metabolizing enzymes as well as phospholipid composition in the disease-related materials have a potential for providing an insight into a pathogenic machinery of IRG- or GBP-related autoimmune diseases (e.g., hyperactivation of these enzymes or accumulation of some phospholipids).

Materials and Methods

Bacterial Strains and Plasmids. The conditions for the growth of *L. pneumophila* strains (*Lp01* wild-type, Δ dotA strains, thymidine auxotroph *Lp02* pentuple and single island deleting strains) were described as previously (13, 24, 43–45). The *Lp01* Δ lpg2552 mutant was constructed by allelic exchanges as described previously (44, 46). mGBPs and hGBPs constructs are described previously (47, 48). For expression of proteins as a GFP fusion constructs in mammalian cells, complementary DNA (cDNA) encoding Lpg2552 or PA binding domain of Nir2 (amino acid: 911 to 1,244), or mGBP1 was inserted into pEGFP-vector (Clontech). For bacterial expression, cDNAs encoding full-length or C-terminal domain (amino acid: 483 to 589) of mGBP2 or PA binding domain of Nir2 (amino acid: 911 to 1,244) were inserted into pGEX4T-3 vector (Cytiva). Site-directed mutagenesis to construct K585A of mGBP2 and truncation of N-terminal (amino acid: 1 to 303), middle (amino acid: 304 to 482), or C-terminal (amino acid: 483 to 589) domain of mGBP2 were conducted by inverse PCR. GFP-Rab1B was used as described previously (31).

Antibodies. Anti-FLAG rabbit polyclonal antibody, Sigma, Cat# F7425
Anti-GFP rabbit polyclonal antibody, Thermo Fisher Scientific, Cat# A-6455
Anti-GAPDH mouse monoclonal antibody, BioDesign, Cat# H86504M
Anti-GST rabbit polyclonal antibody, Santa Cruz Biotechnology, Cat# sc-549
Anti-hGBP1 rabbit polyclonal antibody, Proteintech Cat# 15303-1AP

Anti-hGBP2 rabbit polyclonal antibody, Proteintech Cat# 11854-1AP
Anti-*Legionella* rabbit antiserum, (see "production of anti-*Legionella* antiserum")

Anti-*Legionella* mouse antiserum, (see "production of anti-*Legionella* antiserum")
Peroxidase-conjugated goat anti-rabbit antibody, Bio-Rad, Cat# 170-6515
Peroxidase-conjugated goat anti-mouse antibody, Bio-Rad, Cat# 170-6516
Alexa Fluor 488-conjugated goat anti-rabbit antibody, Thermo Fisher Scientific, Cat# A11034

Alexa Fluor 594-conjugated goat anti-rabbit antibody, Thermo Fisher Scientific, Cat# A11037

Alexa Fluor 594-conjugated goat anti-mouse antibody, Thermo Fisher Scientific, Cat# A11032

Cell Culture, Transfection and IFN- γ Treatment. HeLa cells stably expressing Fc γ RII (HeLa-Fc γ RII cells) were maintained as described previously (15, 49–52). Plasmid transfection into the cells was performed using polyethylenimine (Polysciences; 24765). Then, 1 μ g of plasmid was used for transfection. For induction of endogenous hGBP1 or hGBP2 expression, recombinant human IFN- γ was purchased from R&D systems (285-IF). THP-1 cells were maintained with RPMI-1640 medium supplemented with 10% fetal calf serum and 50 IU/mL penicillin–50 μ g/mL streptomycin, at 37 °C under a 5% CO₂ atmosphere. THP-1 cells were differentiated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA, Wako Chemical; 162-23591) for 3 d and rested for 2 d by replacing the differentiated medium without PMA. Transfection of siRNA was performed using lipofectamine RNAiMax (Thermo Fisher Scientific; 13778150) according to the manufacturer's protocol before *L. pneumophila* infection 72 h and the cells were stimulated for 24 h before infection in complete medium with 1.0 ng/mL human IFN- γ was purchased from R&D systems (285-IF).

Preparation of *L. pneumophila*-Infected Cells. HeLa-Fc γ RII cells were seeded at a density of 1 $\times$ 10⁶ cells per well of a six-well plate on a previous day of infection or transfection. *L. pneumophila* opsonized with rabbit (dilution: 1/3,000) or mouse (dilution: 1/1,000) anti-*L. pneumophila* antisera was used for infection of the cells at the required multiplicity of infection (MOI). At 10 min after infection, the plate was centrifuged at 200 $\times$ g for 5 min at room temperature (Eppendorf, centrifuge 5430). After centrifugation, the plate was placed in a CO₂ incubator for the required infection time, and the cells were extensively washed with phosphate-buffered saline prior to processing. THP-1-derived macrophages were seeded at a density of 1 $\times$ 10⁶ cells per well of a 6-well plate on a previous day of infection. The cells were infected with *L. pneumophila* at the MOI of 50.

Immunofluorescence. Cells were fixed with 4% paraformaldehyde/phosphate-buffered saline (PBS) for 30 min at room temperature. In the imaging analyses, distinction of extracellular and intracellular *L. pneumophila* was performed as described previously (15, 50–52). The KEYENCE BZ-X810 microscope equipped with a 100 $\times$ oil-immersion lens (NA 1.45) was used to obtain images. Microscopic observations were performed at room temperature.

Protein Purification. Affinity purification of GST-tagged proteins was conducted as described (53). Proteins were dialyzed in buffer containing 100 mM NaCl, 1 mM MgCl₂, and 20 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES)-KOH (pH 7.2).

PIP strip and liposome floatation assay. PIP strip (P-6002; Echelon) was performed using 1 μ g/mL GST proteins according to the manufacturer's protocol. For liposome floating assay, lipids were purchased from Echelon (1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC); L-1182, 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE); L-2182, 1,2-Dipalmitoyl-sn-glycero-3-phosphatidic acid (DPPA); L-4116, 16:0 CL; L-C160) and Avanti (1,2-dioleoyl-sn-glycero-3-phosphatidic acid (DOPA); 840875P). The lipids were mixed indicated ratio and dried at 100 °C for 10 min using a heat block. The lipid film was resuspended at a concentration of 10 mM in 100 mM NaCl and 20 mM

MES (pH 7.0), vortexed for 1 h, and subjected to freeze–thaw cycle five times using liquid nitrogen and a 42 °C hot-water bath. Unilamellar liposomes were generated via extrusion through a nanopore membrane with pore size of 10 μ m. This process was repeated 21 times. Then, 5 mM liposomes were mixed with indicated GST-proteins (0.5 μ g) and incubated at room temperature with gentle mixing for 1 h. The GST-proteins–liposome mixture was diluted in 2 M sucrose/20 mM MES (pH 7.0) and placed at the bottom of Ultra-Clear Centrifuge Tubes (BECKMAN COULTER; 344057). Then, we overlaid 2.9 mL of 1.25 M sucrose/20 mM MES (pH 7.0) and 0.85 mL of 0.25 M sucrose/20 mM MES (pH 7.0). The sucrose gradient was centrifuged at 287,000 $\times$ g at 4 °C for 2 h. After centrifugation, the sucrose gradient was fractionated from the top in volumes of 0.5 mL, 0.75 mL, 1.75 mL, 1.75 mL, and 0.25 mL. Equal volume of each fraction analyzed sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Immunoblotting. Quantification of samples was conducted as described previously (54).

SDS-PAGE, Western Blotting, Immunoblotting, and Antibodies. Samples were loaded onto 12% polyacrylamide gels, and electrophoresis was conducted at 180 V for 60 min. After electrophoresis, proteins were transferred onto polyvinylidene fluoride membranes (Merck Millipore, IPVH00010) at 400 mA for 25 min. The membranes were blocked with 2% bovine serum albumin (Wako, 017-15124) or 5% skim milk in tris buffered saline-tween 20 (TBS-T) buffer (20 mM Tris-HCl, 137 mM NaCl and 0.1% Tween-20) for 1 h at room temperature and then incubated with primary antibodies. After incubation, the membranes were washed with TBS-T buffer and incubated with secondary antibodies conjugated to horseradish peroxidase for 1 h at room temperature. The horseradish peroxidase signal on the membranes was detected by enhanced chemiluminescence (Merck Millipore, WBLUC0500). Images of immunoblots were obtained using LumiVision Pro image analyzer (TAITEC AISIN). The quantification of immunoblots was conducted using ImageJ/Fiji software.

Quantification and Statistical Analysis. GraphPad Prism version 10 was used to draw all graphs and conduct all statistical analyses.

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

ACKNOWLEDGMENTS. We thank Mr. Taketo Hirasawa for technical assistance. This work was supported in part by Grant-in-Aid for Scientific Research, #18H02656, #20H05772 (to K.A.), #23H02716/23K27407 (to H.N.), #22H02867/23K241299, #25K02493 (to T.K.), and #25K02494 (to I.N.); Grant-in-Aid for Transformative Research Area (B) (Establishment of PLAMP as a new concept to determine self and nonself for obligatory intracellular pathogens; 20B304) (to M.Y.); Fusion Oriented Research for Disruptive Science and Technology (JPMJFR206D), Moonshot research & development (JPMJMS2025) from Japan Science and Technology Agency (to M.Y.); the Research Program on Emerging and Re-emerging Infectious Diseases (JP23fk0108682) from the Agency for Medical Research and Development (to M.Y.); and The Naito Foundation (to H.N. and M.S.) and The Takeda Science Foundation (T.K.).

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