Preventing bacterial DNA release and absent in melanoma 2 inflammasome activation by a *Legionella* effector functioning in membrane trafficking

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***Legionella pneumophila*, the causative agent of Legionnaires' pneumonia, resides in a distinct vacuole structure called *Legionella*-containing vacuole (LCV). The LCV resists fusion with the lysosome and permits efficient bacterial replication in host macrophages, which requires a Dot/Icm type IVB secretion system. Dot/Icm-translocated effector SdhA is critical for *L. pneumophila* intracellular growth and functions to prevent host cell death. Here, we show that the absence of SdhA resulted in elevated caspase-1 activation and IL-1β secretion as well as macrophage pyroptosis during *Legionella* infection. These inflammasome activation phenotypes were independent of the established flagellin-NAIP5-NLRC4 axis, but relied on the DNA-sensing AIM2 inflammasome. We further demonstrate that *Legionella* DNA was released into macrophage cytosol, and this effect was significantly exaggerated by the absence of SdhA. SdhA bears a functional Golgi-targeting GRIP domain that is required for preventing AIM2 inflammasome activation. Ectopically expressed SdhA formed a unique ring-shape membrane structure, further indicating a role in membrane trafficking and maintaining LCV membrane integrity. Our data together suggest a possible link, mediated by the function of SdhA, between LCV trafficking/maturation and suppression of host innate immune detection.**

Gram-negative *Legionella pneumophila*, the causative agent of Legionnaires' disease, infects and replicates within alveolar macrophages. *L. pneumophila* resides in a distinct vacuole structure called *Legionella*-containing vacuole (LCV). Dynamic and extensive engagement with host membrane transport system diverts LCV trafficking from normal eukaryotic endocytic pathway and, thereby, results in resistance to fusion with the lysosome (1), a process that requires a Dot/Icm type IVB secretion system (2, 3). The primitive function of the Dot/Icm system is to transfer DNA by bacterial conjugation (4), but its predominant role in infection is to translocate protein substrates across the LCV membrane into host cells. A prevalent function for known Dot/Icm effectors is to subvert eukaryotic vesicular trafficking (1, 5, 6). A large number of >250 Dot/Icm effectors are experimentally confirmed (7). Few Dot/Icm effectors are genetically required for LCV avoidance of lysosomal fusion, suggesting a functional redundancy. However, the Dot/Icm system is critical for establishment of the LCV. The LCV serves as an intracellular niche, where *L. pneumophila* can efficiently replicate, as has been observed in human U937 monocytes (8).

The inflammasome pathway, generally mediated by cytosolic NOD-like receptors (NLRs), is a critical component in macrophage innate immunity (9). Similar to the Toll-like receptor pathway that senses pathogen-associated molecular patterns (PAMPs), inflammasome also responds to microbial products for counteracting infection. Inflammasomes are large cytoplasmic complexes that induce activation of downstream inflammatory caspases, mainly caspase-1. Caspase-1 activation leads to maturation and secretion of IL-1β and IL-18 as well as macrophage pyroptosis. NAIP5, a mouse NLR protein, functions as a receptor for bacterial flagellin and forms an inflammasome complex with NLRC4 and the ASC adaptor (10, 11). Upon infection, *L. pneumophila* flagellin triggers potent NAIP5-dependent caspase-1 activation and pyroptosis in C57/BL6-derived macrophages that are nonpermissive for *L. pneumophila* growth. Both interference with host vesicular trafficking and modulation of the innate immune response are crucial for Dot/Icm-dependent *L. pneumophila* intracellular survival/replication, but it is not known whether the two pathogenic aspects of *L. pneumophila* are functionally connected.

Different from the NAIP family of inflammasome receptors (11), the non-NLR protein AIM2 recognizes cytosolic double-strand DNA (dsDNA), such as that from intracellular bacteria, and forms an active inflammasome complex with ASC (12–15). Here, we discover that SdhA, the sole Dot/Icm effector whose mutation leads to severe *L. pneumophila* growth defects in macrophages (16), negatively regulates AIM2 inflammasome activation during infection. *L. pneumophila* Δ*sdhA*-stimulated AIM2 activation correlates with much-elevated bacterial DNA release from the Δ*sdhA* mutant into macrophage cytosol. Further characterization of SdhA function suggests that SdhA is intimately linked to the membrane trafficking system, including the presence of a functionally important Golgi-targeting GRIP domain. Thus, the function of SdhA represents a possible link between LCV trafficking/maturation and suppression of inflammasome-mediated innate immune detection.

Results

***L. pneumophila* ΔsdhA Infection Triggers Pyroptosis and Caspase-1 Activation in Human Macrophage Cells.** Dot/Icm-translocated SdhA has been shown to function to prevent macrophage death (16). To investigate the mechanism underlying SdhA function, PMA-differentiated U937 human macrophage cells were infected with *L. pneumophila* strain proficient (Lp02) or deficient in Dot/Icm-mediated secretion (Δ*dotA*, Lp03) or the isogenic Δ*sdhA* strain. By 5 h of infection, U937 cells infected with Δ*sdhA* exhibited evident osmotic pressure-induced cell swelling and nuclear condensation (Fig. 1*A*), two typical morphological features of macrophage pyroptosis. In contrast, cells infected with Lp02 and Lp03 strains underwent little such morphological changes. Trypan blue staining further showed extensive nuclear staining signals in cells infected with *L. pneumophila* Δ*sdhA* but not in Lp02 and Lp03 infected cells (Fig. 1*B*). Δ*sdhA*-triggered loss of cell membrane integrity was confirmed by massive releases of lactose dehydrogenase (LDH) and high-mobility group protein B1 (HMGB1) (Fig. 1 *C* and *D*). Quantification of LDH release further indicated that nearly 50% of Δ*sdhA*-infected cells underwent pyroptosis within 5 h of infection, whereas the

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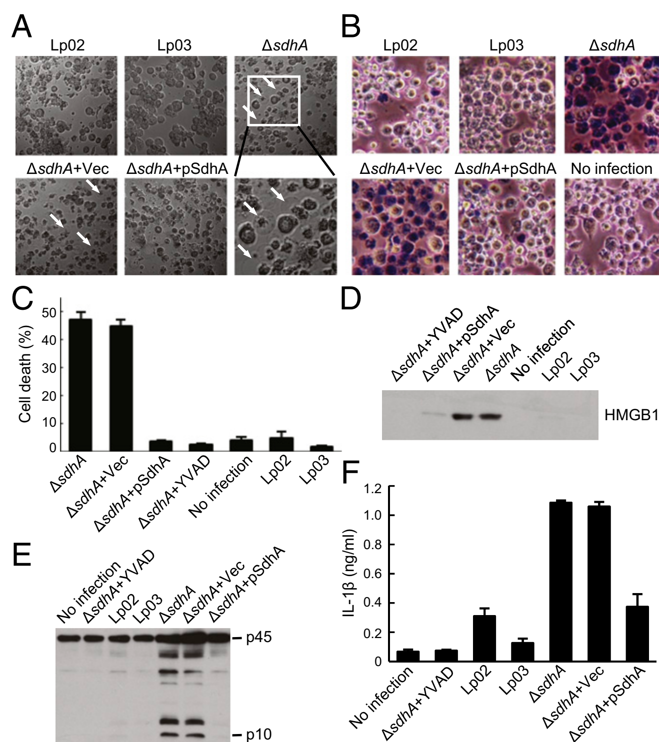

Fig. 1. Deletion of *sdhA* triggers pyroptosis and caspase-1 activation in U937 cells. (*A* and *B*) Morphological examination of effects of *sdhA* deletion during *L. pneumophila* infection of PMA-differentiated U937 cells (MOI = 10). Lp02 is used as the wild-type strain; Lp03 is a *dotA* mutant. pSdhA is a complementation plasmid expressing SdhA. Differential interference contrast images (5 h after infection) are shown with cells stained with Trypan blue in *B*. Arrows indicate pyroptotic cells in *A*. (*C* and *D*) Lactate dehydrogenase (LDH) (*C*) and HMGB1 (*D*) release assays of effects of *sdhA* deletion. Shown in *C* are percentages of cell death as mean values ± SD (error bars) from four independent experiments. Anti-HMGB1 immunoblot of culture media is shown in *D*. YVAD, a caspase-1 inhibitor. (*E* and *F*) Caspase-1 activation and IL-1β release assays of *sdhA* deletion. Shown in *E* is anti–caspase-1 immunoblot of culture supernatants. p45, procaspase-1; p10, the processed mature form of caspase-1. IL-1β ELISA data shown in *F* are as mean values ± SD (error bars) from three independent experiments.

percentages of cell death observed with Lp02 and Lp03 infections were comparable to that in uninfected cells (Fig. 1*B*).

Consistent with the morphological feature of inflammasome activation, robust caspase-1 activation was detected in the culture supernatant of Δ*sdhA*-infected U937 macrophages, but not in that of uninfected or Lp02/Lp03-infected macrophages (Fig. 1*E*). Δ*sdhA*-induced caspase-1 activation and macrophage pyroptosis were completely diminished by YVAD, a caspase-1 specific inhibitor (Fig. 1 *C* and *E*). Furthermore, compared with Lp02 and Lp03 infection, infection of U937 cells with *L. pneumophila* Δ*sdhA* resulted in secretion of a much larger amount of mature IL-1β, which was also sensitive to YVAD pretreatment (Fig. 1*F*).

We then tested whether loss of SdhA is responsible for the above observed increased inflammasome activation. An SdhA-expressing plasmid was found to be able to completely reverse Δ*sdhA*-induced morphological changes (Fig. 1 *A* and *B*), LDH and HMGB1 release (Fig. 1 *C* and *D*), caspase-1 activation (Fig. 1*E*), and IL-1β production (Fig. 1*F*), whereas a control empty vector showed so such rescue effects. These analyses clearly establish that SdhA prevents human macrophage cell death by blocking inflammasome activation.

Flagellin Is Dispensable for ΔsdhA-Induced Inflammasome Activation. Cytosolic flagellin triggers strong caspase-1 activation during *L. pneumophila* infection (17–19). To test whether SdhA prevents or negatively regulates flagellin-induced inflammasome activation, flagellin-deficient (Δ*flaA*) and SdhA/flagellin (Δ*flaA*Δ*sdhA*) double deletion strains were constructed and assayed for inflammasome activation. In human U937 macrophages, Δ*flaA*Δ*sdhA* induced significantly higher levels of LDH release (Fig. 2*A*), caspase-1 activation (Fig. 2*B*), and IL-1β maturation (Fig. 2*C*) than the background Δ*flaA* strain, which could be fully rescued by plasmid-encoded SdhA. Flagellin-independent inflammasome activation observed with Δ*sdhA* infection agrees with our recent discovery that human macrophages lack the flagellin-sensing NAIP5 inflammasome receptor (11). Consistently, deletion of flagellin from wild-type *L. pneumophila* also did not alter caspase-1 activation in U937 cells (compare Δ*flaA* with Lp02 in Fig. 2 *A*–*C*).

Different from human cells, mouse macrophages express the NAIP5 receptor and are responsive to *Legionella* flagellin stimulation of caspase-1 activation. Several types of mouse macrophages, including primary peritoneal macrophage (PM) and primary/immortalized bone marrow-derived macrophage (BMM), were then assayed for the inhibitory effects of SdhA on caspase-1 activation. Consistent with that observed in U937 macrophages, ablation of *sdhA* from flagellin-deficient *L. pneumophila* could still lead to elevated caspase-1 activation and, to a lesser extent, IL-1β production (Fig. 2 *D* and *E*). These results suggest that the function of SdhA in inhibiting caspase-1 activation is flagellin-independent and SdhA likely blocks inflammasome activation triggered by other *Legionella*-derived PAMPs.

ΔsdhA-Induced Caspase-1 Activation and Pyroptosis Require AIM2 and ASC. To determine Δ*sdhA*-induced inflammasome activation, primary peritoneal macrophages deficient in various known inflammasome components including NLRP3, NLRC4, and ASC were subjected to infection with Δ*sdhA* strain. As shown in Fig. 3*A*, $Nlrp3^{-/-}$ and $Nlrc4^{-/-}$ macrophages behaved similarly as wild-type macrophages and showed increased caspase-1 activation in response to *sdhA* deletion. In contrast, no caspase-1 activation was detected in $Asc^{-/-}$ macrophages infected with either Δ*flaA* or Δ*sdhA*Δ*flaA* strains (Fig. 3*A*). ASC often aggregates to form a single speck-like large structure called pyroptosome that functions to mediate caspase-1 autoprocessing (20). We also observed ASC pyroptosome formation in ≈25% of U937 cells infected with Δ*sdhA*, but not in cells infected with Lp02, Lp03, or the Δ*flaA* strain (Fig. 3*B*).

Among known inflammasome complexes, ASC is strictly required for NLRP3 and AIM2 mediated caspase-1 activation due to pyrin–pyrin interaction between NLRP3/AIM2 and ASC. The insensitivity of Δ*sdhA*-induced caspase-1 activation to *Nlrp3* deficiency directed our focus to AIM2. In fact, small interference RNA (siRNA) knockdown of AIM2 expression in 129S mice-derived BMMs (Fig. 3*C*) could largely diminish Δ*sdhA*-induced caspase-1 activation and IL-1β production (Fig. 3 *D* and *E*). Knockdown of AIM2 abolished poly(dA-dT)-induced caspase-1 activation, but not that by lipopolysaccharide (LPS) plus ATP (Fig. 3*E*), verifying the functional specificity of the siRNA oligoes used. The requirement of AIM2 for Δ*sdhA*-induced caspase-1 activation was further confirmed in primary BMMs derived from *Aim2* knockout C57/BL6 mice (Fig. 3*F*). Caspase-1 activation in C57/BL6-derived primary BMMs, compared with that in 129S-derived BMMs, appeared to be less responsive to Δ*sdhA* infection likely due to the strain difference. Taken together, our data suggest that elevated caspase-1 activation induced by *L. pneumophila* Δ*sdhA* infection is a result of AIM2 inflammasome activation.

Bacterial DNA Is Released into Macrophage Cytosol, which Is Enhanced by the Absence of sdhA. We then examined whether SdhA could directly target the AIM2–ASC–caspase-1 signaling axis. Coimmunoprecipitation of AIM2 and ASC in the ectopic expression system was not affected by SdhA (Fig. 4*A*). The AIM2/ASC complex produced in 293T cells could induce IL-1β maturation in the presence of caspase-1, but this effect was also

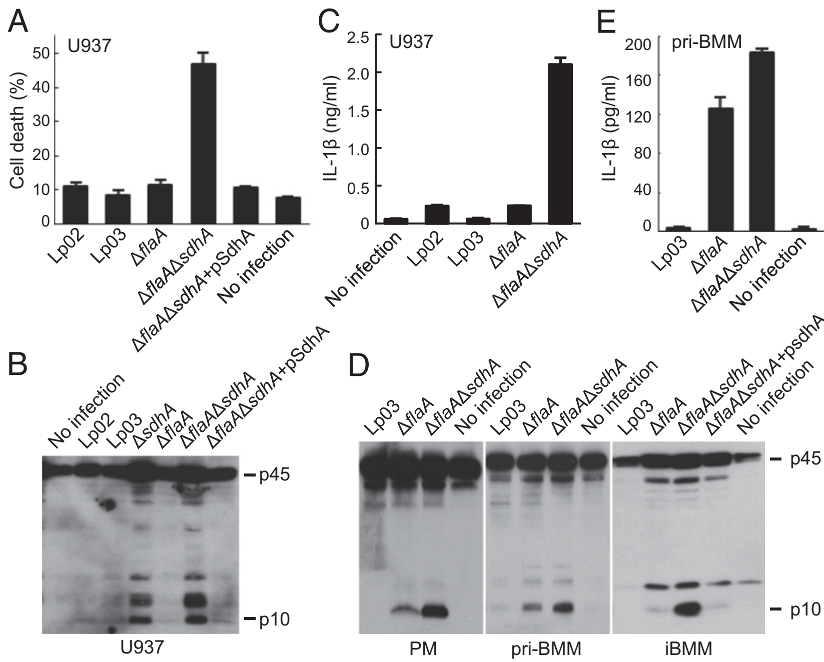

Fig. 2. Δ*sdhA*-induced inflammasome activation is flagellin-independent. U937 cells were used for infection in *A*–*C* and mouse macrophages were assayed in *D* and *E*. LDH release in *A* shows percentages of cell death as mean values ± SD (error bars) from four independent experiments. Shown in *B* and *D* are anti–caspase-1 immunoblots of culture supernatants. IL-1β ELISA data shown in *C* and *E* are mean values ± SD (error bars) from three independent experiments. iBMM, immortalized bone marrow macrophages (C57/BL6 background); PM, peritoneal macrophages (129S background); pri-BMM, primary bone marrow macrophages (129S background). Culture supernatants collected from 2 to 6 h after infection were subjected to analyses in *D* and *E*.

not subjected to inhibition by SdhA (Fig. 4*B*). Because SdhA is unlikely to be a direct inhibitor of the AIM2 inflammasome complex, we then hypothesized that loss of SdhA might lead to an increased bacterial DNA release into macrophage cytosol. To this end, an ampicillin-resistant *Escherichia coli*/*Legionella* shuttle plasmid (pJB908) was transformed into SdhA-positive or -negative strains. After 4 h of infection of U937 cells or mouse BMMs, macrophages were lysed in a hypotonic buffer and cytosolic DNA containing the released plasmid was extracted. Before DNA extraction, macrophage cytosol was supplemented with a kanamycin-resistant plasmid (pEGFP-N1) that served as an internal control for subsequent experimental procedures. The extracted DNA was subjected to both quantitative real-time PCR (qRT-PCR) analysis and a colony-counting assay after *E. coli* transformation. The ratio of pJB908 quantification to pEGFP-N1 quantification calculated from both assays clearly showed that *Legionella* DNA was released into macrophage cytosol and deletion of *sdhA* resulted in a significant enhancement of such effects (Fig. 4 *C* and *D*). The DNA release pattern for the different *L. pneumophila* strains assayed correlated with their profiles of inducing caspase-1 activation and IL-1β release (Fig. 2 *C* and *D*). Thus, SdhA blocks AIM2 inflammasome-mediated innate immune response by preventing bacterial DNA release into the cytosol of infected macrophages. The pJB908 reporter

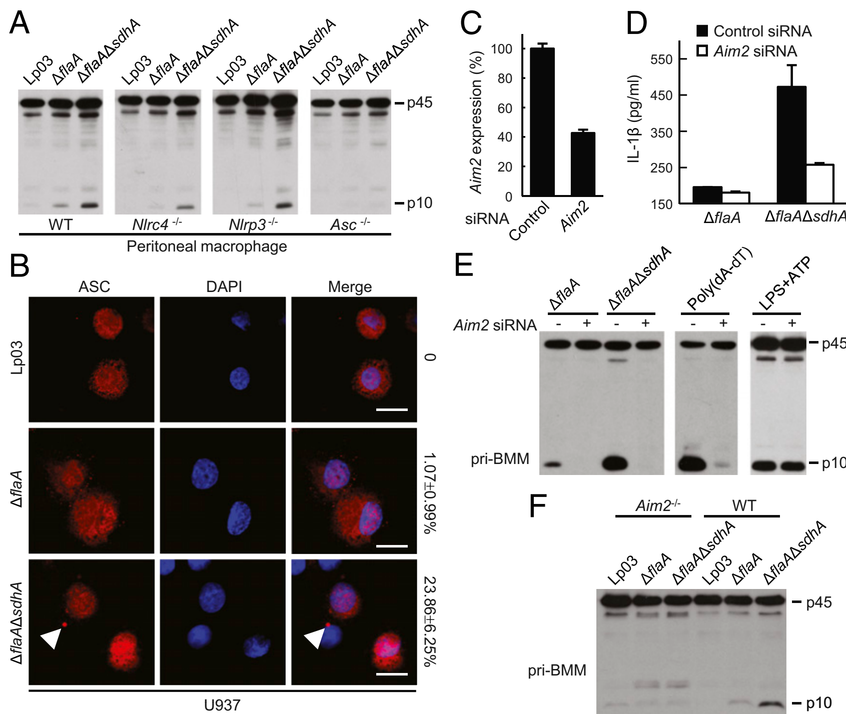

Fig. 3. Δ*sdhA*-induced inflammasome activation requires *Aim2* and *Asc*. (*A*) Δ*sdhA*-induced caspase-1 activation in *Nlrc4*$^{-/-}$, *Nlrp3*$^{-/-}$, and *Asc*$^{-/-}$ macrophages. Peritoneal macrophages prepared from C57/BL6 (WT) or indicated knockout mice were infected with indicated *L. pneumophila* strains. (*B*) ASC foci formation in U937 cells infected with *sdhA*$^{+}$ or *sdhA*$^{-}$ strains (MOI = 10). Statistics of percentages of cells showing the ASC pyroptosome (arrowhead) are listed on the right to the merged confocal images. (Scale bars: 7.5 μm.) (*C*–*E*) Effects of *Aim2* knockdown on Δ*sdhA*-induced inflammasome activation. BMMs from 129S mice were transfected with *Aim2* targeting or a control siRNA before indicated infections. qRT-PCR measurements of knockdown efficiency (*C*) are shown as mean values ± SEM (error bars) from three independent experiments. The supernatants were subjected to IL-1β ELISA (*D*) or anti–caspase-1 immunoblotting (*E*). Mean values ± SEM (error bars) from two independent experiments are shown in *D*. (*F*) Δ*sdhA*-induced caspase-1 activation in *Aim2*$^{-/-}$ macrophages. Peritoneal macrophages prepared from C57/BL6 (WT) or *Aim2*$^{-/-}$ mice were infected with indicated *L. pneumophila* strains.

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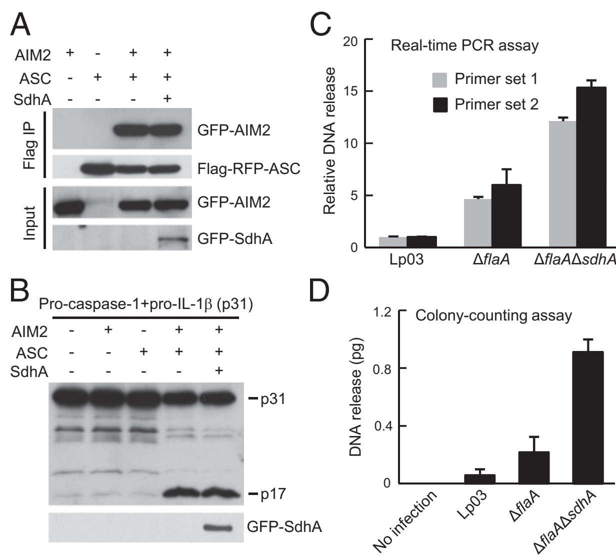

Fig. 4. SdhA inhibits *Legionella* DNA release into macrophage cytosol. (*A* and *B*) SdhA does not directly target the AIM2–ASC–caspase-1 pathway. 293T cells transfected with indicated plasmid combination were subjected to anti-Flag immunoprecipitation and/or immunoblotting. p17, the processed mature form of IL-1β. (*C* and *D*) Bacterial DNA release into macrophage cytosol during infection and effects of *sdhA* deletion. PMA-differentiated U937 cells were infected with indicated *L. pneumophila* strains harboring a reporter plasmid. DNA fractions extracted from infected macrophage cytosol were subjected to qRT-PCR analysis (*C*) or colony-counting assay (*D*) to measure relative plasmid DNA release (detailed in *SI Materials and Methods*). Mean values ± SEM (error bars) from three independent experiments are shown.

plasmid used here lacks the oriT origin and was defective for Dot/Icm-mediated conjugal transfer (4), regardless of the presence and absence of *sdhA* (Table S1). This result suggests that increased DNA release induced by *ΔsdhA* is independent of Dot/Icm-mediated plasmid conjugation.

L. pneumophila infection also potently activates IRF3-dependent type I IFN response, which plays a role in restricting *Legionella* intracellular growth (21, 22). A genetic screen also identifies SdhA as a suppressor of *L. pneumophila*-stimulated type I IFN response (23). Our model of SdhA function is consistent with these observations because cytosolic bacterial DNA is known to be a potent stimulator of type I IFN response. It has been debated whether *Legionella* DNA or RNA is responsible for activation of the type I IFN response (23, 24). Our data indicate that at least bacterial DNA is released into host cytosol and exposed to host innate immune receptors.

ΔsdhA-Induced AIM2 Inflammasome Activation Is Independent of IFN and TNFα Signaling. *Francisella tularensis* also releases its DNA into host cytosol and triggers both type I IFN response and AIM2-dependent caspase-1 activation (25). Full inflammasome activation requires *Francisella* DNA-stimulated type I IFN signaling (26, 27). We then examined whether such requirement also applies to *L. pneumophila* Δ*sdhA*-induced inflammasome activation. To this end, $Ifnar^{-/-}$, $Ifngr^{-/-}$, $Irf3^{-/-}$, $Irf7^{-/-}$, or $Irf3^{-/-}Irf7^{-/-}$ peritoneal macrophages that are deficient in either type I or type II IFN response were infected with $sdhA^{+}$ or $sdhA^{-}$ strains. Similarly to that observed in wild-type macrophages, deletion of *sdhA* still resulted in elevated caspase-1 activation in all these IFN signaling-deficient macrophages (Fig. S1*A*). Moreover, pretreatment of mouse BMMs or U937 cells with IFN-β did not vary the extent of Δ*sdhA*-induced caspase-1 activation (Fig. S1*B*). Thus, *L. pneumophila* differs from *F. tularensis* and induces AIM2 activation independently of the type I IFN response.

Bacterial infection often triggers production of the proinflammatory cytokine TNFα that widely regulates macrophage innate immunity. We also observed that Δ*sdhA*-induced caspase-1 activation remained unchanged in $Tnfrsf1a^{-/-}Tnfrsf1b^{-/-}$ macrophages (Fig. S1*C*). Moreover, TNFα pretreatment of U937 cells had little effects on caspase-1 activation in response to Δ*sdhA* infection (Fig. S1*C*). These data suggest that TNFα signaling is not involved in *L. pneumophila* Δ*sdhA*-induced AIM2 inflammasome activation.

SdhA Is Targeted to the Endomembrane Structure and Membrane Targeting Is Required for SdhA Function in Preventing Inflammasome Activation. SdhA contains 1,429 amino acids and sequence homology analysis revealed a C-terminal GRIP domain (residues 1341–1390) (Fig. 5 *A* and *B*). The GRIP domain of ≈50 residues is present in a group of large coiled-coil membrane proteins, usually at their carboxyl termini (28, 29). The GRIP domain, such as that from many golgins, is necessary and sufficient for Golgi targeting (30). Consistently, the GRIP domain of SdhA was found to be exclusively localized to the Golgi apparatus in mammalian cells as indicated by immunostaining of a Golgi marker GM130 (Fig. 5*C*). Notably, a GRIP domain deletion mutant of SdhA (SdhAΔGRIP) failed to reverse Δ*sdhA*-induced caspase-1 hyperactivation (Fig. 5*D*) despite the mutant was expressed at a higher level than endogenous SdhA (Fig. S2). (A SidC translocation signal was added to SdhAΔGRIP for Dot/Icm-dependent translocation.)

Interestingly, we also observed that GFP-SdhA formed distinct ring-shape membrane structures with a diameter ≈0.5–2 μm in HeLa cells (Fig. 6*A*). These membrane structures did not adopt *cis*- (GM130) and *trans*-Golgi (TGN38) markers (Fig. 6*B*) and were not sensitive to Golgi-disassembling drugs such as brefeldin A, colchicine, and nocodazole (Fig. S3). The SdhA ring-shape structures were also negative for a panel of subcellular organelle markers including GFP-PTS1 for peroxisome (Fig. S4*A*), VDAC1, cytochrome *c*, and Mitotracker for mitochondria (Fig. S4 *B–D*), Lysotracker and LAMP1 for lysosome (Fig. S5), calreticulin for endoplasmic reticulum (Fig. S6*A*), Rab5 and EEA1 for early endosome (Fig. S6 *B* and *C*), and Rab7 for late endosome (Fig. S6*D*). Disruption of lysosome biogenesis by bafilomycin A1, a vacuolar-type H^{+}-ATPase inhibitor, had no effects on SdhA formation of the ring-shape structure (Fig. S5*A*). This membrane structure formed by N-terminal Flag-tagged SdhA-GFP could be visualized by anti-Flag immunofluorescence staining even when digitonin was used for cell permeabilization (Fig. S7*A*). This result suggests that the amino terminus of SdhA is likely exposed to the cytosol because digitonin was known to be incapable of permeabilizing the endomembrane (Fig. S7*B*). These extensive analyses, although not revealing for the identity of SdhA-marked membrane structures, provide another support to the potential function of SdhA in membrane trafficking.

Truncation analyses were further performed to map the regions in SdhA that are required for formation of the ring-shape structure. Three SdhA truncation mutants with deletions of various amino-terminal regions (SdhA_A, SdhA_B, and SdhA_C) lost the ability to form the unique ring-shape structure and became dispersed in the cytoplasm (Fig. 6 *C* and *D*). All three mutants were expressed at a much higher level than endogenous SdhA (Fig. S2) but failed to reverse Δ*sdhA*-induced pyroptosis and caspas-1 activation in infected U937 cells (Fig. 6 *E* and *F*). Similar results were obtained in mouse BMM cells (Fig. S8). Consistent with our proposed role of SdhA in preventing bacterial release into macrophage cytosol, the relative amount of bacterial DNA released from the three mutants-complemented strains was at a similarly higher level as that from the Δ*sdhA* deletion strain (Fig. S9). These data, together with the functional requirement of the Golgi-targeting GRIP domain, indicate that Dot/Icm-translocated SdhA likely targets the membrane system to prevent bacterial DNA release and activation of AIM2 inflammasome, which is possibly through maintaining LCV membrane integrity.

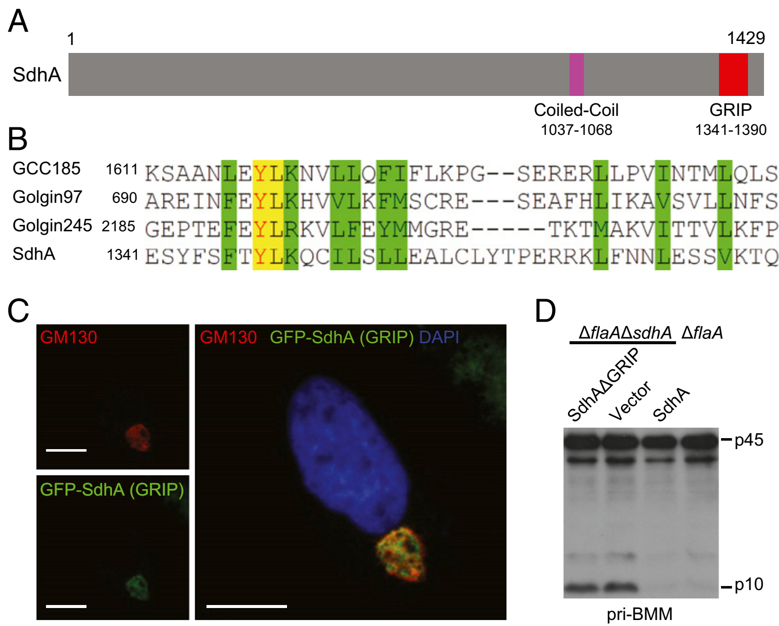

Fig. 5. SdhA harbors a Golgi-targeting GRIP domain that is required for inhibiting inflammasome. (*A*) The domain structure of SdhA. (*B*) Sequence alignment of residues 1341–1381 in SdhA with GRIP domains from several mammalian golgins. Similar residues are highlighted in green, and identical ones are in yellow. (*C*) SdhA GRIP domain localization in HeLa cells. The Golgi was visualized by GM130 staining. Representative confocal immunofluorescence images are shown. (Scale bars: 7.5 μm.) (*D*) Complementation of Δ*flaA*Δ*sdhA* strain by plasmid-encoded SdhA or the SdhAΔGRIP mutant. BMMs from 129S mice were infected with *L. pneumophila* deletion or rescue strains as indicated. Caspase-1 immunoblot of culture supernatant is shown.

Discussion

In this study, we show that *L. pneumophila* triggers inflammasome activation in human monocyte-derived macrophages. Consistent with the absence of NAIP5 inflammasome receptor for bacterial flagellin in human system (11), we identify the DNA-sensing AIM2 inflammasome that is responsible for *L. pneumophila*-induced caspase-1 activation in human macrophages. *Legionella* activation of AIM2 also occurs in mouse macrophages, but is relatively hysteretic compared with the more predominant activation of the NAIP5 inflammasome by flagellin. The difference between the human and mouse inflammasome system might explain the long known permissiveness to *L. pneumophila* intracellular replication observed with U937 cells, but not mouse macrophages that harbor wild-type *Naip5*. We further provide direct evidences that *L. pneumophila* DNA leaks out of the bacteria into macrophage cytosol, which can also explain the previously observed induction of the type I IFN response (21–23). Release of bacterial DNA to trigger AIM2 inflammasome activation and type I IFN response has been observed with other bacteria such as *F. tularensis* and *L. monocytogenes* (27, 31). Thus, DNA derived from intracellular bacteria is likely a general PAMP for host innate immune system. Meanwhile, host-derived DNA, particularly mitochondrial DNA, may also possibly contribute to *L. pneumophila* stimulation of the innate immune response. In fact, mitochondria are extensively

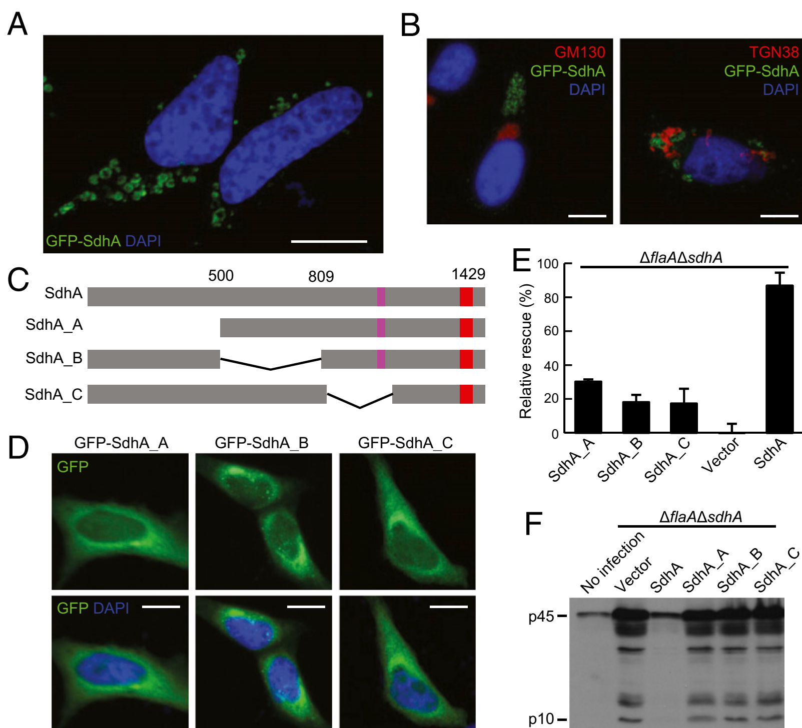

Fig. 6. SdhA forms a distinct ring-shape membrane structure and mutants deficient in ring formation fail to complement Δ*sdhA* strain. (*A* and *B*) GFP-SdhA forms a ring-shape membrane structure that is negative for Golgi markers. GM130 and TGN38 antibodies stain the Golgi structure. (Scale bars: 7.5 μm.) (*C*) A schematic drawing of SdhA truncation mutant assayed in (*D*–*F*). SdhA_A, Δ1–499aa; SdhA_B, Δ501–809aa; SdhA_C, Δ847–1104aa. (*D*) Localization of GFP-tagged SdhA truncation mutants in HeLa cells. (Scale bars: 7.5 μm.) (*E* and *F*) Complementation assays of SdhA truncation mutants. PMA-differentiated U937 cells were infected with Δ*flaA*Δ*sdhA* strain transformed with a plasmid expressing SdhA or the indicated truncation mutants. Cell death measured by LDH release was assayed in *E*, and rescue effects relative to vector-complemented strain are shown as mean values ± SD (error bars) from three independent experiments.

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recruited to the LCV and mitochondria disruption has been noted in Δ*sdhA*-infected macrophages (16).

SdhA is critically required for *L. pneumophila* growth in macrophages (16). Our genetic studies demonstrate that SdhA functions to prevent bacterial DNA release into macrophage cytosol. Loss of SdhA, therefore, results in much increased activation of AIM2 inflammasome and the type I IFN response observed (23). Caspase-1–mediated secretion of IL-1β /IL-18 trigger strong inflammatory responses that alert the immune system for pathogen clearance. Caspase-1–induced pyroptosis can also clear intracellular bacteria independently of cytokine secretion (32). Nuclear acid-activated type I IFN response has been established as a crucial immune defense mechanism to restrict intracellular bacteria including *L. pneumophila* (21, 33, 34). Thus, our discovery of SdhA function in preventing DNA release provides a possible mechanistic explanation for the severe growth defect observed with Δ*sdhA* mutant in macrophage hosts. Our observation may also explain that Δ*sdhA* mutant has no intracellular growth defects in amoebae hosts that do not have the nuclear acid-sensing innate immune system.

SdhA contains a functionally important GRIP domain that alone is targeted to the Golgi apparatus. Our extensive analyses establish that SdhA is most likely involved in membrane trafficking. This observation is not unexpected given that a large majority of Dot/Icm-secreted substrates appear to modulate the membrane system for appropriate LCV trafficking (6). A plausible model for SdhA function is that it is involved in a certain aspect of vesicular trafficking and thereby maintains LCV membrane integrity to prevent bacterial DNA from leaking out of the LCV. Supporting our model, a recent study that came out during the revision process of our manuscript provides direct evidences that LCV containing Δ*sdhA* mutant is unstable and, therefore, accessible to host cytosol (35). Despite that it is not known whether preventing DNA release by SdhA is a bacterial "intentional" virulence mechanism, the mode of SdhA action through maintaining the LCV membrane integrity represents another paradigm in bacterial effector-mediated counteraction of host innate immune defense (36).

Materials and Methods

L. pneumophila strains were cultured on buffered charcoal yeast extract agar plates supplemented with 0.1 mg/mL thymidine. *L. pneumophila* Δ*flaA*, Δ*sdhA*, and Δ*flaA*Δ*sdhA* strains were generated by standard homologous recombination using the suicide plasmid pSR47s. pJB908-based complementation plasmids were introduced into *L. pneumophila* by electroporation (2.5 Kv, 200 Ω, 25 μF, and 5 ms). The complementation stains were cultured on buffered charcoal yeast extract agar without thymidine. For macrophage infection, fresh single bacterial colonies were streaked onto culture plates 2 d before infection. Bacteria were scraped off, diluted in sterile water, and added to cells at a multiplicity of infection (MOI) of 10. Infection was facilitated by a centrifugation of 300 × *g* for 10 min. All infection assays were performed in the media without serum and antibiotics. Caspase-1 activation and cell death assays were performed as described (11, 37). The rest of information about plasmid, antibodies, mice, cell culture and siRNA transfection, DNA release, and immunofluorescence assays is presented in *SI Materials and Methods*.

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