Activation of Ran GTPase by a *Legionella* Effector Promotes Microtubule Polymerization, Pathogen Vacuole Motility and Infection

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Abstract

The causative agent of Legionnaires’ disease, *Legionella pneumophila*, uses the Icm/Dot type IV secretion system (T4SS) to form in phagocytes a distinct “*Legionella*-containing vacuole” (LCV), which intercepts endosomal and secretory vesicle trafficking. Proteomics revealed the presence of the small GTPase Ran and its effector RanBP1 on purified LCVs. Here we validate that Ran and RanBP1 localize to LCVs and promote intracellular growth of *L. pneumophila*. Moreover, the *L. pneumophila* protein LegG1, which contains putative RCC1 Ran guanine nucleotide exchange factor (GEF) domains, accumulates on LCVs in an Icm/Dot-dependent manner. *L. pneumophila* wild-type bacteria, but not strains lacking LegG1 or a functional Icm/Dot T4SS, activate Ran on LCVs, while purified LegG1 produces active Ran(GTP) in cell lysates. *L. pneumophila* lacking *legG1* is compromised for intracellular growth in macrophages and amoebae, yet is as cytotoxic as the wild-type strain. A downstream effect of LegG1 is to stabilize microtubules, as revealed by conventional and stimulated emission depletion (STED) fluorescence microscopy, subcellular fractionation and Western blot, or by microbial microinjection through the T3SS of a *Yersinia* strain lacking endogenous effectors. Real-time fluorescence imaging indicates that LCVs harboring wild-type *L. pneumophila* rapidly move along microtubules, while LCVs harboring *LegG1* mutant bacteria are stalled. Together, our results demonstrate that Ran activation and RanBP1 promote LCV formation, and the Icm/Dot substrate LegG1 functions as a bacterial Ran activator, which localizes to LCVs and promotes microtubule stabilization, LCV motility as well as intracellular replication of *L. pneumophila*.

Introduction

The amoebae-resistant environmental bacterium *Legionella pneumophila* is the causative agent of a severe pneumonia termed Legionnaires’ disease [1,2]. In free-living amoebae as well as in macrophages of the innate immune system, *L. pneumophila* employs an apparently conserved mechanism to form a replication-permissive membrane-bound compartment, the “*Legionella*-containing vacuole” (LCV) [3,4,5]. LCVs avoid fusion with bactericidal lysosomes, and instead interact in a bi-phasic process with early secretory vesicles budding from endoplasmic reticulum (ER) exit sites and with the ER. Microtubules play a role in the initial trafficking events of LCVs, prior to the acquisition of the early secretory vesicle marker GFP-HDEL and the resident ER marker calnexin-GFP [6]. A proteomics analysis of purified intact LCVs revealed more than 560 host proteins, including α- and β-tubulin, as well as a number of small GTPases and GTPase-interacting factors [7]. In addition to Arf1 and Rab GTPases implicated in the secretory and endosomal vesicle trafficking pathways, Ran and its effector Ran binding protein 1 (RanBP1) were identified in this study as LCV host components.

The small GTPase Ran is implicated in a variety of cellular processes, such as nuclear pore translocation [8], or mitotic spindle assembly and post-mitotic nuclear envelope formation [9,10]. Furthermore, Ran plays an important role in cytoplasmic events involving non-centrosomal microtubules, e.g. endocytic receptor trafficking and retrograde signaling along microtubules in nerve axons [11]. Ran can be activated by a nuclear (or in mitotic cells: chromatin-bound) Ran guanine nucleotide exchange factor (GEF) termed regulator of chromosome condensation 1 (RCC1) [12].
**Author Summary**

*Legionella pneumophila* is an environmental bacterium that grows within free-living amoebae and, upon inhalation, in human lung macrophages, thus causing the severe pneumonia Legionnaires’ disease. Within amoebae or macrophages the bacteria form a distinct membrane-bound replication niche, the “*Legionella*-containing vacuole” (LCV). To this end, *L. pneumophila* injects via a dedicated secretion apparatus about 300 different “effector” proteins directly into host cells, where they interfere with cellular processes. LCV formation is poorly understood, and the function and targets of most bacterial effector proteins are unknown. In this study, we characterize an *L. pneumophila* effector protein that activates the small host GTPase Ran, which is essential for crucial cellular processes, such as spindle assembly and cytokinesis, nucleo-cytoplasmic transport, as well as nuclear envelope formation. We discovered that Ran promotes intracellular replication of *L. pneumophila* and its activation on the LCV membrane by LegG1 causes the polymerization of microtubules, along which cellular vesicles as well as LCVs move within cells. Our study defines a novel strategy how pathogenic bacteria subvert host processes to promote intracellular survival and replication.

Ran(GTP) is inactivated by the cytoplasmic Ran GTase-activating protein 1 (RanGAP1) in concert with RanBP1 harboring a Ran(GTP)-binding domain [11].

The common mechanism of Ran activity involves sequestration of a transport complex compound by Ran(GTP), which is liberated upon GTP hydrolysis. Thus the displacement of Ran leads to the assembly of functional transport complexes and process activation [13]. A prominent example of this mechanism is the direct binding of Ran(GTP) to β-importin, preventing the formation (or leading to disassembly) of cargo transport complexes during nucleo-cytoplasmic transport, axonal retrograde signaling and post-mitotic nuclear membrane reconstitution. In addition, Ran has been implicated in endocytic receptor trafficking [14] and cytoplasmic organization of non-centrosomal microtubules [15]. A role for Ran in pathogen vacuole formation has not yet been described.

The formation of the *Legionella*-containing pathogen vacuole requires the Icm/Dot type IV secretion system (T4SS), which translocates at least 275 different “effector proteins” into eukaryotic cells [4,16,17]. The function of most Icm/Dot substrates is unknown, yet recent studies by several groups shed light on the intricate manner by which some effector proteins interfere. To this end, we demonstrated that LegG1 acts as a Ran activator in infected cells and cell lysates. Moreover, the activation of Ran on LCVs promotes microtubule stabilization, LCV motility and intracellular replication of *L. pneumophila*.

**Results**

The small GTPase Ran and the Icm/Dot substrate LegG1 localize to LCVs

The small GTPase Ran and its effector RanBP1 have been identified on purified LCVs by proteomic analysis [7,39]. To directly investigate the presence of these proteins on LCVs by fluorescence microscopy, *Dictyostelium discoideum* producing the corresponding GFP fusion proteins was infected with red fluorescent *L. pneumophila*. Ran was found to localize to the LCV membrane in *D. discoideum* infected with *L. pneumophila* wild-type or ΔlegG1 but not with Δicm1 mutant bacteria (Figure 1A). Moreover, RanBP1 localized to LCVs harboring wild-type *L. pneumophila* (see below). These results confirm the proteomic data and show that Ran and RanBP1 localize to LCVs in an Icm/Dot-dependent manner.

To assess whether Ran or RanBP1 play a role for intracellular replication of *L. pneumophila* we depleted the proteins by RNA interference. To this end, A549 lung epithelial cells were treated with siRNA oligonucleotides targeting Ran, RanBP1 or Arf1, and intracellular replication of *L. pneumophila* was monitored over 24 h (Figure 1B). Upon depletion of either Ran or RanBP1 the number of intracellular *L. pneumophila* was reduced two-fold, indicating that the small GTPase as well as its effector RanBP1 are required for efficient intracellular growth of *L. pneumophila*. As expected, the depletion of Arf1 also reduced intracellular growth of *L. pneumophila*, albeit less efficiently than depletion of Ran or RanBP1. The treatment with siRNA oligonucleotides efficiently depleted Ran or RanBP1, yet had no effect on A549 cell viability (Figure S1). Therefore, the depletion of Ran or RanBP1 impedes the intracellular replication of *L. pneumophila* without dramatically affecting the host cell physiology.

The LegG1 gene is conserved among the *L. pneumophila* strains sequenced to date (Philadelphia-1, Paris, Lens, Corby, Alcoy, 130b/AA100, Lorraine, HLO0641035), but apparently not present in other *Legionella* spp. LegG1 is translocated by the Icm/Dot T4SS as a TEM-β-lactamase fusion protein into J774 or RAW264.7 macrophages ([36]; Figure S2), or as an...
adenylate cyclase fusion protein into CHO cells [37]. Upon infection of D. discoideum producing L. pneumophila wild-type, ΔlegG1 or ΔicmT harboring pSW001 and immuno-stained for the LCV membrane marker SidC. LCVs in lysates of infected cells are shown. (B) Depletion of Ran or RanBP1 inhibits intracellular growth of L. pneumophila. A549 lung epithelial carcinoma cells were treated with AllStars siRNA (negative control) or with siRNA oligonucleotides targeting Ran, RanBP1 or Arf1 (positive control) for 2 d, and intracellular replication of GFP-producing L. pneumophila harboring pNT28 was quantified by fluorescence measurements after 24 h. Data represent mean and standard deviation of three independent experiments considering the 3 most effective out of 4 different oligonucleotides (Fig. S1B). Student’s t-test; ***, p < 0.001. (C) ΔicmT-dependent localization of M45-LegG1 on LCVs in cell homogenates. D. discoideum producing calnexin-GFP was infected (MOI 50) with L. pneumophila wild-type, ΔicmT or ΔlegG1 harboring pSU19 (M45-LegG1) or with ΔlegG1 harboring pCR033 (vector), homogenized and immuno-stained with an anti-M45 antibody and with DAPI. The percentage of M45-LegG1-positive LCVs (n = 100/strain, 3 independent experiments) was scored in lysates of infected cells (*, p < 0.05). Bars (A, C), 0.5 μm.

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Figure 1. The small GTPase Ran and the Icm/Dot substrate LegG1 localize to LCVs. (A) Ran accumulates on LCVs. D. discoideum producing RanA-GFP was infected (MOI 50, 1 h) with DsRed-producing L. pneumophila wild-type, ΔlegG1 or ΔicmT harboring pSW001 and immuno-stained for the LCV membrane marker SidC. LCVs in lysates of infected cells are shown. (B) Depletion of Ran or RanBP1 inhibits intracellular growth of L. pneumophila. A549 lung epithelial carcinoma cells were treated with AllStars siRNA (negative control) or with siRNA oligonucleotides targeting Ran, RanBP1 or Arf1 (positive control) for 2 d, and intracellular replication of GFP-producing L. pneumophila harboring pNT28 was quantified by fluorescence measurements after 24 h. Data represent mean and standard deviation of three independent experiments considering the 3 most effective out of 4 different oligonucleotides (Fig. S1B). Student’s t-test; ***, p < 0.001. (C) ΔicmT-dependent localization of M45-LegG1 on LCVs in cell homogenates. D. discoideum producing calnexin-GFP was infected (MOI 50) with L. pneumophila wild-type, ΔicmT or ΔlegG1 harboring pSU19 (M45-LegG1) or with ΔlegG1 harboring pCR033 (vector), homogenized and immuno-stained with an anti-M45 antibody and with DAPI. The percentage of M45-LegG1-positive LCVs (n = 100/strain, 3 independent experiments) was scored in lysates of infected cells (*, p < 0.05). Bars (A, C), 0.5 μm.

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L. pneumophila wild-type but not ΔlegG1 activates Ran on LCVs

To analyze the function of LegG1 genetically, an L. pneumophila strain lacking legG1 (ΔlegG1, Table S1) was constructed by deleting the gene from the chromosome by double homologous recombination. LCVs harboring ΔlegG1 mutant bacteria stained for the GTPase Ran faintly but to the same extent as wild-type L. pneumophila (Figure 1A), suggesting that LegG1 is dispensable for the recruitment of the small GTPase to the pathogen vacuole. In
**Figure 2.** *L. pneumophila* wild-type but not Δ*legG1* activates Ran on LCVs. (A) LegG1 promotes RanBP1 accumulation on LCVs. *D. discoideum* producing RanBP1-GFP (green) was infected (MOI 50, 1 h) with DsRed-producing *L. pneumophila* wild-type, ΔicmT or Δ*legG1* harboring pCR077 (red), or with Δ*legG1*/pERS (M45-LegG1) and immuno-stained for SidC (blue). The percentage of RanBP1-GFP-positive LCVs (n = 100/strain, 5 independent experiments) was scored in lysates of infected cells (**, *p* < 0.01; ***, *p* < 0.001). (B) Production of Ran(GTP) in infected macrophages. RAW264.7 macrophages were infected (MOI 25, 1 h) with *L. pneumophila* wild-type, ΔicmT or Δ*legG1* harboring pCR033 (vector) or with Δ*legG1*/pSU19 (M45-LegG1). The infected macrophages were lysed, activated Ran was immuno-precipitated with an antibody specifically recognizing Ran(GTP) and visualized by Western blot using an anti-Ran antibody. Lysates of uninfected cells incubated with GTP or GDP in presence of EDTA were used as positive or negative controls for endogenous GEF activity. Loading control: Western blot of Ran in samples before immuno-precipitation. (C) Production of Ran(GTP) in cell lysates. A549 epithelial cells were lysed and incubated with purified His6-LegG1 (native or heat-inactivated, h. i.) in...
The ΔlegG1 mutant strain was as cytotoxic for RAW264.7 macrophages as wild-type bacteria, since macrophages infected with either strain were vacuolized to the same extent (Figure 3C), and the percentage of cells permeable for propidium iodide was similar (Figure 3D). However, the overproduction of LegG1 reduced cytotoxicity significantly. The reduction of cytotoxicity was specific for LegG1, as overproduction of other effector proteins such as SidC or SidM significantly increased cytotoxicity (Figure S6A). Cytotoxicity reduction by LegG1 did not seem to be due to an impairment of type IV secretion, since translocation of the Icm/Dot substrate SidC was not affected (Figure S6B). Furthermore, L. pneumophila lacking or overexpressing legG1 was taken up with the same efficiency as wild-type bacteria by amoebae (Figure S7). The uptake of L. pneumophila is promoted by the Icm/Dot T4SS [40], and therefore, this finding also indicates that the overproduction of LegG1 does not simply obstruct the T4SS.

To test further effects of LegG1 on host cells, we assessed the fragmentation of the Golgi apparatus by L. pneumophila by using transmission electron microscopy (Fig. 3E). Upon infection of RAW264.7 macrophages with wild-type L. pneumophila the Golgi cisternae were almost completely disrupted, while in cells infected with a ΔicmT mutant strain the Golgi was preserved to an extent similar to uninfected cells. In macrophages infected with L. pneumophila ΔlegG1 the Golgi cisternae were conserved to an intermediate degree and appeared shorter than in uninfected cells.

Finally, in absence of legG1, the same number of LCVs accumulated the ER/LCV marker calnexin, suggesting that LegG1 does not affect the fusion of the pathogen vacuole with the ER (Figure S7C). Together, these results indicate that LegG1 is an Icm/Dot-translocated L. pneumophila virulence factor that localizes to the LCV membrane, is dispensable for bacterial uptake, and promotes intracellular replication in protozoan and mammalian phagocytes.

LegG1 stabilizes microtubules in L. pneumophila-infected phagocytes

Ran controls a number of cellular processes, some of which involve microtubule assembly and microtubule-dependent trafficking processes [11,13]. To study a possible role of microtubule polymerization and LegG1 for intracellular replication of L. pneumophila, macrophages were treated with the microtubule-depolymerizing agent nocodazole, and the fate of GFP-producing wild-type, ΔicmT or ΔlegG1 mutant bacteria was monitored (Figure 3F). The growth rate of wild-type L. pneumophila in nocodazole-treated cells was somewhat lower compared to control cells treated with DMSO only. However, the difference in the growth rate of the ΔlegG1 strain in nocodazole-treated cells compared to control cells was much more pronounced, indicating that in the absence of the Ran activator LegG1 the depolymerization of microtubules is more deleterious for intracellular bacterial growth. ΔicmT mutant bacteria were unable to grow and were killed to the same extent in macrophages treated with nocodazole. These results suggest that the depolymerization of microtubules by nocodazole and the absence of the Ran activator LegG1 synergistically compromise intracellular growth of L. pneumophila.

contrast, however, significantly fewer LCVs containing ΔlegG1 acquired detectable levels of the Ran effector RanBP1 compared to wild-type L. pneumophila (Figure 2A). Less than 50% of LCVs containing ΔlegG1 stained positive for RanBP1 compared to wild-type LCVs, and the phenotype of the ΔlegG1 mutant strain was fully complemented by expressing plasmid-encoded M45-legG1. This finding indicates that LegG1 promotes the accumulation of RanBP1 on LCVs and thus activates Ran on LCV membranes.

To determine more directly whether L. pneumophila activates Ran and whether LegG1 plays a role in this process, we assayed the production of activated Ran in pulldown experiments using an antibody specifically recognizing Ran(GTP), as detailed in the Materials and Methods section. To this end, RAW264.7 macrophages were infected with L. pneumophila wild-type, ΔicmT or ΔlegG1, or with ΔlegG1 expressing M45-legG1. The infected macrophages were lysed and Ran(GTP) was immuno-precipitated and visualized by Western blot using an anti-Ran antibody. Ran(GTP) was detected following infection with L. pneumophila wild-type but not with ΔicmT or ΔlegG1, and the phenotype of the ΔlegG1 mutant strain was complemented by providing legG1 on a plasmid (Figure 2B). Therefore, LegG1 is required to catalyze the activation of Ran by L. pneumophila.

A similar pulldown experiment was performed by adding purified His6-LegG1 to lysates of A549 cells (Figure 2C). Under these conditions, Ran(GTP) was produced in cell lysates upon addition of native (but not heat-inactivated) LegG1 in presence of GTP (but not GDP). Moreover, purified LegG1-His6 but not the mutant protein LegG1-N223A-His6 also activated Ran in cell lysates (Fig. S3C).

Using purified N- or C-terminally His-tagged LegG1 fusion constructs, we also attempted to directly measure Ran GEF activity in vitro with fluorescent mantGDP (2′,3′-O-(N-methylanthraniloyl)-guanosine-5′-diphosphate). As a positive control, the human Ran GEF RCC1 significantly stimulated mantGDP-release from Ran(mantGDP) in the presence of excess GTP as indicated by a rapid decrease in mantGDP-fluorescence. However, under various conditions tested the purified LegG1 fusion constructs did not show Ran GEF activity in vitro even at elevated concentrations (Fig. S3; data not shown). In summary, these results demonstrate that L. pneumophila activates Ran in infected protozoan and mammalian host cells by means of a translocated effector and that LegG1 is required to activate Ran.

LegG1 promotes intracellular replication of L. pneumophila

L. pneumophila lacking legG1 grew in broth at the same rate as the isogenic wild-type strain (data not shown). Yet, the ΔlegG1 strain was slightly impaired for intracellular replication in RAW264.7 macrophages (Figure 3A) and Acanthamoeba castellanii (Figure S4A), but not in D. discoideum (Figure S4B). Upon co-infection of ΔlegG1 with wild-type L. pneumophila at a 1:1 ratio, the mutant strain was efficiently out-competed by wild-type bacteria and eradicated within 6 days (Figure 3B). Thus the Ran activator LegG1 is essential for competition against wild-type L. pneumophila upon co-infection of amoebae. In contrast, the Rab1 GEF SidM was not required for competition under the same conditions, since a mutant strain lacking sidM did not show a competition defect (Figure S5).
Next, we used confocal laser scanning fluorescence microscopy to test whether Ran activation by LegG1 promotes microtubule polymerization in L. pneumophila-infected phagocytes. For this purpose, a D. discoideum strain producing GFP-α-tubulin was infected with red fluorescent L. pneumophila wild-type, ΔicmT, ΔlegG1 or ΔlegG1 expressing M45-legG1 (Figure 4A). In amoebae infected with wild-type L. pneumophila microtubules were polymerized to a greater extent compared with cells infected with the ΔlegG1 or ΔicmT mutant strains. More than 50% of the cells infected with ΔlegG1 showed a less dense microtubule network, and the phenotype was complemented by the legG1 gene. In D. discoideum infected with the complemented strain, the majority of microtubules emanated from the centrosome and reached the cell cortex, similar to amoebae infected with wild-type L. pneumophila.

Similarly, in RAW264.7 macrophages infected with red fluorescent L. pneumophila wild-type, microtubules were polymerized to a greater extent, compared to cells infected with the ΔlegG1 mutant strain (Figure 4B). Approximately 50% of the cells infected with ΔlegG1 showed a less dense microtubule network, and again the phenotype was complemented upon providing the legG1 gene on a plasmid. In macrophages infected with ΔicmT, microtubules were polymerized to a similar extent as in cells infected with wild-type L. pneumophila. Uninfected macrophages treated with taxol or nocodazole served as controls for microtubule polymerization or depolymerization, respectively.

The effect of the above L. pneumophila strains on microtubule polymerization in macrophages was further analyzed by stimulated emission depletion (STED) microscopy. This super-resolution immuno-fluorescence microscopy analysis confirmed that microtubules were polymerized to a greater extent in macrophages infected with green-fluorescent wild-type L. pneumophila or ΔicmT, compared to cells infected with ΔlegG1, and the phenotype was complemented by providing the legG1 gene on a plasmid (Figure 4C). Moreover, a high magnification inspection of the microtubule network in the vicinity of LCVs revealed an α-tubulin accumulation on 11% or 49% of LCVs harboring either wild-type L. pneumophila or the complemented ΔlegG1 strain, but not on LCVs harboring ΔicmT or ΔlegG1 mutant bacteria (Figure 4C, inset; Figure 4D).

The amount of polymerized or non-polymerized microtubules in L. pneumophila-infected macrophages was also assessed by Western blot using an anti-tubulin antibody (Figure 4E). Lysates of infected macrophages were subjected to a low and a high speed centrifugation step, and the quantity of microtubules in the pellet and the supernatant was compared. This approach revealed that macrophages infected with L. pneumophila lacking legG1 contained smaller amounts of polymerized microtubules compared to cells infected with wild-type bacteria, and the phenotype was complemented by overexpression of legG1. In summary, different approaches revealed that L. pneumophila promotes microtubule polymerization in amoebae and macrophages in a LegG1-dependent manner to efficiently replicate intracellularly.

Microbial microinjection of LegG1 promotes microtubule polymerization

As an alternative approach to analyze the effect of a single effector protein, LegG1, on host cells, we delivered the effector into host cells by microbial microinjection using the Yersinia enterocolitica strain WA (pT3SS). This Yersinia “toolbox” strain produces the Ysc type III secretion system (T3SS), yet lacks all endogenous T3SS effectors [41, 42]. N-terminal fragments of the Y. enterocolitica RhoG/Rac1 GAP YopE (YopE1-53, YopE1-138) are not cytotoxic but mediate secretion and translocation of hybrid proteins through the T3SS. Fusions of YopE1-53 or YopE1-138 with LegG1 or SidM were produced and secreted into the bacterial supernatant via the T3SS upon calcium depletion with 5 mM EGTA (Figure 5A). Moreover, dependent on the T3SS and sensitive to the protonophore CCCP (carbonyl cyanide m-chlorophenyl-hydrazone) heterologously produced YopE1-53-LegG1 was translocated into HeLa cells by Y. enterocolitica WA (pT3SS) (Figure 5B).

To analyze the translocation of heterologously produced effector fusion proteins, HeLa cells were infected for 2 h with Y. enterocolitica WA (pT3SS) producing YopE1-138-LegG1, YopE1-138-SidM or YopE, and the morphology and microtubules were analyzed by immuno-fluorescence microscopy (Figure 5C). Uninfected cells or cells infected with WA (pT3SS) producing YopE1-138-LegG1 showed a similar morphology and microtubule network. In contrast, cells infected with WA (pT3SS) producing YopE1-138-SidM or full length Y. enterocolitica YopE, a RhoG/Rac1 GAP [43], rounded up and the microtubules disintegrated. Uninfected HeLa cells treated with 30 µM taxol or nocodazole served as controls.

In order to visualize more subtle effects of LegG1 on the microtubule network, HeLa cells were pretreated for 1 h with 1 µM nocodazole prior to infection for 2 h with Y. enterocolitica WA (pT3SS) producing YopE1-53 or YopE1-53-LegG1 (Figure 5D). Immuno-fluorescence microscopy indicated that under these conditions, cells infected with Y. enterocolitica producing YopE1-53-LegG1 contained a denser microtubule network, compared with cells infected with bacteria producing YopE1-53 or uninfected cells. Throughout the cell body, the injection of YopE1-53-LegG1 caused the formation of a larger number of microtubule bundles, which emanated from the peri-nuclear region and radiated towards the cell cortex.

Microtubule polymerization triggered by Y. enterocolitica WA (pT3SS) producing YopE1-53 or YopE1-53-LegG1 was also quantified by Western blot using an anti-α-tubulin antibody (Figure 5E). This approach confirmed that LegG1 injected into
Ran Activation by a Legionella Effector

**A**

| L. pn. | DAPI | SidC | α-Tubulin | Merge |
|--------|------|------|-----------|-------|
| WT     | ![Image](image1) | ![Image](image2) | ![Image](image3) | ![Image](image4) |
| ΔicmT  | ![Image](image5) | ![Image](image6) | ![Image](image7) | ![Image](image8) |
| ΔlegG1 | ![Image](image9) | ![Image](image10) | ![Image](image11) | ![Image](image12) |
| ΔlegG1/LegG1 | ![Image](image13) | ![Image](image14) | ![Image](image15) | ![Image](image16) |

**B**

- WT
- ΔicmT
- ΔlegG1
- ΔlegG1/LegG1

**C**

- Uninfected
- Taxol
- Nocodazole

**D**

| L. pn. | SidC | α-Tubulin | Merge |
|--------|------|-----------|-------|
| WT     | ![Image](image17) | ![Image](image18) | ![Image](image19) |
| ΔicmT  | ![Image](image20) | ![Image](image21) | ![Image](image22) |
| ΔlegG1 | ![Image](image23) | ![Image](image24) | ![Image](image25) |
| ΔlegG1/LegG1 | ![Image](image26) | ![Image](image27) | ![Image](image28) |

**E**

- Taxol
- Nocodazole
- WT
- ΔicmT
- ΔlegG1
- ΔlegG1/LegG1

**Graphs**

- Cells with polymerized microtubules [%]
- α-Tubulin/Actin ratio
- Polymerized α-tubulin [%]
HeLa cells significantly increased the amount of insoluble tubulin in the pellet and thus caused microtubule polymerization. Taken together, these results indicated that heterologously produced LegG1 delivered into eukaryotic cells via microbacterial microinjection promotes polymerization of microtubules.

Finally, to assess whether LegG1 requires the Ran GTPase to exert its effect on microtubule polymerization, A549 cells (Fig. S1) were treated with siRNA oligonucleotides silencing Ran for 2 days, followed by 1 μM nocodazole for 1 h and infection with Y. enterocolitica WA pT3SS5 producing YopE1-53 or YopE1-53-LegG1 for 2 h (Figure 5F). Under these conditions, LegG1 triggered microtubule condensation only in cells treated with allStars negative control siRNA, but not in cells depleted for Ran. While 93% of the control cells infected with Y. enterocolitica producing YopE1-53-LegG1 contained a dense tubulin network, only 45% of the Ran-depleted cells did so. This experiment indicates that Ran is essential for LegG1 to promote microtubule polymerization.

LCVs harboring L. pneumophila ΔlegG1 are stalled

Shortly after formation, LCVs rapidly move within *D. discoideum* cells, and the pathogen vacuoles are transported along microtubules [6]. The dynamics of LCVs harboring either *L. pneumophila* wild-type (Movie S1) or ΔlegG1 mutant bacteria (Movie S2) was assessed by real-time confocal laser scanning fluorescence microscopy using calnexin-GFP-producing *D. discoideum* or DsRed-producing *L. pneumophila*. Two hours post infection the LCV migration was recorded for 5 min with images taken every 15 s (Figure 6A). While LCVs harboring wild-type *L. pneumophila* were very motile and rapidly moved along microtubules, LCVs harboring ΔlegG1 mutant bacteria were drastically slowed down and hardly moved. The velocity of LCVs was quantified by tracking the migration distance of LCVs over time. These experiments revealed that LCVs harboring wild-type *L. pneumophila* moved with a speed of about 40 nm/s within the *D. discoideum* cells, while LCVs harboring ΔlegG1 mutant bacteria moved with a 3–4 times lower speed and were almost stalled (Figure 6B).

Discussion

In this work, we demonstrate that the small GTPase Ran, its effector RanBP1 and the Icm/Dot substrate LegG1 localize to LCVs and promote intracellular replication of *L. pneumophila*. Moreover, the legG1 gene is required for Ran activation in infected macrophages, and purified LegG1 protein functions as a Ran activator. Finally, activation of Ran on LCVs promotes microtubule stabilization, LCV motility and intracellular growth of *L. pneumophila*. LegG1 represents the first prokaryotic Ran activator known in a non-GTPase system, and likely regulates in a spatiotemporal manner the production of a Ran(GTP) gradient originating from (a) subcellular membrane-bound compartment(s). While LegG1 localizes to the LCV membrane, the effector might not only act as a Ran GAP inhibitor, thus preventing the inactivation of Ran. Finally, the level of Ran(GTP) can also be modulated by nucleotide release proteins such as Mog1 [44], which might be targeted by LegG1.

The Ran GEF RCC1 is chromatin-bound and nuclear in interphase cells, or chromosome-associated in mitotic or postmitotic cells. Regardless of the cell cycle phase, RCC1 produces a gradient of activated Ran originating from cellular DNA [10,13]. In contrast, the cytoplasmic Ran GEF RanBP10 has been shown to directly bind tubulin and activate Ran [15], and therefore might provide a scaffold for cytosolic Ran activation and microtubule polymerization. Analogously to RanBP10, LegG1 localizes to the cytosol by accumulating on the cytosolic face of the LCV in *L. pneumophila*-infected cells (Figure 1C). Furthermore, LegG1 co-localizes with (but does not disrupt) the Golgi apparatus upon ectopic production in mammalian cells [37,38]. Thus, LegG1 likely regulates in a spatiotemporal manner the production of a Ran(GTP) gradient originating from (a) subcellular membrane-bound compartment(s). While LegG1 localizes to the LCV membrane, the effector might not only act as a Ran activator in cis (on LCVs) but also in trans (in a distance from LCVs) to promote the formation of a replication-permissive compartment and/or to affect other cellular processes regulated by Ran. In this context it is interesting to note that the *L. pneumophila* Icm/Dot substrate RomA (Regulator of methylation A) is targeted to the host cell nucleus, where the methyltransferase modifies chromatin and gene expression by producing a novel histone mark [45]. It will be interesting to assess, whether by activating Ran LegG1 regulates nucleo-cytoplasmic transport, and thereby, the activity of RomA.

Membrane localization of LegG1 is modulated by prenylation (likely geranyl-geranylation) of a C-terminal CAAX motif [38], implying that the bacterial Ran activator subverts essential host lipidation machinery to direct its subcellular localization. Prenylation might contribute to the specific subcellular distribution among different membranous compartments and thus play an important role for the function of LegG1. Instead of the prenylation (likely geranyl-geranylation) of a C-terminal CAAX motif [38], implying that the bacterial Ran activator subverts essential host lipidation machinery to direct its subcellular localization. Prenylation might contribute to the specific subcellular distribution among different membranous compartments and thus play an important role for the function of LegG1. Instead of the prenylation (likely geranyl-geranylation) of a C-terminal CAAX motif [38], implying that the bacterial Ran activator subverts essential host lipidation machinery to direct its subcellular localization. Prenylation might contribute to the specific subcellular distribution among different membranous compartments and thus play an important role for the function of LegG1.
Figure 5. Microbial microinjection of LegG1 promotes microtubule polymerization. (A) Yersinia enterocolitica strain WA (pT3SS), encoding the Ysc T3SS but lacking type III-secreted effectors, produced and secreted fusion proteins of YopE1–53 or YopE1–138 with the Legionella Ran activator LegG1 or the Rab1 GEF SidM. The proteins in the pellet (P) or supernatant (S) were precipitated by chloroform/methanol treatment and visualized by Western blot using an antibody against YopE. (B) HeLa cells were infected (MOI 10, 2 h) with Y. enterocolitica WA (pT3SS) producing YopE1–53-LegG1, washed several times and lysed with 1% digitonin. After centrifugation, proteins in the pellet (intact bacteria, debris) and in the supernatant (translocated bacterial/soluble host proteins) were precipitated, and the fusion protein was visualized by Western blot using an anti-YopE antibody.
Controls: HeLa cells alone, bacteria treated with 1% digitonin, 1% SDS or with the T3SS inhibitor CCCP (50 μM). (C) Fluorescence microscopy of YopE1–138-LegG1 translocation into HeLa cells infected (MOI 10, 2 h) with Y. enterocolitica WA (pT3SS) producing YopE1–138-LegG1, YopE1–138-SidM or YopE. Controls: uninfected cells or cells treated with 30 μM taxol or nocodazole. The cells were immuno-stained for α-tubulin and YopE, and nuclei were labeled with DAPI. Bars, 10 μm. (D) Fluorescence microscopy of YopE1–53-LegG1 translocation into HeLa cells treated with nocodazole (1 μM, 1 h) and infected (MOI 10, 2 h) with Y. enterocolitica WA (pT3SS) producing YopE1–53 or YopE1–53-LegG1. The cells were immuno-stained for α-tubulin (green) and YopE (red), and nuclei were labeled with DAPI (grey). Bars, 10 μm. (E) Western blot of YopE1–53-LegG1 translocation into HeLa cells treated with nocodazole (1 μM, 1 h) and infected (MOI 10, 2 h) with Y. enterocolitica WA (pT3SS) producing YopE1–53 or YopE1–53-LegG1. The soluble microtubule fraction in the cell supernatant (S) and the pellet (P) was analyzed with an anti-YopE. Controls: uninfected cells or cells treated with 30 μM taxol or nocodazole. (F) Fluorescence microscopy of A549 cells treated or not with siRNA against Ran and infected (MOI 10, 2 h) with Y. enterocolitica WA (pT3SS) producing YopE1–53 or YopE1–53-LegG1. The cells were immuno-stained for α-tubulin (green) and YopE (red), and nuclei were labeled with DAPI (grey). Bars, 10 μm or 5 μm (insets). The data shown is representative of 3 independent experiments (A–F).

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as membrane anchors for bacterial GEF effector proteins targeting distinct eukaryotic small GTPases.

Ran activation in mitotic or post-mitotic cells controls the assembly of microtubule spindles and the reconstitution of the nuclear membrane envelope, a process which requires vesicle trafficking, recruitment and fusion [10,13]. Similarly, Ran activation by translocated LegG1 positively regulates microtubule polymerization (Figure 4, 5), and LCV motility (Figure 6). Microtubule-dependent motility of LCVs might reposition the pathogen vacuole in the infected phagocytic cell and serve to locate the vacuole in the vicinity of interacting compartments such as the ER. Alternatively or in addition, LegG1-dependent microtubule polymerization might promote vesicle trafficking processes in a distance from the pathogen vacuole, in order to promote fusion and fission events of vesicles communicating with the vacuole. Thus, LegG1-dependent microtubule polymerization likely plays a crucial role in defining the membrane dynamics and equilibrium of LCVs. In any case, the LegG1-catalyzed microtubule dynamics are essential for L. pneumophila infection, as in absence of LegG1, Ran or RanBP1 intracellular bacterial growth is compromised (Figure 1B, 3A, 3B).

Ran accumulates on LCVs in an Icm/Dot-dependent manner (Figure 1A). Yet, LegG1 apparently does not affect the recruitment of Ran to LCVs, and it is unknown which protein or lipid receptor(s) on LCVs the Ran GTPase binds to. In contrast, LegG1 promotes the activation of Ran on LCVs, since in absence of legG1 less RanBP1 accumulates on LCVs in D. discoideum, and Ran/GTP production is reduced in infected macrophages (Figure 2). In

![Figure 6. LCVs harboring L. pneumophila ΔlegG1 show impaired motility. (A) Real-time fluorescence microscopy of LCV motility in D. discoideum producing calnexin-GFP and infected (MOI 10, 2 h) with DsRed-producing L. pneumophila wild-type or ΔlegG1 mutant bacteria harboring pSW001. Two hours post infection, trafficking of LCVs was recorded by laser confocal scanning microscopy for 5 min with images taken every 15 s. Bars, 1 μm. (B) The velocity of LCVs was quantified by tracking the migration distance of LCVs over time (n=50/strain; ***, p<0.001).](image-url)

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is suitable to functionally deliver into host cells not only T3SS substrates but also heterologous T3SS substrates.

In summary, we document here a characterization of the first bacterial Ran activator, \textit{L. pneumophila} \textit{LegG1}. This finding paves the way for the future analysis of the signal transduction pathways activated by \textit{RanGTP} in \textit{L. pneumophila}-infected phagocytes, which are implicated in microtubule polymerization, LCV motility and intracellular bacterial replication.

\textbf{Materials and Methods}

\textbf{Bacteria, cells, growth conditions and infection}

Bacteria and cells are listed in Table S1. \textit{L. pneumophila} strains were grown for 3 days on CYE agar plates containing charcoal yeast extract, buffered with N-(2-acetamido)-2-amino-ethanesulfonic acid (ACES). Liquid cultures were inoculated in AYE medium at an OD_{600} of 0.1 and grown at 37°C to an OD_{600} of 3.0 (21–22 h). Chloramphenicol (Cam; 5 μg/ml) and IPTG (1 mM) were added when needed.

Murine RAW264.7, as well as human HeLa and A549 lung epithelial carcinoma cells were cultivated in RPMI 1640 medium amended with 10% heat-inactivated fetal bovine serum and 1% glutamine (all from Life Technology). \textit{D. discoideum} strains (Table S1) were grown and transfected by electroporation as described [47,49], and \textit{A. castellanii} (ATCC 30234) was propagated as described [50].

The infection of phagocytes by \textit{L. pneumophila} wild-type, Δ\textit{icmT} or Δ\textit{LegG1} mutant strains producing GFP was analyzed as described using \textit{A. castellanii}, \textit{D. discoideum} or murine RAW264.7 macrophages as host cells [32,47,48,50,51]. Briefly, the phagocytes were infected with \textit{L. pneumophila} grown for 21–22 h in AYE broth (MOI 1–50), the infection was synchronized by centrifugation (450 × g, 10 min, RT), and the infected phagocytes were incubated at 37°C, 30°C or 25°C (\textit{D. discoideum}) for the time indicated.

\textbf{Chromosomal deletion of \textit{legG1} and plasmid construction}

All plasmids and oligonucleotides used are listed in Table S1 or Table S2, respectively. DNA manipulations were performed according to standard protocols, and plasmids were isolated using commercially available kits from Macherey-Nagel. All PCR fragments were sequenced.

The chromosomal deletion of \textit{legG1} was performed as described [50]: 800 bp upstream and downstream fragments of \textit{legG1} (lpg176) were amplified by PCR using the primer pairs oSU94/oSU95 and oSU96/oSU97, respectively, and chromosomal \textit{L. pneumophila} DNA as a template. Both fragments were inserted by a four way ligation into a pGEM-T easy vector with a Kan^R cassette in-between using \textit{BamHI} sites and adenosine overhangs, yielding plasmid pSU1. Plasmids were analyzed by restriction digestion and sequencing. The Kan^R cassette flanked by upstream and downstream fragments was transferred into the \textit{pLAW344} suicide plasmid using \textit{NdeI}, yielding plasmid pSU2. \textit{L. pneumophila} JR32 was transformed by electroporation with pSU2 and selected for Kan^R/Suc^R and Cam^R colonies. Positive clones were tested by PCR, using the primers oSU94, oSU97, oKan3' and oKan3'', and by sequencing.

Translational M45-fusion proteins of \textit{legG1} were constructed by PCR amplification using the primer pairs: oER158/oER160 or oER3/oER7 and chromosomal DNA of \textit{L. pneumophila} JR32 as a template. The fragments were cut with the appropriate restriction enzymes and inserted into pMMB207-RBS-C-M45 (pCR33), pMMB207-C-RBS-gfp-RBS (pCR76), pMMB207-C-RBS-DeRed-RBS (pCR77), yielding the plasmids pSU19, pER4 and pER5, respectively. The plasmids pSU17 and pSU26 (encoding RanA-GFP or RanBPI-GFP) were constructed by PCR-amplification of the corresponding genes using the oligonucleotides listed in Table S2. \textit{D. discoideum} cDNA was used as a template, cut with \textit{Nol} and inserted into pSW102 cut with the same restriction enzyme.

Plasmids encoding \textit{LegG1}-His\textsubscript{6} \textit{(pER2)} or \textit{His\textsubscript{6}-LegG1} \textit{(pER3)} were constructed using the primers oER4/oER6 or oCR158/oER5, respectively, and chromosomal DNA from \textit{L. pneumophila} strain JR32 as a template. The PCR fragment was cut with \textit{Nol}/SacII or with \textit{BanHII/SalI} and cloned into the vector pET-28a. Plasmid pER35 encoding \textit{LegG1}\textsubscript{N223A}-His\textsubscript{6} was obtained with the QuickChange protocol using the primers oER22 and oER23, and plasmid pER2 as a template.

To construct plasmids encoding YopE\textsubscript{1–53} (5.5 kDa) or YopE\textsubscript{1–138} (14.8 kDa) fusion proteins produced in \textit{Y. enterocolitica}, the \textit{legG1} gene was amplified by PCR using the oligonucleotides oER158/oER3 and chromosomal DNA of \textit{L. pneumophila} JR32 as template. The PCR fragment was cut with \textit{BanHII/SalI} and cloned into pCJYE53-G3 or pCJYE138-G3 cut with the same enzymes, yielding pGP3 and pGP4, respectively. The genes \textit{sidM}, \textit{sidC} or \textit{sidC_P4C} were released from plasmid pEB189, pCR6 or pHP56, respectively, by digestion with \textit{BanHII/SalI} and cloned into pCJYE53-G3 or pCJYE138-G3 cut with the same enzymes. To liberate \textit{gfp} from pCJYE53-G3 or pCJYE138-G3, the plasmids were cut with \textit{BanHII/SalI}, filled in with Klenow polymerase and re-ligated.

\textbf{Transfection assay}

To determine \textit{Icm}/\textit{Dot}-dependent translocation into host cells of \textit{LegG1}, 5 × 10\textsuperscript{7}/ml RAW264.7 macrophages were seeded onto 96-well plates in a final volume of 100 μl/well and incubated at 37°C overnight. The macrophages were infected with \textit{L. pneumophila} (MOI 20, 1 h, 37°C) producing TEM β-lactamase fusion proteins (kindly provided by X. Charpentier), grown for 21 h in AYE supplemented with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside). 20 μl of 6-fold CCF4/AM substrate (Invitrogen) was added to each well. After 90 min incubation, the fluorescence was measured with a fluorescence plate reader (FluoStar Optima, BMG Labtech) using an excitation wave length of 410 nm, and an emission of 450 nm or 520 nm, respectively. The \textit{Icm}/\textit{Dot} substrate LepA served as a positive control and the cytoplasmic protein FabI as a negative control.

\textbf{Purification of His-tagged \textit{LegG1}}

The constructs pER2 or pER3 were transformed into \textit{E. coli} BL21(DE3) grown aerobically in LB medium at 37°C and induced with 1 mM IPTG during exponential growth for 4 h at 30°C. Alternatively, pER2 was transformed into \textit{E. coli} BL21-CodonPlus (DE3)-RIL and grown in 1 L LB medium at 37°C to an OD\textsubscript{600} of 0.5 to 0.7 before expression was induced with 0.5 mM IPTG at 20°C overnight. Cells were harvested by centrifugation (7'000 × g, 30 min, 4°C), suspended and homogenized in lysis buffer (50 mM Tris/HCl, pH 7.5, 10% glycerol, 10 mM β-mercaptoethanol (BME), 0.5 mM PMSF, 30 ng ml\textsuperscript{-1} DNase), disrupted at 10,000 psi by a French Press, and the suspension was cleared by centrifugation (11'000 × g, 30 min, 4°C).

\textit{His\textsubscript{6}-tagged LegG1} was purified by affinity chromatography using nickel-nitrilotriacetic acid-agarose (Qiagen) equilibrated with buffer E (50 mM Tris/HCl, pH 8.0, 10% glycerol, 10 mM BME, 10 mM imidazole), eluted with buffer F containing 250 mM imidazole and dialyzed overnight against elution buffer lacking imidazole (4°C). Some preparations of \textit{His\textsubscript{6}-LegG1} were further purified by gel filtration (Superdex 75 16/600; GE Healthcare, Munich, Germany) using a buffer containing 20 mM HEPES.
pH 7.5, 50 mM NaCl, 1 mM MgCl₂, and 2 mM DTE (dithioerythritol).

**Guanine nucleotide exchange assay**

For the GEF assay Ran GTPase was pre-preparedly loaded with fluorescent mantGDP (2′3′-O-(N-methyl-anthraniloyl)-guanosine-5′-diphosphate). To this end, purified human Ran was incubated for 2 h RT in the presence of EDTA in five times molar excess over MgCl₂ and mantGDP in five times molar excess over Ran protein. Unbound nucleotides were removed by buffer exchange using a NAP-5 column (GE Healthcare, Munich, Germany) eluting the protein with exchange buffer (20 mM HEPES pH 7.5, 50 mM NaCl, 2 mM DTE, 1 mM mantGDP). Collected fractions containing the protein were pooled and concentrated using a Spin-X UF 500 (Corning, Munich, Germany) concentrator.

The GEF-assay was performed in 1 ml fluorescence buffer (20 mM HEPES pH 7.5, 50 mM NaCl, 2 mM MgCl₂, 2 mM DTE, 1 mM GTP) at 25 °C using a fluorescence spectrometer (Fluoromax-4, Horiba Jobin Yvon). The excitation and emission wavelength of mantGDP is 345 and 440 nm, respectively. The release of mantGDP was monitored via the change in mant-fluorescence after addition of recombinant LegG1 or RCC1, respectively, in the presence of 100 μM GTP. The fluorescence traces have been corrected for dilution.

**Pulldown and Western blot**

Ran(GTP) was identified in cell lysates by pulldown experiments using reagents from New East Biosciences. To this end, RAW264.7 macrophages in T75 tissue culture flasks were infected (MOI 20, 1 h), lysed with lysis buffer (50 mM Tris-HCl, pH 8.0, 130 mM NaCl, 10 mM MgCl₂, 1 mM EDTA, 1% Triton X-100) containing a protease inhibitor tablet (complete; Roche) and incubated with an anti-Ran(GTP) antibody. As positive and negative controls, uninfected cells were treated with γ-S-GTP or GDP in presence of 20 mM EDTA before incubation with the anti-Ran(GTP) antibody. Alternatively, A549 cells were washed three times with cold PBS before lysis, incubated for 30 min at 30 °C with γ-S-GTP (100 μM) or GDP (1 mM), together with purified His₆-LegG1, heat-inactivated His₆-LegG1, LegG-His₆, LegG₁₁₂₃₃₃-His₆, or human RCC1, and Ran(GTP) was immuno-precipitated as described above.

To separate polymerized and soluble tubulin, RAW264.7 macrophages seeded in 6-well plates were infected with *L. pneumophila* strains (MOI 10, 4 h), lysed with microtubule stabilization buffer (0.1 M PIPES, pH 7.6, 2 M glycerol, 5 mM MgCl₂, 2 mM EGTA, 0.5% Triton X-100, 4 mM taxol, protease inhibitors) and centrifuged at low speed (240 g, 6 min). The supernatant containing detached cells was combined with the stored media and centrifuged (240 g, 10 min). The cells were suspended in 0.5 ml PBS containing 1 μg/ml propidium iodide (15 min, 25 °C, in the dark) and quantified by flow cytometry.

**Intracellular replication and competition**

To analyze intracellular replication of *L. pneumophila*, exponentially growing *D. discoideum* were washed with Sorensen phosphate buffer (2 mM Na₂HPO₄, 15 mM KH₂PO₄, pH 6.0) containing 50 μM CaCl₂ (SacC), seeded into a 96-well plate (1×10⁵ cells/ml MB medium) and allowed to adhere for 1–2 h. RAW264.7 macrophages were seeded (1×10⁵ cells/ml RPMI medium) one day before infection. *L. pneumophila* grown for 21 h in AYE broth was diluted in MB or RPMI and used for infection (MOI 1). For centrifugation, the infected phagocytes were incubated at 30 °C (*A. castellanii*) or 37 °C (macrophages), a bacterial input control was plated, and samples were taken at the time points indicated (t₀ = 10 min post infection).

Single round intracellular growth of GFP-producing *L. pneumophila* was assayed in *A. castellanii* or RAW264.7 macrophages as described [52]. Fresh medium was added to the cultures two days before the experiment. One day before the experiment, the cells were suspended and seeded into a black 96-well clear bottom plate (Perkin-Elmer) at a density of 2×10⁴ (amoebae) or 3×10⁴ (macrophages) cells/well and allowed to adhere overnight. Overnight cultures of *L. pneumophila* harboring pNT28 (GFP) were grown in AYE/Cam or an OD₆₀₀ of 3.0 (~2×10⁹ bacteria/ml) and diluted to 8×10⁵ bacteria/ml in LoFlo low fluorescence medium (Formedium). The cells were infected (MOI 20) with 100 μl of diluted *L. pneumophila*, centrifuged and incubated at 30 °C (amoebae) or 37 °C (macrophages) for 48 h or longer. Nocodazole was added directly to the infection at the concentrations indicated. The GFP fluorescence was quantified at multiple time points using a plate reader (FluoStar Optima, BMG Labtech). To correlate fluorescence readings with bacterial viability, the cells were lysed at set time points using 0.8% saponin, dilutions were plated on CYE plates, and CFU were counted.

For the competition assays, *A. castellanii* (2×10⁴ per well, 96-well plate) in A缓冲 was co-infected (MOI 0.01) each with wild-type *L. pneumophila* and the Kan-resistant mutant strain to be tested. The infected amoebae were grown for 21 days at 37 °C. Every third day the supernatant was combined with amoebae lysed with 0.8% saponin, diluted 1:1000, and fresh amoebae were infected (50 μl homogenate per 200 μl amoebae culture volume). Aliquots were plated on CYE agar plates containing Kan (10 μg/ml) or not to determine CFU.

**RNA interference**

For the RNA interference experiments, A549 cells were grown in 96-well plates and treated for 2 days with a final concentration of 10 nM of the siRNA oligonucleotides indicated (Table S3). To this end, the siRNA stock (10 μM) was diluted 1:15 in RNAase-free water, and 3 μl of diluted siRNA was added per well. Allstars siRNA (Qiagen) was used as a negative control. Subsequently, 24.25 μl RPMI medium without FCS was mixed with 0.75 μl intensity of the cells. Equal fluorescence intensities of different *L. pneumophila* strains were checked by a plate reader.

To determine cytotoxicity of different *L. pneumophila* strains, 2.5×10⁵ RAW264.7 macrophages were seeded onto a 24-well plate. The cells were infected with *L. pneumophila* (MOI 10, 4 h), and a bacterial input control was plated on CYE plates. After the infection, the medium was collected and replaced with PBS. To detach the infected macrophages, the plate was shaken vigorously at 1400 rpm for one minute on an Eppendorf plate incubator. The supernatant containing detached cells was combined with the stored media and centrifuged (240 g, 10 min). The cells were suspended in 0.5 ml PBS containing 1 μg/ml propidium iodide (15 min, 25 °C, in the dark) and quantified by flow cytometry.
HiPerFect transfection reagent (Qiagen), added to the well, mixed and incubated for 5–10 min at RT. In the meantime, cells were diluted in RPMI medium with 10% FCS, 175 μl of the diluted cells (2 £ 10^6 cells) were added on top of each siRNA-HiPerFect transfection complex and incubated for 4 h. The cells were then infected (MOI 10) with GFP-expressing L. pneumophila wild-type grown for 2 h, diluted in RPMI, centrifuged and incubated for 1 h. The infected cells were washed 3 times with pre-warmed medium containing 10% FCS and incubated for 24 h (well plate was kept moist with water in extra wells). To determine intracellular growth of L. pneumophila, GFP-fluorescence was measured using a plate reader (FluoStar Optima, BMG Labtech).

Microbial microinjection

For microbial microinjection the Y. enterocolitica strain WA (pT3SS) was used [41]. This Yersinia “toolbox” strain harbors a mini pYV virulence plasmid encoding the Ysc T3SS and the YadA adhesin, yet lacks all endogenous type III-secreted effectors [42,53]. The T3SS recognizes an N-terminal secretion/translocation signal of Yersinia effectors. In particular, N-terminal fragments of the RhoG/GacA GAP YopE (YopE153, YopE1-138) are not cytotoxic but sufficient to mediate secretion and translocation of hybrid proteins. Fusion proteins composed of YopE1-53 or YopE1138 and L. pneumophila LegG1, SidM, SidC or its PtdIns(4)-binding domain SidC_P1AC were produced in Y. enterocolitica WA (pT3SS) and analyzed by Western blot using a polyclonal anti-YopE antibody (1:5000; gift from J. Heesemann). The production of YopE served as a positive control.

T3SS-dependent protein secretion was triggered by calcium depletion with EGTA essentially as described [53]. Briefly, Y. enterocolitica cultures grown overnight in BHI broth at 27°C were diluted 1:20 in 10 ml BHI and grown at 37°C for another 1.5 h, followed by the addition of 10 ml of a solution containing 15 mM MgCl₂, 5 mM EGTA and 0.2% glucose for 2 h (final OD600 0.6–0.7). Subsequently, the cells were centrifuged (5000 g, 10 min), and the pellet was washed with PBS and suspended in 500 μl SDS PAGE sample buffer. The supernatant was sterile filtered, precipitated with methanol/chloroform, and YopE1-53, YopE1-138, SidC or LegG1 were visualized by Western blot using an anti-YopE antibody (1:5000; gift from J. Heesemann). The production of YopE served as a positive control.

To obtain homogenates, infected amoebae were washed with cold SorC one hour post infection, suspended in homogenization buffer (20 mM HEPES, 250 mM sucrose, 0.5 mM EGTA, pH 7.2) [57] and lysed by seven passages through a ball homogenizer (Isobiotech) using an exclusion size of 8 μm. The homogenate was centrifuged onto coverslips coated with poly-L-lysine, fixed with 4% paraformaldehyde (PFA) for 30 min at 4°C and blocked with 1% BSA in SorC for 30 min.

The coverslips containing homogenates were incubated for 1 h at RT on parafilm with 40 μl of primary antibody diluted in blocking buffer (affinity purified rabbit anti-SidC (1:100) [48]; anti-M45 1:300, Genovac AG) and washed 3 times. Appropriate secondary antibodies were diluted 1:200 in blocking buffer and incubated for 1 h at RT. Finally, the coverslips were washed and mounted using Vectashield (Vector Laboratories) supplemented with 1 μg/ml DAPI to stain DNA. The samples were viewed with a Leica TCS SP5 confocal microscope (HCX PL APO CS, objective 63x/1.4-0.60 oil; Leica Microsystems, Mannheim, Germany).

Microtubule cytoskeleton analysis was performed with RAW264.7 macrophages or D. discoideum infected with DsRed or GFP-producing L. pneumophila (MOI 10, 4 h, 37°C). Subsequently, the infected phagocytes were washed once with Brb80, and fixed (50% Brb80, 0.1% Triton X-100, 0.5% glutaraldehyde) for 5 min. After washing with SorC, samples were blocked with 1 mg/ml sodium borohydrate in SorC for 10 min. The samples were stained with the anti-a-tubulin antibody W3 (gift from M. Schleicher) and anti-SidC (1:100) on parafilm for 1 h. Appropriate secondary antibodies were used at a dilution of 1:200 (confocal microscopy) or 1:400 (STED microscopy). 10 cells per coded sample were assessed for the degree of tubulin polymerization.

For real-time fluorescence microscopy exponentially growing D. discoideum amoebae producing calnexin-GFP were seeded in life-cell imaging dishes (Ibidi) in LoFlo medium containing G418 (20 μg/ml) to a total cell number of 5 £ 10^7 cells one day prior to the experiment. Before infection with L. pneumophila strains producing DsRed (MOI 10), the cells were washed with LoFlo. Ascorbic acid was added to the cells (20 mg/ml). After two hours incubation at 25°C with no centrifugation, an agar overlay was prepared and the infected cells were observed (recorded) for 5 min. Images were taken every 15 s.
STED microscopy

Stimulated emission depletion (STED) microscopy was employed for sub-diffraction resolution fluorescence imaging on a custom-made in-house setup. The system’s basic principle is described elsewhere [58]. The system used is capable of acquiring one channel with confocal and two channels with STED resolution quasi-simultaneously. For imaging GFP-producing *Legionella* and Atto 635-coupled anti-β-tubulin, we used excitation/emission wavelengths of 408±3 nm/520±14 nm and 637±5 nm/685±20 nm, respectively. The STED wavelength for Atto 635 was 750±10 nm. Beam powers for acquisition were 0.5–4.5 μW, and 7 μW for GFP and Atto 635, respectively, as measured in front of the objective. STED beam powers were 1.4 mW for Atto 635. To reduce crosstalk, pulses for various channels were separated in time by varying optical path lengths. A home-built electronic gating device transmitted detector signals occurring at the correct time to the acquisition hardware, and rejected crosstalk signals occurring at other times. Dichroes and filters were purchased from AHF, Tübingen. The supercontinuum laser source was a SC450-PP-HE system running at 1 MHz, manufactured by Fianium Ltd, Southampton. For beam-scanning, we used a YANUS IV scan head from Till Photonics, Munich. The objective was a Leica 100/1.4.

STED image processing: For acquiring images, movies comprised of 5–20 frames were acquired at an exposure time per pixel of 20–50 μs. All frames were then added up, and a non-local means algorithm (Buades et al., 2005) was applied via a matlab script (Peyré et al., 2007) with algorithm parameters being radius of search window: 1, radius of similarity window: 10, degree of filtering: 10.

Transmission electron microscopy

For transmission electron microscopy cells were fixed by adding 2% glutaraldehyde (GA) in 100 nM PIPES, pH 7.0, to the culture medium at a 1:1 volume ratio. After 5 min, the supernatant was discarded, replaced with fresh 1% GA in the same PIPES buffer and incubated overnight at RT. For the subsequent epoxy resin embedding, either (i) cells were scraped, spun down and resuspended in non-supplemented PIPES buffer, or (ii) the fixative of adherent cells was exchanged with non-supplemented PIPES buffer.

For epoxy resin embedding, the suspended or adherent cells were washed two times with 100 mM cacodylate buffer, post-fixed with 2% OsO₄ solution containing 1.5% potassium ferricyanide for 1 hour, and stained en block with 1.5% aqueous uranyl acetate for 30 min. The suspended cells were then dehydrated using a graded ethanol series and propylene oxide, and embedded in epoxy resin (Sigma-Aldrich; St. Louis/MO, USA). The cell monolayer was dehydrated using a graded ethanol series and detached from the bottom of the culture dish by dissolving the plastic with propylene oxide followed by rapid, vigorous pipetting. The cells were then washed four times with propylene oxide to remove the remains of dissolved plastic and thereafter embedded in epoxy resin. Ultrathin sections of 60–70 nm were cut with an ultramicrotome (Leica Ultracut UCT), stained with 0.2% lead citrate (Taраб; Berks, England) in 0.1 M NaOH for 20 s and examined in a Philips CM100 transmission electron microscope.

For the stereological analysis of Golgi cisternae, two whole samples from two blocks of each sample were systematically sampled at 900× magnification in order to estimate the cytoplasmic area (volume). Within these micrographs all areas containing identifiable Golgi cisternae were selected and imaged at 8,900× magnification. To analyze the micrographs, a stereological test grid with horizontal and vertical lines was used. The total volume of the cytoplasm, which represented a reference space, was estimated by counting the number of test points over the cytoplasm. In order to estimate the total length of the Golgi cisternal membrane, the number of intersections of all identifiable Golgi membrane with horizontal test lines was counted. In addition, the number of Golgi cisternae - defined as an elongated enclosed membrane profile with a length minimum twice its breadth - was counted in these images. From these values the ratio of the total number of intersections (length) of Golgi cisternal membranes to the total volume of cytoplasm (relative surface density of cisternae) was estimated, as was the relative number of cisternae per cytoplasmic volume for the different conditions. At least 80 cell profiles were analyzed for each sample, and two independent experiments were performed. Within each experiment indices of the estimated values were calculated as ratios compared to the wild-type sample.

Supporting information

Supporting information includes eight figures (Figure S1, S2, S3, S4, S5, S6, S7, S8), three tables (Table S1, S2, S3) and two movies (Movie S1, S2) and can be found with this article online.

Accession numbers

The GenBank [http://www.ncbi.nlm.nih.gov/Genbank] accession numbers for the proteins discussed in this paper are *L. pneumophila* Icm/Dot T4SS (Y13044), LegG1/Lpg1976 (YP_095992), SidC (AY504673), *D. discoideum* calnexin (AF073037), human Ran GTPase (CAG29934) and RanBP1 (CAG30442).

Supporting Information

**Figure S1** Effects of siRNA treatment on intracellular replication, cytotoxicity or protein depletion efficiency. (A) Untreated A549 lung epithelial cells immuno-stained for β-tubulin (green); nuclei were labeled with DAPI (grey). Microtubule polymerization was analyzed by immuno-fluorescence microscopy. Bars, 10 μm or 5 μm (insets). A549 cells were treated for 2 days with 10 nM of four different siRNA oligonucleotides per target (Table S3). AllStars negative control siRNA (Qiagen) served as negative control. (B) Intracellular replication of GFP-producing *L. pneumophila* harboring pNT28 was monitored over 2 days and quantified by fluorescence measurement. The data represent mean and standard deviation of three independent experiments. (C) Cytotoxicity of siRNA treated cells was assessed by adding propidium iodide (1 μg/ml, 15 min) to detached cells, followed by flow cytometry analysis. (D) The depletion efficiency of the siRNA treatment was assessed by Western blot using antibodies against Ran or RanBP1. Loading control: glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

(TIF)

**Figure S2** Icm/Dot-dependent translocation of LegG1. RAW264.7 macrophages were infected (MOI 20) with *L. pneumophila* wild-type strain JR32 or ΔicmT harboring pXDC61-legG1, pXDC61-ΔlegG1 or pXDC61-fahl encoding TEM β-lactamase fusion proteins. Enzymatic activity was assayed through hydrolysis of the fluorogenic substrate CCF4/AM (emission ratio 460/530 nm).

(TIF)

**Figure S3** Analysis of Ran GEF activity of LegG1 in vitro. (A) LegG1 does not show GEF activity toward Ran:mantGDP in vitro. Purified His₆-LegG1 (620 nM) and RCC1 (100 nM) were sequentially added as indicated to 1 μM purified human Ran.
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GTPase loaded with fluorescent mantGDP. The addition of His6-LegG1 did not stimulate mantGDP release from Ran:mantGDP in presence of excess GTP (100 μM), whereas the human Ran GEF RCC1 significantly accelerated mantGDP-GTP exchange, as indicated by a rapid exponential change in mant-fluorescence. Fluorescence has been corrected for dilution effects. (B) Production of Ran/GTP in lysates of A549 cells treated with purified His6-LegG1 or RCC1, or (C) purified LegG1-His6 or LegG1-N223A-His6, in presence of excess GTP (100 μM). Activated Ran was immuno-precipitated with an antibody specifically recognizing Ran/GTP (and visualized by Western blot) and used by Western blot using an anti-Ran antibody. Loading control: Western blot of Ran in samples before immuno-precipitation. (TIF)

Figure S4  Replication of L. pneumophila ΔlegG1 in amoebae. (A) A. castellanii amoebae were infected (MOI 20) with L. pneumophila wild-type, ΔicmT, or ΔlegG1 harboring pNT28 (GFP), and intracellular growth (“single round replication”) was monitored by GFP fluorescence. Representative time course from a single experiment is shown (12 samples per strain), indicating mean fluorescence and 95% confidence intervals; data are representative of at least 3 independent experiments. (B) D. discoideum was infected (MOI 1) with L. pneumophila wild-type strain JR32, ΔlegG1 or ΔicmT, and bacteria released into the supernatant were quantified by CFU. (TIF)

Figure S5  Amoebae competition assay of L. pneumophila ΔsidM. L. pneumophila ΔsidM is not outcompeted by wild-type bacteria in the amoebae competition assay. A. castellanii was co-infected (1:1 ratio, MOI 0.01) in 96-well plates with L. pneumophila wild-type and the ΔsidM mutant strain, and grown at 37°C for 21 d. Every third day the supernatant and lysed amoebae were diluted 1:1000, fresh amoebae were infected (50 μl homogenate per 200 μl culture), and aliquots were plated on CYE agar plates containing kanamycin or not to determine CFU. The data shown are means and standard deviations of triplicates and representative of 3 independent experiments. (TIF)

Figure S6  Toxicity and effector translocation of L. pneumophila ΔlegG1 strains overproducing SidM or SidC. (A) For toxicity assays RAW264.7 macrophages were infected (MOI 10, 4 h) with L. pneumophila wild-type harboring pCR033 (vector), pCR034 (M45-SidC) or pEB201 (M45-SidM), detached from the wells by scraping, stained with propidium iodide (1 μg/ml) and analyzed by flow cytometry. (B) To assay translocation efficiency, HeLa cells were infected (MOI 100, 1 h) with L. pneumophila wild-type, ΔicmT or ΔlegG1 harboring the vector pCR033, or with ΔlegG1/ pSU19 (M45-LegG1), washed several times and lysed with 1% digitonin, 25 μl lysate of uninfected or infected HeLa cells or wild-type L. pneumophila were separated by SDS PAGE and stained with Coomassie Brilliant Blue, or were subject to Western blot using an anti-SidC antibody to quantify the amount of translocated effector protein. (TIF)

Figure S7  Uptake and LCV formation of L. pneumophila ΔlegG1. L. pneumophila ΔlegG1 is not impaired for uptake and LCV formation. (A) A. castellanii or (B) D. discoideum was infected (MOI 20, 45 min) with GFP-producing L. pneumophila wild-type, ΔlegG1 or ΔicmT harboring pCR076, or with ΔlegG1/pPER4 (M45-LegG1), and uptake was determined by flow cytometry. (C) D. discoideum producing calnexin-GFP was infected (MOI 50, 1 h) with DsRed-producing L. pneumophila wild-type, ΔlegG1 or ΔicmT harboring pCR077, or with ΔlegG1/pER5 (M45-LegG1). The percentage of calnexin-GFP-positive LCVs (n = 100/strain, 4 independent experiments) was scored in lysates of infected cells. (TIF)

Figure S8  Comparison of L. pneumophila LegG1 with human RCC1. (A) Amino acid sequence of the 31.2 kDa L. pneumophila protein LegG1/Lpg1976 (280 amino acids). The three RCC1 domains, which are predicted by the PROSITE program (http://prosite.expasy.org/), are highlighted in red. (B) Schematic overview and position of RCC1 domains in L. pneumophila LegG1 and human RCC1 Ran GEF. (C) Alignment of the three RCC1 domains of LegG1 with a single RCC1 domain of RCC1. (D) Predicted structure of LegG1 (Plyre2; http://www.sbg.bio.ic.ac.uk/plyre2) and comparison with the X-ray crystallography structure at 1.7 Å resolution of human RCC1 forming a seven-bladed propeller (Renault et al. 1998) Nature 392: 97–101. (TIF)

Movie S1  Motility of LCVs in D. discoideum infected with L. pneumophila wild-type. D. discoideum amoebae producing calnexin-GFP were infected (MOI 10) with L. pneumophila wild-type harboring pSW6001 (DsRed). Two hours post infection, the trafficking of LCVs was recorded by laser confocal scanning microscopy for 5 min, and images were taken every 15 s. The speed of LCVs was determined by tracking the migration distance of LCVs over time. Bars, 1 μm. (AVI)

Movie S2  Motility of LCVs in D. discoideum infected with L. pneumophila ΔlegG1. LCVs harboring L. pneumophila ΔlegG1 show impaired motility. D. discoideum amoebae producing calnexin-GFP were infected (MOI 10) with L. pneumophila ΔlegG1 mutant bacteria harboring pSW6001 (DsRed). Two hours post infection, the trafficking of LCVs was recorded by laser confocal scanning microscopy for 5 min, and images were taken every 15 s. The speed of LCVs was determined by tracking the migration distance of LCVs over time. Bars, 1 μm. (AVI)

Table S1  Strains and plasmids. (DOCX)

Table S2  Oligonucleotides used in this study. (DOCX)

Table S3  Oligonucleotides used for RNA interference. (DOCX)

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Author Contributions

Conceived and designed the experiments: ER CH CFH HH. Performed the experiments: ER GP CH CFH HG UR MH. Analyzed the data: ER GP CH CFH HG UR AMT AI HH. Contributed reagents/materials/analysis tools: SW AB GG. Wrote the paper: ER CH CFH HH.
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