Translocated *Legionella pneumophila* small RNAs mimic eukaryotic microRNAs targeting the host immune response

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*Legionella pneumophila* is an intracellular bacterial pathogen that can cause a severe form of pneumonia in humans, a phenotype evolved through interactions with aquatic protozoa in the environment. Here, we show that *L. pneumophila* uses extracellular vesicles to translocate bacterial small RNAs (sRNAs) into host cells that act on host defence signalling pathways. The bacterial sRNA RsmY binds to the UTR of *ddx58* (RIG-I encoding gene) and cRel, while tRNA-Phe binds *ddx58* and *irak1* collectively reducing expression of RIG-I, IRAK1 and cRel, with subsequent downregulation of IFN-β. Thus, RsmY and tRNA-Phe are bacterial trans-kingdom regulatory RNAs downregulating selected sensor and regulator proteins of the host cell innate immune response. This miRNA-like regulation of the expression of key sensors and regulators of immunity is a feature of *L. pneumophila* host-pathogen communication and likely represents a general mechanism employed by bacteria that interact with eukaryotic hosts.
Intracellular bacterial pathogens communicate with their hosts to allow pathogen entry, persistence, and replication within the host cells. This is mainly achieved by translocating bacterial protein effectors into the host cell through dedicated secretion systems. The translocated proteins manipulate different host cell processes and signalling pathways. The Gram-negative bacterium Legionella pneumophila, a parasite of aquatic protozoa and a feared pathogen when reaching the human lungs, is one of these pathogens. In the environment, L. pneumophila replicates intracellularly in freshwater protozoa and during human disease, a severe pneumonia called Legionnaires’ disease, it replicates in alveolar macrophages. The capacity to replicate in these immune cells is thought to stem from the ability to replicate within protozoan cells, as intracellular replication is similar in both hosts.

Genome sequence analysis has revealed a unique feature of this pathogen, namely the presence of many proteins similar to eukaryotic proteins suggesting that mimicry of eukaryotic functions allows L. pneumophila to subvert host pathways and to replicate in these cells. Indeed, many studies thereafter showed that these proteins are secreted effectors of the Dot/Icm type IV secretion system and are part of the over 330 translocated effector proteins of L. pneumophila. Evolutionary analyses strongly suggested that they had been acquired by horizontal gene transfer from their protozoan hosts during co-evolution.

The translocation of these different bacterial proteins in the host cell is a vital part of a successful infection. They are subverting numerous host pathways such as ubiquitin signalling, the sphingolipid metabolism and autophagy. Rab proteins and target different organelles such as the nucleus or mitochondria.

Given the high number of protein effectors that mimic eukaryotic functions that have been identified in the Legionella genomes, it is tempting to hypothesize that L. pneumophila might also mimic eukaryotic RNAs such as miRNAs to interfere with eukaryotic regulatory mechanism. If it is the case, this raises another question, how the bacterial RNA is transported into the eukaryotic cell? One possibility could be that it is mediated by extracellular vesicles (EVs) shed from bacteria. EVs produced by bacteria, archaea, and eukaryotic cells, are increasingly recognized as important mediators of intercellular communication via transfer of a wide variety of molecular cargoes. They have been implicated in many aspects of cell physiology such as stress response, intercellular competition, lateral gene transfer (via RNA or DNA), pathogenicity, and detoxification. The pathophysiological roles of EVs are beginning to be recognized in diseases including cancer, infectious diseases, and neurodegenerative disorders, highlighting potential novel targets for therapeutic intervention.

Furthermore, recent studies highlight that the role of EVs in intercellular communication and in pathogenicity might have been largely under-valued so far. Recently, EVs have gained great attention for their proposed roles in cell-to-cell communication, and as biomarkers for disease. Indeed, EVs have been implicated in many functions as mediators of near and long distance communication between eukaryotic cells and as mediators of cell–cell and trans-kingdom communication in host-pathogen interactions.

L. pneumophila is well known to release EVs when grown in laboratory media. These L. pneumophila-derived EVs (Lp-EVs) can be internalized by macrophages during cellular co-incubation and are able to fuse with eukaryotic membranes in vitro, as suggested by analyses of Lp-EVs with model-membranes. Furthermore, when mouse macrophages were infected with such Lp-EVs the fusion of the phagosome with the lysosome was inhibited, like during infection of human macrophages with L. pneumophila bacteria. Recently, it has been shown that Lp-EVs, when incubated with THP-1 macrophage-like cells are potent pro-inflammatory stimulators of macrophages. At later time points, EVs seem to facilitate L. pneumophila replication by miR-146a-dependent IRAK-1 suppression. It was suggested that EVs might thereby promote the spreading of L. pneumophila in the host. A first characterization of the proteome content of Lp-EVs identified 33 proteins enriched in the EVs, some of which possess proteolytic and lipolytic enzyme activities, which may contribute to the destruction of the alveolar lining during infection. However, it is not known whether Lp-EVs contain also RNAs.

Here we show that L. pneumophila releases EVs in vitro and in cellulo during infection of the human U2OS cell line, human THP-1 monocytic cells, and human monocye-derived macrophages (hMDMs). These Lp-EVs contain bacterial RNAs that are transported into the host cell where they downregulate IRAK1 and RIG-I, most likely by mimicking eukaryotic miRNAs.

Results

L. pneumophila extracellular vesicles are enriched in bacterial small RNAs. Like many bacteria, L. pneumophila produces EVs during extracellular and intracellular growth. To analyze the shape and structure of these Lp-EVs we purified them from broth culture and imaged them by uranyl acetate negative staining transmission electron microscopy and Cryo-TEM. On average, we purified 0.6–1.0 × 10⁸ Lp-EVs from a 500 ml L. pneumophila culture grown to post-exponential phase. As shown in Fig. 1A, the Lp-EVs are mostly spherical structures harbouring single membrane bilayers ranging from around 20 to 200 nm in diameter. Moreover, the Cryo-TEM analyses revealed the presence of tube-shaped vesicles and double membrane bilayer vesicles (Fig. 1B). To analyze whether they contain RNA molecules, we used Vybrant™ DiD solution, a lipid dye to stain the membranes of the EVs, and Syto®RNA-Select to label the RNA content if present. Indeed, Lp-EVs stained with the DiD membrane stain (red) and the selective RNA dye (green) could be visualized (Fig. 1C).

To quantify the amount of purified Lp-EVs and estimate their size nanoparticle tracking analyses (NTA) was used after staining the putative Lp-EVs with Vybrant™ DiD to stain the membranes of the EVs. Although we used a size filtration column to remove excess of free dye, the presence of aggregated dye within our samples, or the presence of other large protein/lipid aggregates emanating from L. pneumophila could not be completely ruled out. Thus, we added a well-established sucrose floatation step to the isolation procedure. We first analyzed the size distribution and the number of particles pre- and post-floatation, through light scattering mode revealing that the number of particles was moderately decreased after floatation likely due to the three additional ultracentrifugation steps required for the floatation procedure (Fig. 1D, E). Particles in both samples showed a median size of ~130 nm (Fig. 1E). In addition, we compared particles size and concentration when measured in fluorescence mode to analyze Lp-particles labelled with the red-lipophilic dye. Size distribution was similar (Fig. 1F, right panel), and ~85% of the particles were positive for the membrane dye, consistently with our FACS data (Supplementary Fig. 1) and previous studies. Possible differences in size between the analyses are probably due to the different accuracy of the various methods used here, a phenomenon described in detail by Bachurski and colleagues who have compared the different methods. Together these results suggest that most of the Lp-derived nanoparticles considered in our study are indeed Lp-EVs.

However, we could not use the NTA to measure the percentage of Lp-EVs positive for the RNA dye as the set-up of the machine was not compatible with the fluorescent properties of the RNA-dye. Thus, we used conventional flow cytometry analyses, as the NTA results with respect to the size distribution and the
Quantification were comparable to determine the percentage of Lp-EVs that contains RNA molecules (Supplementary Fig. 1A). For standardization, we used Megamix-Plus SSC (BioCytex) beads, a mix of fluorescent beads of varied diameters selected to cover a major part of the theoretical microparticle size range (0.1–0.5 µm), as a size-related parameter. Bead acquisition allowed setting the cytometer to study microparticles within a constant size region and obtaining reproducible microparticle counts by flow cytometry (Supplementary Fig. 1B). Quantification showed that approximately 30% of the examined Lp-EVs contained detectable amounts of RNA (Supplementary Fig. 1C). We thus isolated the RNA from the Lp-EVs and analyzed the size
L. pneumophila sRNAs contained in Lp-EVs show similarity to human microRNAs. To characterize the RNA molecules present, and to identify those enriched in Lp-EVs we performed RNAseq analyses. Four independent RNAseq libraries were constructed from RNA isolated from purified Lp-EVs and as control from the bacterial pellets. The RNAseq libraries were compared to those sequenced using an Illumina platform. The sequences obtained were mapped to human, L. pneumophila and E. coli genomes using the usearch software (http://www.mirbase.org/). We conducted the search with a cut-off E-value <1245. This revealed that segments of 15 of the 20 L. pneumophila sRNAs showed similarity to different hsa-miRs (Table 1). Most interestingly, the nucleotides between 48 and 65 of RsmY showed similarity to the hsa-miR144-3p, a microRNA predicted to interact with the UTR of mRNAs coding for proteins involved in the host immune response to pathogens, such as ddx58 mRNA (coding for RIG-I), rel mRNA (coding for c-Rel, a NFkB subunit) or mapk8 mRNA (coding JNK1). The nucleotides located either in the coding region or the untranslated region (UTR) of genes (Table 1).

To predict possible functions in the host cells we investigated whether certain of these sRNA might show similarities to human microRNA (hsa-miR) sequences using the miRBase database (http://www.mirbase.org/). We conducted the search with a cut-off E-value <1245. This revealed that segments of 15 of the 20 L. pneumophila sRNAs showed similarity to different hsa-miRs (Table 1). Most interestingly, the nucleotides between 48 and 65 of RsmY showed similarity to the hsa-miR144-3p, a microRNA predicted to interact with the UTR of mRNAs coding for proteins implicated in the host immune response to pathogens, such as ddx58 mRNA (coding for RIG-I), rel mRNA (coding for c-Rel, a NFkB subunit) or mapk8 mRNA (coding JNK1). The nucleotides located either in the coding region or the untranslated region (UTR) of genes (Table 1).

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Table 1 The twenty most enriched RNA molecules in the Lp-EVs as determined by RNAseq analyses.

| Label/DNA region | Annotation | log2FC | norm Lp-EV reads | Similarity |
|------------------|------------|--------|-----------------|------------|
| lpp0294 (f):     | UTR/start  | 13     | 13 184          | hsa-miR 329-3p |
| lppnc374         | tRNA (anti lpp1558) | 12     | 77 201          |             |
| lpt29            | tRNA-Phe   | 12     | 304 066         | hsa-miR 5001-3p + 4451 |
| lpt25            | tRNA-Pro   | 12     | 280 164         |             |
| lpt08            | tRNA-Pro   | 10     | 29 498          | hsa-miR 6813-5p + 152-5p |
| lpt37            | tRNA-Met   | 9      | 33 524          |             |
| lpt0956 (f):     | UTR        | 9      | 6 110           | hsa-miR 4775 |
| lppnc0001        | tRNA RsmY  | 9      | 349 673         | hsa-miR 144-3p |
| lpt04            | tRNA-Tyr   | 9      | 81 954          | hsa-miR 4687-3p + 681-5p |
| lpt39            | tRNA-Val   | 8      | 104 797         | hsa-miR 323a-5p + 619-3p |
| lpt38            | tRNA-Pro   | 8      | 44 788          | hsa-miR 6816-3p |
| lpt12            | tRNA-Leu   | 8      | 26 718          |             |
| lppnc0692        | sRNA (intergenic lpp2966/67) | 8      | 25 277          | hsa-miR 6807-5p + 4773 |
| lpt34            | tRNA-Leu   | 8      | 327 716         |             |
| lpt43            | tRNA-Leu   | 7      | 134 92          | hsa-miR 6742-3p |
| lpt0111 (f):     | CDS        | 7      | 17 070          | hsa-miR 3619-5p |
| lpt0103 (f):     | CDS        | 7      | 9 863           | hsa-miR 4637 + 6751-3p |
| lpt06            | tRNA-Thr   | 6      | 648 296         | hsa-miR 302b-5p + 6077 |
| lppnc0047        | sRNA       | 6      | 6 946           | hsa-miR 1290 + 329-5p |
| lpt41            | tRNA-Met   | 6      | 845 116         | hsa-miR 769-5p |

*aLabel/DNA region: sRNA label as defined in Sahr et al., 2017. 
*bLog2FC: Enrichment of RNA molecules in the Lp-EVs as compared to the bacterial culture defined as log 2 fold change (FC). 
*cnorm Lp-EV reads: Number of reads detected for each sRNA within the Lp-EVs after normalization. 
*dSimilarity, similarity found to a human sapiens micro RNAs (has-miR), 5p, 3' end, 3p, 3'end, inverted, similarity with the inverted sequence; CDS coding sequence.
mav, ago1, and mapk9 mRNAs (coding for IRAK1, RIG-I, MAVS, AGO1, and JNK2, respectively). Several of these human target mRNAs, such as the retinoic-acid inducible gene I (RIG-I, encoded by the ddx58 gene) IRAK1, or MAVS are key players in sensing pathogen-associated molecular patterns and their downstream immune signalling.\(^{16}\) Thus, RsmY and tRNA-Phe are promising candidate RNAs contained within Lp-EVs that might impact the innate immune response of host cells.

RIG-I and IRAK1 protein levels are suppressed in the host cell in an Lp-EV and RsmY-dependent manner. To determine whether Lp-EVs and specifically the sRNA RsmY modulates the RIG-I-like receptor (RLR) and the Toll-like (TLR) receptor signalling pathway of infected host cells we analyzed the levels of RIG-I and IRAK1 proteins after \(8\) h of infection of THP-1 cells with \(L.\) pneumophila wt or its isogenic rsmY mutant strain (\(\Delta rsmY\)). In parallel we incubated THP-1 cells with Lp-EVs purified from wt \(L.\) pneumophila or from the \(\Delta rsmY\) strain. We choose RIG-I because of the similarity of RsmY with hsa-miR144-3p, which is predicted to influence RIG-I expression, and IRAK1 because it has been described previously, that \(L.\) pneumophila infection leads to a suppression of IRAK1 protein levels\(^{19}\) and, according to the prediction described above, IRAK1 would be affected by tRNA-Phe but not by RsmY. Indeed, RIG-I protein levels were significantly downregulated in THP-1 cells infected with the wt bacteria, but not when infected with the \(\Delta rsmY\) strain. IRAK1 levels were also down regulated as reported previously, but this regulation was independent of RsmY as supposed (Fig. 1G). As the tRNA-Phe gene has only a single locus in the \(L.\) pneumophila genome, it was not possible to construct a viable knock out mutant to study the impact of tRNA-Phe in this way. Most importantly, RIG-I and IRAK1 protein levels were downregulated in a similar manner when THP-1 cells were incubated with purified Lp-EVs only and the downregulation of RIG-I but not IRAK1 was lost when the Lp-EVs had been purified from the \(\Delta rsmY\) strain (Fig. 1H). This phenomenon was also observed when incubating human monocyte derived macrophages (hMDM) with Lp-EVs purified from wt \(L.\) pneumophila or the \(\Delta rsmY\) strain (Supplementary Fig. 2A). Taken together, the observed downregulation of RIG-I seems to be at least partially RsmY-dependent during infection as well as after incubation with Lp-EVs. In contrast, although IRAK1 levels were lowered after \(8\) h post infection with \(L.\) pneumophila bacteria and after \(3\) h incubation with Lp-EVs, this was independent of RsmY (Fig. 1G, H).

To better understand the impact of the sRNA contained within the Lp-EVs on RIG-I and IRAK1 we aimed to exclude the effects of other factors on the surface of EVs, such as LPS, lipopeptides, or effector proteins that might be inside the Lp-EVs that may influence immune signalling regulated by RIG-I and IRAK1. Thus, we transfected THP-1 cells either with in vitro transcribed RsmY, or in vitro transcribed tRNA-Phe and as control (ctrlRNA) with an unrelated RNA or alternatively, with the sequences complementary to RsmY or tRNA-Phe using lipofectamine or electroporation, respectively. Both methods yielded comparable results. When transfecting with either of the sRNA, the RIG-I levels were up-regulated, which is expected as RIG-I is a major cytosolic RNA sensor in the cell sensing the presence of non-self RNAs. However, when comparing ctrlRNA transfection to RsmY and tRNA-Phe transfection, it becomes evident that RsmY and tRNA-Phe transfection led to a significantly reduced induction of the RIG-I protein level, as predicted (Fig. 1I and Supplementary Fig. 2B). Strikingly, IRAK1 protein levels were suppressed only after transfection with tRNA-Phe-RNA, but not with RsmY-RNA (Fig. 1I and Supplementary Fig. 2B). The above-described results indicated that RsmY and tRNA-Phe are implicated in regulating RIG-I and IRAK1, respectively. Indeed, RNA interaction assays of RsmY and tRNA-Phe with the UTR of ddx58 (RIG-I) or irak1 mRNA revealed that in vitro RsmY interacts with the UTR of ddx58 and tRNA-Phe with the UTR of irak1 and ddx58 (Fig. 1J) further suggesting that RsmY-RNA is not responsible for the Lp-EV-dependent IRAK1 suppression. Interestingly, interaction between RsmY and the UTR cRel used as control, was also observed but not of tRNA-Phe. This goes in hand, with the analyses of the protein levels of cRel as they were only slightly reduced after transfection of RsmY, but not after transfection of tRNA-Phe or ctrlRNA (Fig. 1J and Supplementary Fig. 2B) indicating that RsmY might have additional targets besides RIG-I, such as cRel.

Lp-EVs shed during infection contain the sRNA RsmY. Recently it has been shown that bacterial RNA bound to an RNA binding protein might potentiate interferon-\(\beta\) production by binding to RIG-1\(^{17}\), however direct action of bacterial sRNAs on host genes is not known yet. Our results strongly suggest that RsmY may act as a transkingdom RNA regulator in the host cell, as it interacts with RIG-I and c-Rel mRNA and is involved in the regulation of their expression (Fig. 1G–I) and was also found enriched in Lp-EVs. To perform its regulatory actions in the host cell, RsmY should be present in Lp-EVs shed from \(L.\) pneumophila during infection of human macrophages. Using fluorescent in situ hybridization (FISH) we analyzed if Lp-EVs can enter hMDM, and if we can detect RsmY within these Lp-EVs after internalization. After \(5\) h of incubation of hMDM with Lp-EVs cells were fixed and hybridized with two RsmY-specific FISH probes (Supplementary Table 1). As shown in Fig. 2A, RsmY packed in Lp-EVs were identified in the host cytoplasm. We then infected hMDMs with the \(L.\) pneumophila wt strain, fixed them after \(5\) h of infection, and stained with the RsmY specific FISH probe. Indeed, we also could detect the RsmY signal in Lp-EV-like structures (Supplementary Fig. 2C), suggesting that RsmY is transported during infection in EVs into the host cell.

Lp-EVs are taken up by the host cell and seem to escape from the Legionella containing vacuole (LCV) to the cytosol. Using electron microscopy previous studies have shown that \(L.\) pneumophila produces EVs when grown in broth culture as well as during intracellular growth after \(24\) h of infection of the amoeba Dictyostelium discoideum\(^{40}\). Here we used fluorescent confocal microscopy and imaging in living cells to follow internalized Lp-EVs from early time points of uptake as well as during infection of human cells. First, we incubated U2OS cells stably expressing Sec61β-GFP, an ER marker, with purified Lp-EVs stained with DiD dye as described above. As expected, the Lp-EVs entered the U2OS Sec61β-GFP cells (Supplementary Fig. 2D). To understand, by which pathway the Lp-EVs are taken up, we followed the entry of the purified EVs through the plasma membrane in presence or absence of cytochalasin D an inhibitor of actin polymerization and phagocytosis or in presence or absence of dynasore, a dynamin inhibitor\(^{48}\) acting on clathrin and lipid-raft mediated entry. Cytochalasin D and to a lesser extent also dynasore reduced the entry of Lp-EVs significantly (Supplementary Fig. 2E). Thus Lp-EVs seem to be taken up preferentially via actin-dependent phagocytosis or macropinocytosis. However, also some dynamin-dependent internalization occurs indicating that Lp-EV internalization may involve multiple pathways as proposed for eukaryotic EVs\(^{48}\). To determine the localization of Lp-EVs shed from \(L.\) pneumophila during infection we used confocal microscopy of fixed cells as well as confocal imaging of living cells. \(L.\) pneumophila...
was stained with DiD and washed carefully to avoid any dye excess. These bacteria were used to infect the U2OS Sec61β-GFP cells or hMDMs in 96 well plates. If Lp-EVs were shed during infection they should also be stained with DiD and thus their budding off from the bacteria and their localization in the host cell should be visible. To record such events, we used an automated confocal microscope to acquire images every 30 min after infection with DiD-labelled L. pneumophila thus imaging hundreds of living cells up to 7 h post-infection. As shown in Fig. 2B, after >3 h, little vesicle-like DiD-labelled structures, the Lp-EVs, were visible outside of the predicted LCVs that are closely surrounding the bacteria. The number of Lp-EVs in the host cell cytosol increased over time with L. pneumophila remaining intact during the infection followed over 7 h. We repeated this experiment by infecting hMDMs with DiD-labelled L. pneumophila and fixing the cells at 5 h post-infection. Again, we observed DiD labelled vesicles, indicating that L. pneumophila produces Lp-EVs in both, U2OS cells and primary macrophages. We then used 3D confocal time-lapse imaging of living, infected U2OS-Sec61β-GFP cells, where we acquired Z-stack images every 30 s for 18 min to capture the event of Lp-EV “release” from the bacteria in real time. Indeed, as shown in Movie 1 and Supplementary Fig. 3A we observed the release of DiD-labelled EVs from L. pneumophila, showing that this happens during infection in living, human cells. Taken together, we show that L. pneumophila shed EVs during infection of human host cells of which about 30% contain specific sRNAs that are transported within these Lp-EVs across the plasma and LCV membranes in the host cell cytosol.

**Fig. 2 Lp-EVs are shed during infection and contain RsmY.** A Representative images of three independent experiments from FISH analyses using probes specific for RsmY. hMDM cells incubated with purified DiD-labelled Lp-EVs; Internalized, DiD-labelled Lp-EVs (white) 5 h post-infection. Blue, nucleus fluorescently stained using Hoechst 33342. B Human bone osteosarcoma epithelial cells stably expressing Sec61β-GFP for ER labelling (U2OS- Sec61β cells; first three panels) and human monocyte-derived macrophages (hMDM) (right panel) were infected with DiD-labelled L. pneumophila grown until post-exponential phase (OD4.2). Images of living infected U2OS- Sec61β cells after 1, 4, and 7 h of infection at 37 °C (5% CO2) taken with an automated confocal microscope (Opera) are shown. Upper panel shows the cells with the green fluorescence labelling of the ER, lower panel shows the same cells without the green channel. Representative images of three independent experiments. White: DiD-labelled L. pneumophila and DiD-labelled Lp-EVs shed during infection; Green: ER; Blue: Nucleus (Hoechst 33342). Right panel shows fixed hMDM cells, 5 h post-infection, analyzed by confocal microscopy. White: L. pneumophila and Lp-EVs; Blue: Nucleus (Hoechst 33342).
cells were stained with Hoechst 33342 to visualize the DNA/nucleus, with MitoTracker to visualize the mitochondria, or with LysoTracker to reveal acidic organelles. We then used fluorescence-based high content dynamic imaging to follow the internalized Lp-EVs in living cells over time (Fig. 3A). The Lp-EVs seemed to co-localize with the ER and to acidic organelles, but not with mitochondria (Fig. 3A). To quantify the events, we applied High-Content Analyses (HCA) of single cells. The DiD signal was used to detect Lp-EVs in the cytoplasm of U2OS-Sec61β-GFP cells and the fluorescence of Mitotracker, Lyso-tracker, Sec61β-GFP were measured to analyze in hundreds of living cells at the single cell level if Lp-EVs co-localize with
mitochondria, acidic organelles, or the ER. As shown in Fig. 3B the quantitative analyses confirmed that starting from 1.5 h post incubation, automatically detected Lp-EVs have statistically significantly enriched mean fluorescence intensity overlapping with GFP and lysoTracker signal, but not with Mitotracker, suggesting that Lp-EVs co-localize with the ER and acidic organelles, not with mitochondria. Similarly, when using hMDM cells and pHrodo to label acidic structures and tracking the Lp-EVs within these cells for 17 h the Lp-EVs clearly co-localize with acidic subcellular structures (Supplementary Fig. 3B), but overall intensities and differences were less pronounced than in the U2OS cell lines. High content analyses also allowed to analyze the number of cells that contained Lp-EV. This revealed that an average of 15% of the U2OS cells contained Lp-EVs and thus an important background noise is present in in cellulo experiments (Supplementary Fig. 3C). For a more in depth analyses, we labelled U2OS cells with CellLight Fluorescent Protein Labelling to visualize Rab5, a marker for early endosomes and Rab7, a marker for late endosomes, and incubated cells with purified DiD-labelled Lp-EVs (Fig. 3C). Again, we used automatic confocal microscopy to acquire images of hundreds of living, infected cells every 30 min. We observed that single vesicles originally co-localizing with Rab5-early endosomes (EE) seem to exclusively colocalise with Rab7-late endosomes (LE) at later time points, thus indicating that the Lp-EVs might follow the endosomal pathway. When using an HCA approach to analyze Lp-EVs in more than 3000 single, infected cells our results indicated that Lp-EVs colocalise at early time points slightly more frequently with the EE whereas at later time points (>3.5 h) correlation rates seem to shift more to LE (Fig. 3D). To further investigate the path of Lp-EVs within living cells, we followed labelled Lp-EVs in single living cells by confocal microscopy in time-lapse experiments and recorded their localization over time. Time-lapse confocal 3D reconstructed movies showed clearly that Lp-EVs transit from EE-related to LE-related EVs over time (Movie 2 and Supplementary Fig. 3D). Thus, we propose that about 10% of the total Lp-EVs analyzed are transported within the endosomal network towards the ER presumably following a similar pathway as observed for exosome-mediated miRNA transfer between eukaryotic cells49-51.

To examine whether Lp-EVs release their content in the host cell cytosol, we developed a content release assay based on a recent study that followed the delivery of a soluble EV-cargo (HSP70, human homologue of GroEL) within the cytosol of the acceptor cells52. This assay was upgraded by taking advantage of split-luciferase complementation system52. Briefly, an EV cargo was tagged with HiBiT (split luciferase 1/2) and isolated EVs were incubated on acceptor cells expressing LgBiT (Split luciferase 2/2) within their cytosol. Luciferase complementation only occurs when EV deliver their cargo into the cytosol of acceptor cells. We had previously shown that the bacterial protein GroEL (lp00743) is present in the Lp-EVs. Thus, we tagged GroEL with a HiBiT-tag and in parallel, we transfected THP-1 cells with a LgBiT construct (pCMV-Tag2-LgBiT) under the control of the CMV promoter to express the LgBiT protein in the host cell. If the EV-content is released, Luciferase complementation occurs, and this can be measured. To determine whether the Lp-EVs had released their content, we measured luciferase activity in cellulo with the NanoGlo(R) Live Cell Assay System (Promega) after 3 h of incubation with purified Lp-EVs containing GroEL-HiBiT. To estimate the total input of Lp-EVs containing GroEL-HiBiT, the luciferase activity was quantified after lysis of the Lp-EVs using the NanoGlo (R) HiBiT Lytic detection System (Promega). Lp-EVs not containing GroEL-HiBiT were used as negative control. As shown in Fig. 3E, we could detect around 3% luciferase activity of the total GroEL-HiBiT input after 3 h post-infection. This result also corresponds to our co-localization experiments, where after 3 h pi around 5% of Lp-EVs co-localized with early or late endosomes, respectively (Fig. 3D). When adding 10 μM of cytochalasin D to the cells, the uptake and/or release was completely abolished (Fig. 3D), suggesting that actin-dependent processes play a crucial role in the endocytosis and/or membrane fusion events.

Strikingly, after the addition of 200 nM Bafilomycin A1, which inhibits endosomal acidification, less than 1% of the total luciferase activity was detected after 3 h pi meaning that less than a third of GroEL-HiBiT proteins were reacting with cytosolic LgBiT protein of the THP-1 cells compared to non-BA1-treated samples. As it was shown recently that Bafilomycin A1 does not change the general uptake of EVs into the host cell9, this is another hint that the acidification in late endosomes or lysosomes might be an important factor for Lp-EV content release.

Taken together, we propose that internalized Lp-EVs can follow the endosomal pathway of the host cell leading to transient contacts with the ER ("ER scanning") where the bacterial sRNA could be released ("endosomal or lysosomal escape")53-57, at least partially dependent on an acidic environment, as previously proposed for eukaryotic EVs58. There, the bacterial sRNA like RsmY might be processed and/or is directly loaded into the RISC complex leading to the downregulation of the protein expression of Rig-1 and IRAK1, and probably also other target proteins, in a miRNA-like manner.
RsmY targets Rig-I in a miRNA-like manner. Cells incubated with Lp-EVs purified from L. pneumophila wt but not those purified from L. pneumophila lacking RsmY showed decreased levels of Rig-I protein. Furthermore, RsmY shows some similarity with has-miR144-3p that is predicted to influence Rig-I expression. Thus, we propose that RsmY might act like a human miRNA by base pairing with the 3’ untranslated region (UTR) of Rig-I and thereby influence its expression. To test this hypothesis, we performed a dual luciferase reporter gene assay, a powerful tool for confirming gene regulation by miRNAs. We introduced the UTR regions of the ddx58 (Rig-I), irak1 (IRAK1), or rel (cRel) genes downstream of the luc2 (Firefly) luciferase and a nonspecific (non-silencing), negative ctrRNA, RsmY, or tRNA-Phe under the control of the H1 promoter. The hRluc (Renilla) expression and activity were used as an internal control and the changes in the ratios of normalized Firefly- versus Renilla-luciferase activities at the different conditions were determined by comparing to the negative control. As shown in Fig. 4A, the relative luciferase activity is significantly reduced when Luc2 was fused to the UTR of ddx58/Rig-I and RsmY and tRNA-Phe were present, indicating again that both regulatory RNAs down-regulate Rig-I expression. In contrast, only tRNA-Phe impacts the activity of the IRAK1-UTR, and only RsmY significantly affects the expression of the luciferase gene fused to cRel-UTR. Furthermore, when conducting the same assay with the complementary RNA sequence of RsmY (as-RsmY) and tRNA-Phe as-tRNA-Phe) luciferase activity remained unchanged, highlighting that only the correct strand sequence impacts expression (Supplementary Fig. 4A). Additionally, we undertook the dual luciferase reporter gene assay described above also in primary cells, to rule out the possibility...
that the result is due to the cell line used. Indeed, when repeating the above-described experiment in CD14+ cells isolated from human blood we obtained the same result as in THP-1 cells (Supplementary Fig. 4B). These results are also in agreement with the results obtained after RNA transfection (Fig. 11 and Supplementary Fig. 2B), further supporting our results that RsmY interacts with the UTR of the RIG-1, and tRNA-Phe with the UTR of the irak1 encoding gene and indeed can behave like eukaryotic micro-RNAs. Additionally, we analyzed the effect of Lp-EVs purified from the L. pneumophila wt or the L. pneumophila ΔrsmY strain on the different UTRs by measuring the luciferase activity (Fig. 4B). Also, here we observed a clear dependency of the luc2 activity on the presence of Lp-EVs and of RsmY similar to what we have seen by western blot (Fig. 1H) supporting the idea that Lp-EVs participate in manipulating the immune response.

Argonaute family proteins play a crucial role in RNA-induced silencing complex (RISC). Thus, to further analyze if RsmY acts in a miRNA like manner we investigated whether argonaute-2 (Ago2), the only member with catalytic activity and an essential role within the RISC complex to regulate small RNA guided gene silencing processes impacts RsmY and/or tRNA-Phe activity. Indeed, the suppressive effect of RsmY, tRNA-Phe or of Lp-EVs on the relative luciferase activity of Luc2 fused to the UTR of ddx58/Rig-1 or the UTR of irak1 was significantly reduced when Ago2 inhibitor was added (Fig. 4C, D). This suggests that the presence of a functional Ago2 is necessary for Lp-EV RNAs to interact with ddx58 and irak1 UTRs in cellulo and show that Ago2 and thus probably RISC-mediated silencing are involved in ddx58 and irak1 expression during L. pneumophila infection. However, secondary effects of Ago2 inhibition may also influence this result, since endogenous human miRNA are described to play an important role in the regulation of the expression of a variety of immune-related proteins, including Rig-1 and IRAK1 (Supplementary Fig. 4C). Yet, as transfection of RsmY-RNA did not influence luciferase activity of Luc2 fused to the irak1-UTR independently of Ago2 inhibition, the observed impact of tRNA-Phe on IRAK1 cannot solely depend on the effect of endogenous has-miRNA-silencing, but it further suggests that indeed L. pneumophila RsmY has a significant impact on protein expression in a miRNA-like and Ago2-dependent manner.

To determine if Ago2 plays a direct role in Lp-sRNA-mediated gene silencing, we analyzed whether Lp-sRNAs and in particular RsmY directly interact with human Ago2 during infection. We infected THP-1 cells with L. pneumophila and used has-Ago2 antibodies for Ago2-immunoprecipitation experiments followed by sequencing. To validate our approach, we first analyzed whether has-miRNAs known to interact with Ago2 were among the sequences obtained. Indeed, we identified 72 known Ago2-interacting has-miRNAs (Supplementary Table 2), but most excitingly we also identified RNAs derived from L. pneumophila, in particular, we identified RsmY in two of our three pull downs. Given the fact that only about 50% of the THP-1 cells are infected by L. pneumophila, that only 30% of Lp-EVs contain RNA (Supplementary Fig. 1C), and that according to our assays only about 3% of the Lp-EVs cargo is released in the tested condition (Fig. 3E), the probability to identify Lp-derived RNAs in the bulk of human RNAs is very low. Thus, although the results are not statistically significant, our Ago2-CLIP indicated that RsmY seem to directly interact with Ago2 during infection. These results further support our model that RsmY and other L. pneumophila RNAs can act in a miRNA-like manner in the host cell.

Thus, Lp-EVs not only follow similar pathways as observed for exosomes, but the bacterial sRNA transported within Lp-EVs seem to have a similar mode of action as eukaryotic miRNAs. They can bind to UTRs of target mRNAs leading to a reduced translation of the corresponding protein and/or to a destabilization of the mRNA and consequently to a downregulation of the protein levels.

**RsmY decreases RIG-I protein expression in the host cell and modulates downstream signalling after uptake of Lp-EVs or during bacterial infection.** To investigate the impact of RsmY contained within Lp-EVs on the downstream activation of the RLR and TLR signalling pathway, we analyzed the protein levels of cRel and the phosphorylated forms of p-TBK1, p-IRF3, p-IRF7, p-IκBα, and Co in THP-1 cells incubated with Lp-EVs purified either from wt L. pneumophila or from the ΔrsmY-mutant strain. After 3 h post incubation the protein levels and/or phosphorylation status were compared to that of non-infected cells. As shown before, in cells incubated with the Lp-EVs purified from the ΔrsmY-mutant protein level of Rig-I were significantly increased as compared to the wt- Lp-EV incubated cells (Fig. 1H). Furthermore, the amounts of activated (phosphorylated) forms of IRF3, IRF7, and TBK1, which are part of the downstream signalling cascade of the RLR and TLR immune signalling pathways, were significantly increased in the cells incubated with Lp-EV from the RsmY-mutant (Fig. 5A and Supplementary Fig. 4D). Similar results were obtained when THP-1 cells were infected with L. pneumophila wt and the protein levels of the above-mentioned proteins were compared to the type IV secretion mutant ΔletA, the ΔrsmY-mutant, and the ΔdotA strain after 1, 3, 6, and 8 h of infection (Supplementary Fig. 4E). The LetA mutant strain was also analyzed as the two-component system LetA/LetS regulates the expression of RsmY. As noted above, tRNA-Phe has only a single locus in the L. pneumophila genome, hence it was not possible to construct a viable knock-out mutant. Protein levels of Rig-I and cRel decreased significantly in a time-dependent manner in cells infected with the wt but increased in cells infected with ΔdotA- and ΔletA-mutant strains. Strikingly, in cells infected with the ΔrsmY-mutant strain protein level of Rig-I were significantly increased as compared to the wt-infected cells (Supplementary Fig. 4E). These results further show that RsmY indeed impacts the RLR signalling pathway. In contrast, the levels of the phosphorylated forms of IκBα, a negative regulator of NF-κB, were not significantly different when compared to the wt infected cells, indicating that RsmY, although targeting cRel after transfection, does not have a significant influence on the activation of the initial NF-κB response (Fig. 5A and Supplementary Fig. 4E).

**RsmY contained in Lp-EVs and RsmY alone decrease the IFN-β response of the host cell.** The RLR response, but also TLR signalling via IRAK1 are linked to interferon type I expression and secretion. Thus, RsmY mediated downregulation of RIG-I and the attenuation of the downstream transcription factors should influence IFN-β secretion of the host cells. We thus first analyzed the IFN-β concentration in the supernatants of THP-1 cells infected with the wt L. pneumophila or the ΔrsmY-mutant strain. We quantified the amount of extracellular IFN-β by performing an ELISA with the supernatants at different time points of the infection. However, no significant differences were found when IFN-β concentrations of cells infected wt L. pneumophila were compared to cells infected with the ΔrsmY-mutant strain (Supplementary Fig. 5A), suggesting that this approach does not reveal the influence of RsmY on IFN-β secretion as additional factors may also influence IFN-β levels as known for several bacterial and viral infections, and these combined effects are measured. Thus, to measure specifically the impact of Lp-EVs containing RsmY, we analyzed the extracellular concentration of IFN-β in the supernatant of THP-1 cells after incubation with Lp-
EVs purified either from wt *L. pneumophila* or from the Δ*rsmY* mutant strain. Indeed, as shown in Fig. 5B, internalization of *Lp*-EV containing RsmY (purified from the wt strain) induces less IFN-β secretion by the host cells, than those infected with *Lp*-EVs from which RsmY is absent (purified from the Δ*rsmY* strain). These results suggest that *Lp*-EVs containing RsmY dampen IFN-β secretion of infected human cells. To further substantiate this finding, we transfected THP-1 cells with RNA to activate RIG-I-dependent IFN-β secretion. The transfection of RsmY-RNA and to a lesser extent of tRNA-Phe lead to a significant lower IFN-β secretion of the host cells when compared to the nonspecific control RNA (Fig. 5C).

To further investigate the impact of RsmY on the host immune response, we performed RNAseq analyses comparing THP-1 cells incubated with *Lp*-EVs purified from wt bacteria or *Lp*-EVs purified from the RsmY mutant strain at 3 h pi. Our results
revealed that only slight but not significant differences in the host cell transcriptome were present. Indeed, the difference in the IFN-β levels we observed between THP-1 cells treated with Lp-EV purified from the wt strain and Lp-EVs purified from the ΔrsmY strain are apparently not enough to see significant changes in the transcription of Interferon stimulated genes (ISGs) at transcript level. However, this is not surprising as accumulating evidence indicates that ISG expression is not solely dependent on IFN-β, but that ISGs can also be up-regulated directly after a pathogen infection independent of IFN-β signalling, thus the network underlying the regulation of the ISG is much more complex64–66. Furthermore, RsmY and tRNA-Phe seem to act in concert on the host immune response and a knockout of both, which is unfortunately not possible to achieve, might lead to more important effects on the transcriptional level. Overall, the differences on transcript level between wt Lp-EV and ΔrsmY-EV treated cells are small and not significant including the transcripts of ddx58 (Rig-I) and irak1 suggesting also that post-transcriptional effects may play a more dominant role in the regulation of protein expression by Lp-EVs.

Taken together, our results indicate that the absence of RsmY in Lp-EVs resulted in an up-regulation of IFN-β in the supernatant, whereas the transfection of RsmY-RNA lead to a reduced IFN-β secretion compared to the control RNA, in agreement with our model that RsmY contained within the Lp-EVs is biological active in the host cell and plays a role in dampening the immune response of host cells to infection.

IFN-β levels, Ago2 inhibition, and Rig-I silencing impact replication of L. pneumophila that is modulated by Lp-EVs. To investigate the influence that IFN-β secretion, partly induced by RsmY, has on infection, we treated THP-1 cells with different concentrations of IFN-β and analyzed the replication phenotype of L. pneumophila. We show that increasing concentrations of extracellular IFN-β reduce intracellular replication of L. pneumophila in THP-1 cells, whereas high concentrations of IL-1β have no impact (Supplementary Fig. 5B). We then pre-treated the THP-1 cells with Lp-EVs either purified from wt bacteria or with Lp-EVs purified from the ΔrsmY strain. In cells pre-treated with wt Lp-EVs we observed a significantly higher replication of L. pneumophila than in cells incubated with Lp-EVs purified from the ΔrsmY strain (Supplementary Fig. 5C). To further analyze the mechanism, we specifically down-regulated the expression of ddx58 and irak1 by siRNA-mediated gene silencing. Protein levels of Rig-I or IRAK1, respectively, were reduced by 60–80% at the time point of infection compared to scramble transfected control cells (Fig. 5D). After 24 hpi, no significant differences in the replication of L. pneumophila were detected, but at later time points (48 and 72 hpi), the number of bacteria in cells where DDX58 (Rig-I) was downregulated by siRNA was increased by up to 50% further confirming that suppression of Rig-I is beneficial for intracellular replication of L. pneumophila, as has also been described previously67. Additionally, we transfected THP-1 cells with RsmY RNA or its anti-sense sequence (as-RsmY) and infected these cells. L. pneumophila replicated significantly better in the cells transfected with RsmY-RNA, similar to what was observed after siRNA knockdown of ddx58, again showing that RsmY has a beneficial effect on L. pneumophila replication (Supplementary Fig. 5D). Surprisingly, no differences in L. pneumophila replication between scramble control and IRAK1 knockdown THP-1 cells were observed suggesting that IRAK-1 has no major influence on intracellular growth of L. pneumophila (Fig. 5D). To confirm these results, we pre-treated the THP-1 cells with the IRAK1/4 Inhibitor 1 (BCI-137). Again, no significant changes in intracellular replication of L. pneumophila were observed (Supplementary Fig. 5E), differently to what was reported previously68. In contrast, inhibition of Ago2 in THP-1 cells prior to infection using BCI-137 significantly reduced intracellular replication of L. pneumophila, again indicating that a functioning Ago2 is necessary for its optimal proliferation (Supplementary Fig. 5E).

Finally, to further characterize the impact of Lp-EVs on the host immune response, we incubated THP-1 cells with agonists that mimic pathogen-associated molecular patterns (PAMPs) and measured extracellular IFN-β concentrations after pre-treatment with Lp-EVs that were either purified from wt bacteria or from the ΔrsmY strain. Indeed, the IFN-β response of certain TLR agonists was dampened after pre-incubation with wt Lp-EVs but less with ΔrsmY-EVs further pointing to the influence of RsmY on the host immune response. In particular, the IFN-β response triggered by agonists for TLR1/2/5/6 and TLR8 was significantly reduced when pre-treating the cells with Lp-EVs (Supplementary Fig. 6). The IFN-β response to TLR9 agonist CpG instead was even more pronounced after pre-incubation of the TLR-1 cells with Lp-EVs compared to control experiments, probably due to synergistic effects of multiple ligand stimulations. In contrast, agonist stimulation of TLR3, TLR4, or TLR7 was not affected by Lp-EV-treatment, whereas inhibition of Ago2 slightly induced the extracellular IFN-β levels after Lp-EV-treatment (Supplementary Fig. 6).

Taken together, these results further support our model that the two sRNAs RsmY and tRNA-Phe transferred in the host cell within Lp-EVs, modulate the host innate immune response in an Ago2-dependent and miRNA-like manner for the benefit of intracellular growth of L. pneumophila.

Discussion

The analyses of the RNA content of L. pneumophila extracellular vesicles (Lp-EVs) and the study of its functional role in infection provides important new insights into host-pathogen communication and trans-kingdom signalling. Our dataset demonstrates that L. pneumophila-RNAs, in particular the sRNA RsmY and the tRNA-Phe, can be transported within Lp-EVs into the host cell where they are biological active and can modulate the host immune response in a miRNA-like manner.

We found that Lp-EVs show a unique RNA profile, different from the RNA content of the bacterial cells from which they derived, as 39 sRNAs are highly enriched in the Lp-EVs suggesting that RNA is not randomly incorporated. Indeed, distinct RNA content has also been reported for bacterial EVs shed from Escherichia coli or Pseudomonas aeruginosa45,68. Eukaryotic EVs have also been described to contain distinct microRNA signatures that are proposed as biomarker for disease69 and can even contain mycobacterial transcripts when released from infected macrophages70. The sRNAs enriched in the Lp-EVs share a common feature of EV-RNAs, which is a size of <200 nucleotides and a stable secondary structure for most of them. It was reported for eukaryotic EVs that selectivity of RNA loading might be affected by secondary structures, sequence motives, or binding to certain proteins71. However, secondary structure alone or simply the abundance of certain sRNAs is not sufficient to explain the selective packaging of e.g. RsmY in Lp-EVs as RsmZ, the most abundant L. pneumophila sRNA, exhibits a secondary structure very similar to RsmY62 but it is predominantly present in the bacterial cell, and not enriched within the Lp-EVs. A recent report suggested that the RNA-binding protein Hfq might affect bacterial membranes and might have a role in exporting sRNAs into the periplasmic space and outside of the bacterial cell72, thus Hfq could play a role in transporting sRNAs of L. pneumophila into EVs, but at the moment the mechanism of “RNA selection”
and cargo loading remains unknown and needs further investigation.

To act efficiently, the sRNAs contained in the Lp-EVs need to be delivered to the host cell at specific locations. It is tempting to assume that EV-content delivery is an ancient and common secretion mechanism as EVs are produced by organisms from all domains of life, gram-negative and gram-positive bacteria, archaea, and eukaryotes among those fungi and protozoan parasites. We show that Lp-EVs are released during infection of the host cell where they seem to cross the LCV and the plasma membrane and enter the sorting of the eukaryotic endosomal pathway, during which they establish transient contacts with the ER. Thus, Lp-EV sorting might be comparable to exosome-mediated delivery of miRNAs, where exosomes are sorted into endosomal trafficking circuits that are targeted to scan the ER as a possible site of cargo release. Indeed, it has been shown that the Lp-EV protein content by two-dimensional gel electrophoresis identified several proteases and phospholipases within the Lp-EVs that might participate in such an endosomal escape. It was also shown that Lp-EVs fuse to reconstituted liposomes composed of different phospholipids and it was proposed that membrane fusion might be a general mechanism of EV-cargo delivery. Thus, we cannot exclude that a certain amount of Lp-EVs may also directly fuse with the host membranes and deliver the RNAs into the cytoplasm. However, our data indicate a significant uptake of Lp-EVs depending on actin cytoskeleton rearrangements and support their transport through the endosomal pathway into the host cytosol. Such a mechanism would be consistent with the content delivery of EVs derived from mammalian cells that seems to require protein and pH-dependent fusion between EVs and endosomal membranes. It is possible that this mechanism and the machinery are conserved through evolution.

Our bioinformatics analyses revealed that the sRNA RsmY and the tRNA-Phe show similarity to human microRNAs, for example, RsmY to mir-144 predicted to act amongst others on RIG-I. Most interestingly, that mir-144 is important in infection was recently shown for influenza, as it impacts viral replication in lung epithelial cells by directly regulating the TRAF6 expression and consequently, affects the IRF7-dependent transcriptional network negatively. Thus, our finding was intriguing and suggested that the bacterial sRNAs might act like miRNAs in the host cell during infection. Indeed, we show that two of these sRNAs, RsmY and tRNA-Phe, can base pair with the UTR of eukaryotic target genes in a miRNA-like manner significantly attenuating the protein expression of at least three proteins that are important players in the RLR and TLR response, RIG-I, cRel, and IRAK1. It was reported previously that Lp-EVs affect the infectivity of L. pneumophila by suppressing IRAK1. Here we identify tRNA-Phe as a new player implicated in the downregulation of IRAK1 expression and reveal that RIG-I is an additional target.

RIG-I is a key sensor that upon recognizing foreign RNA in the host cell mediates the transcriptional induction of type I interferons. Similarly, our analyses of the secretion of INF-β after transfection of RsmY or tRNA-Phe into host cells or incubation of wt or RsmY depleted Lp-EVs showed decreased INF-β secretion when RsmY and to a lesser extent also tRNA-Phe were present. This uncovers that translational of L. pneumophila sRNAs contained in EVs leads to an attenuation of the host immune response and aids the survival and replication of this pathogen during infection. Thus, direct miRNA-like regulation of the expression of key sensors and regulators of the innate immune response is a striking feature of L. pneumophila host-parasite communication.

Remarkably, the identified “bacterial miRNAs” have a dual function, not only can they act as trans-kingdom signalling molecules, but they are also important in the bacterial cell. RsmY is a regulatory RNA implicated in regulating the life cycle of L. pneumophila, and tRNA-Phe is involved in protein synthesis. Whether L. pneumophila sRNAs or that of other bacteria embedded in EVs impact eukaryotic cells in diverse ways and if certain common strategies are used to interfere with the host immune response need to be investigated. Indeed, RNA-based trans-kingdom signalling might be a more general mechanism employed by bacteria for establishing virulence as well as symbiosis and other interactions occurring between bacterial and eukaryotic cells.

Methods

Cell lines, bacterial strains, and infection assays. MDMs (THP-1 cell line purchased from ATCC/TIB-202®) were cultivated in RPMI 1640+ GlutaMAX medium (Life Technologies) and human bone osteosarcoma epithelial cells stably expressing Sec61b-GFP for ER labelling (U2OS-Sec61b) published previously, in DMEM + GlutaMAX (Life Technologies), both supplemented with 10% heat-inactivated FBS (Life Technologies). The cells were incubated at 37 °C with 5% CO2 in a humidified atmosphere. Human monocyte-derived macrophages (hMDM) or CD14 + cells were isolated and differentiated as previously described.

L. pneumophila strain Paris and its derivatives were grown on ACES-buffered charcoal yeast-extract (BCYE) medium at 37 °C and E. coli was grown in Luria-Bertani (LB) broth and agar. For knock out and complementation constructs, the corresponding antibiotic was added with following concentrations: apramycin 15 µg/ml, kanamycin 15 µg/ml, or chloramphenicol 10 µg/ml. Mutant strains ΔetA (lpp2699), ΔrsmY and ΔdotA (lpp2740) were constructed previously. For infection, L. pneumophila or knock out mutant strains from above were grown until post-exponential phase (OD42) in BCYE-medium at 37 °C and harvested by centrifugation at 5.000 × g, 4 °C for 15 min, 4 °C for 15 min and the pellet was frozen at −80 °C for further use. The supernatant was twice passed through a 0.2 µm PES membrane Stericup Quick Release (Millipore) and re-centrifuged for 15 min at 15.000 × g, 4 °C. The supernatant was treated with RNaseA/T1 (Thermo Scientific) at a final concentration of 2 µg/ml RNaseA for 1 h at 37 °C followed by centrifugation at 150.000 × g for 2 h at 4 °C to pelleting the Lp-EVs. The Lp-EV-pellet was washed, re-centrifuged, and resuspended in PBS. For incubation experiments with U2OS or hMDM for FISH, the purified Lp-EVs were labelled with Vybrant DiD-dye (Thermo Scientific) for 30 min, and carefully washed five-times in PBS. THP-1 and U2OS cells were infected with an MOI of 50, hMDM cells with an MOI of 10 as published previously.

Flow cytometry, characterization, and incubation of Lp-EVs with host cells. L. pneumophila strains were grown in BYE until post exponential phase (OD42). Bacterial pellets were harvested at 5.000 × g, 4 °C for 15 min and centrifuged at 15.000 × g, 4 °C for 1 h at 4 °C to pelleting the Lp-EVs. The Lp-EV-pellet was washed, re-centrifuged, and resuspended in PBS. For flow cytometry experiments with U2OS or hMDM for FISH, the purified Lp-EVs were labelled with Vybrant DiD-dye (Thermo Scientific), 30 min at 37 °C, and subsequently cleaned-up using Exosome Spin Columns (MW3000, Thermo Scientific) for removal of unincorporated dye. Human cells were incubated with Lp-EVs at an MOI of 10 (according to flow cytometry dye-labelled events). The values we obtained by flow cytometry are much lower than those obtained with the ZetaView analysis, in which we measure a concentration of Lp-EVs about 1000 times higher as compared to the flow cytometry data. As we adjusted our MOI in all experiments to the conventional flow cytometry data, we indicate throughout the manuscript that the MOI was estimated according to conventional flow cytometry. It also needs to be noted, that we analyzed how many cells indeed take up of Lp-EVs.
EVs to estimate the amounts of Lp-EVs impacting the host cell. We used high content image analyses to analyze the number of EVs detectable in infected U2OS cells. Western blot analysis of the Arp2/3 complex that contained detectable EVs. Thus, even with the relative high amount of EVs used according to ZetaView quantification the cell cultures were not saturated with Lp-EVs but a high background of noninfected cells that moderate the measurable output is present. Also, the amount of EVs we used did not lead to cell death of U2OS or hMDM cells, even after 12 h pi. Supplementary Fig. 1C. All experiments were performed at least in n = 3 independent biological replicates. Transmission electron microscopy. Lp-EVs purified as described above, were resuspended in ultrapure water, and visualized by negative staining TEM and cryo-EM. For negative staining TEM, 4 µl of the Lp-EVs were incubated for one minute on a glow discharged carbon grid (Agar Scientific) and contrasted with 1% uranyl acetate solution. Subsequently, the grids were viewed on a Tecnai T12 microscope (Thermo Scientific) and images were obtained using an Eagle camera (Thermo Scientific). For cryo-EM imaging, 3 µl of the Lp-EVs (corresponding roughly to 8 x 10^7) were placed on a glow discharged Lacey grid (Agar Scientific) and cryofixed at ~180 °C in liquid ethane using an EMGP (Leica). Grids were imaged on a Tecnai F20 using a Falcon II direct detector (Thermo Fisher) operating at 200 kV. Flow cytometry experiments. For flow cytometry experiments, the EVs were labelled with Vybrant DiO or DiD cell dye (Life Technologies) and Syto®RNASelect (Molecular Probes), respectively, according to the manufacturer’s instruction. Experiment standardization was done using Megamix-Plus SSC beads (BioCyten). Megamix-Plus SSC is a mix of fluorescent particles of varied diameter (0.16 - 0.24, 0.5 µm), using SSC a size-related parameter to standardize the setting of the cytometer to study biological vesicles within a constant size region. For analysis, the MACSQuant flow cytometer (Miltenyi Biotec) was used with changing parameters for optimized Megamix-Plus SSC standard separation (Supplementary Fig. 1). However, the mixing of the vesicles and the cytometers smaller than the wavelength of the lasers, it makes it quite challenging to analyze and to distinguish them from the background noise. Therefore, for our experiments with Syto®RNA-Select we reduced the SSC voltage and adapted the SSC threshold to reduce the background noise. Thus, mainly large vesicles and EV-agglomerates were detected, which frequently appear during the purification process. Flow cytometry data are submitted to flow-repository public database (https://flowrepository.org/) accession numbers FR-FCM-Z2XL and FR-FCM-Z2XM. Floatation assays. For the floatation assays, we proceeded as previously described22. Briefly, Lp-EVs labelled with Vybrant DiD-dye or not and processed through a size exclusion column, were centrifuged at 100,000 x g for 1 h (MLA 50 rotor). Pellets were resuspended in 1 mL 60% sucrose and deposited in the bottom of the tube. 1 mL of 30% sucrose solution and 1 mL of PBS were sequentially loaded on top. Samples were centrifuged at 150,000 x g for 16 h at 4 °C (SW55 rotor). The top fraction was removed, and the 30% sucrose fraction (1 mL) was collected and mixed with 6 mL PBS. Samples were centrifuged at 100,000 x g for 1h30 min (MLA 50 rotor). The resulting supenatant was removed and pellet was resuspended in 100 µL PBS prior to further analyses of the particle concentration and size. Nanoparticle tracking analysis. The L. pneumophila vesicles were purified in the same way as described above. The quantification, size characterization, and fluorescence detection of the EVs were then performed on the ZetaView® QUATT (Particle Metrix). For the size and concentration measurements, the 448 nm laser in scatter mode was used; for the fluorescent measurement of DiD-dye positive particles, the 640 nm laser with a 660 nm long-pass filter was used. In all panels, a dot represents the average of 11 measurements corresponding to the 11 frames. For the size, each dot corresponds to the average of the median size which permits to describe the distribution, whereas the mean is biased by the aggregates’ extreme values. For the concentration, each dot corresponds to the average number of particles detected taking into account the dilution factor. The normality was tested with D’Agostino–Pearson test which was negative for all panels. The data are paired, and a non-parametric test (Wilcoxon test) was used. All statistical analysis were performed with GraphPad Prism version 9.3.0 for Windows. RNA isolation and RNaseq of Lp-EVs or THP-1 cells after uptake of Lp-EVs. Lp-EV pellets, purified from a 300 ml liquid culture as described above and the corresponding bacterial pellets were resuspended in Qiazol and the RNA extraction was performed following the instruction of the miRNeasy®Mini kit (Qiagen). RNA samples were digested with Turbo DNase (Thermo Scientific) and the size distribution of the Lp-EV RNA was evaluated with a Bioanalyzer (Agilent Technologies). The Lp-EV and bacterial RNA (but not Lp-EV-RNA) were rRNA depleted using the RiboZero RNA Removal Kit for Gram-negative bacteria (Illumina) and metal-catalyzed heat-denatured to a size around 100–200 nts using an RNA fragmentation kit (Ambion). The bacterial RNA was further processed according to the TruSeq® small sample preparation protocol. RNA-Seq analysis was done n = 4 independent biological experiments. To analyze the transcriptome of THP-1 cells after uptake of Lp-EVs, THP-1 cells were incubated with Lp-EVs or not for 2 h (according to flow Cytometer measure), or without (non-infected control). After 3 h, the THP-1 cells were pelleted by centrifugation at 500 x g and RNA was extracted as previously described using the miRNeasy®Mini kit (Qiagen). Subsequently, total RNA was treated with Turbo DNase (Thermo Scientific), and RNAs after RNA isolation were processed using the Illumina multiplex preparation guide of Illumina. Before Illumina Nextseq 500/550 (Illumina) multiplex sequencing, the quantity was determined by Qubit 2.0 (Invitrogen) and the quality was checked by Bioanalyzer (Agilent Technologies). RNaseq data analyses. For analyzing the RNA content of Lp-EVs the single-end reads in FASTQ format generated by Illumina sequencing were processed to remove adapters using Cutadapt software version 1.1581. Sickle (https://github.com/najoshi/sickle) version 1.33 for trimming of the reads (quality threshold Phred score 20). After adapter removal and trimming, all sequence reads shorter than 20 nucleotides were eliminated. Bowtie version 1.2.282 was used with a database containing the sequences of ribosomal RNA (rRNA) operons of L. pneumophila strain Paris (NC_006368.1) to map all reads corresponding to the rRNA sequences. All mapped reads were removed and the unmapped reads were aligned to the L. pneumophila Paris sequence using Bowtie version 2 2.3.4.35. Only uniquely mapped reads were kept, to avoid that a read contributes for the coverage value at different positions. We build, sorted, and indexed the BAM files for each mapping result using the Samtools suite version 1.3 (https://github.com/samtools/samtools). To count reads overlapping each feature, we used featureCounts version 2.4.3 using the Subreads package58. The primary option was used to be sure to count primary alignments, size-related parameter to standardize the setting of the freereplicates for the two conditions (Lp-EVs vs bacterial pellet), we used the package Sartools version 1.3.0 including DESeq2 methods. In order to normalize the counts, we used DESeq2 with the « median » option to compute size factors83. The resulting tables containing differentially expressed genes with counts, normalized counts, fold changes (ratio of differential expression), and P-values were used and visualized using the Artemis analyzing tool version 16.1786. The transcriptome of THP-1 cells after uptake of Lp-EVs were analyzed as described above. Western blot and interferon IFN-β quantification by ELISA. THP-1 cells were infected (or not) with the wt or the Δleta, AromY or Adoa strains as described above. At 1, 3, 6, 8, and 24 h post-infection, the THP-1 cells were harvested by centrifugation. For Lp-EV uptake, the Lp-EVs were incubated with THP-1 cells for 3 h before harvesting the cells by centrifugation. While the supernatant was kept in 4 °C or directly used for IFN-β quantification, the pellet was resuspended in RIPA buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% Na-deoxycholate, 0.1% SDS, 5% glycerol) + protease inhibitor (Thermo Scientific) and phosphatase and protease inhibitor (Thermo Scientific) for total protein extraction by sonication. After treatment with benzonase (Sigma) and centrifugation, soluble total protein was quantified and an equal amount from each condition was loaded on loading buffer (4% B-200 Tris HCl pH 6.8, 40% glycerol, 400 mM DTT, 0.01% bromphenol blue), denatured at 80 °C for 10 min and loaded on Criterion TGX Stain-free (4–15%) or Mini-Protein TGX Stain-Free (AnyDyl) gels (Biorad). Separated proteins were transferred on 0.2 µm PVDF membranes using the TransBlot Turbo from Biorad. All antibodies used were from Cell Signalling Technology, except IFN-β (NovusBio) and RhoGDI (Santa Cruz Biotechnology) at a 1:500 dilution. The TransBlot Turbo from Biorad. All antibodies used were from Cell Signalling Technology, except IFN-β (NovusBio) and RhoGDI (Santa Cruz Biotechnology) at a 1:500 dilution. The TransBlot Turbo from Biorad. All antibodies used were from Cell Signalling Technology, except IFN-β (NovusBio) and RhoGDI (Santa Cruz Biotechnology) at a 1:500 dilution. The TransBlot Turbo from Biorad. All antibodies used were from Cell Signalling Technology, except IFN-β (NovusBio) and RhoGDI (Santa Cruz Biotechnology) at a 1:500 dilution. The TransBlot Turbo from Biorad. All antibodies used were from Cell Signalling Technology, except IFN-β (NovusBio) and RhoGDI (Santa Cruz Biotechnology) at a 1:500 dilution.
RNA:RNA interaction and RNA transfection experiments. Template DNA for the T7 RNA polymerase was amplified from wt L. pneumophila Paris DNA or TPH-1 DNA using the corresponding T7-primer indicated in Supplementary Table 1. In vitro RNA was transcribed form the PCR fragments using the T7-Megascript Kit (Life technologies). For the synthesis of in vitro sRNA of RsmY and tRNA-Phe, 1.5 mM CTP, 1.5 mM TTP, and 50 mM MgCl₂, 50 mM Tris-HCl, pH 7.0, 100 µM of aT5 and biotinylated RNA or a mixture of the non-biotinylated RNA and biotinylated RNA was used. The reaction mixture was incubated for 2 h at 37°C. After incubation, pure RNA was isolated and precipitated with ethanol.

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Membranes were blocked in PBS containing 0.1% Tween-20 and 1% ECL blocking agent (GE Healthcare) for 2 h at RT and, incubated for 1 h in the same buffer including mouse anti-biotin antibody (Invitrogen) at a dilution of 1:2,000.

Fluorescence in situ hybridization (FISH) and analysis. Aliquots from the supernatant were filtered through 0.2-µm pore size filter (pore size 0.22 µm). Bacteria were resuspended in the adequate culture medium for the respective cell line (see above). until post-exponential growth phase (OD4.2) in broth culture (BYE). Bacteria were washed and resuspended in PBS for 3 h at 4°C with continuous shaking. For the bacterial infection, THP-1 cells were pre-incubated with different concentrations of 0 µM, 100 nM and 1 µM of IRAK1/4 inhibitor (Thermo Scientific) for 1 h at 37°C. After pre-incubation, bacteria were added to the THP-1 cells at a MOI of 10 and the infection was continued for 3 h at 37°C.

After washing and binding of the secondary antibody (anti-mouse IgG-HRP, Cell Signaling) at a dilution of 1:5,000, the RNA-bio was visualized with WesternBright Sirius HRP Substrate (Diagnostics) and imaged with a G:Box (Syngene). Each experiment was done in three independent replicates. RNA transfection was done with in vitro transcribed RNA non-biotinylated RsmY, tRNA-Phe or different ctrlRNAs (either a random, non-specific region of the L. pneumophila genome or the complementary sequences of RsmY or tRNA-Phe). Table 1. Transfected THP-1 cells following the protocol of Amazia 4D-Nucleofector X Unit Transfection using the SG Cell line Kit (Lonza). Transfected were transfected into THP-1 cells following the protocol of Amaxa 4D-replicates and for statistical analysis, a two-way ANOVA was performed with values of $p < 0.01$ statistically significant ($*$), $p < 0.001$ extremely statistically significant (**), and $p < 0.0001$ extremely extremely statistically significant (***)

Membranes were blocked in PBS containing 0.1% Tween-20 and 1% ECL blocking agent (GE Healthcare) for 2 h at RT and, incubated for 1 h in the same buffer including mouse anti-biotin antibody (Invitrogen) at a dilution of 1:2,000. After washing and binding of the secondary antibody (anti-mouse IgG-HRP, Cell Signaling) at a dilution of 1:5,000, the RNA-bio was visualized with WesternBright Sirius HRP Substrate (Diagnostics) and imaged with a G:Box (Syngene).

Bacterial infection assays. For bacterial infection, L. pneumophila wt was grown until post-exponential growth phase (OD4.2) in broth culture (BYE). Bacteria were resuspended in the adequate culture medium for the respective cell line (see above). Non-differentiated THP-1 cells were infected with an MOI = 1 as described before. To test the influence of cytokines, human interferon β 1A (Sigma) at concentration of 25, 50, and 100 IU/ml or human interleukin-1β (Sigma) at 20 ng/ml were added to the THP-1 cell cultures and incubated for 24 h before infection with L. pneumophila. To analyze the impact of LP-EVs on bacterial infection, purified LP-EVs from L. pneumophila wt or the ΔrsmY mutant strain were added to the THP-1 cells at MOI of 10 (according to flow cytometer measure) for 3 h before bacterial infection. Two hours after the bacterial infection the THP-1 cells were carefully washed three times with PBS to remove extracellular bacteria and resuspended in RPMI medium. If mentioned, the same amount of IFN-β was added above the described LP-EVs at different time points were taken at 24 h, 48 h, and 72 h. For inhibition experiments, 5 µM IRAK1/4 inhibitor (Thermo Scientific) was added 2 h before the bacterial infection and the infection was continued for 3 h at 37°C.

For statistical analysis with values of $p < 0.05$ significant ($*$), $p < 0.001$ extremely statistically significant (**), and $p < 0.0001$ extremely extremely statistically significant (***)

Fluorescence in situ hybridization (FISH) and analysis. LAPMDs were incubated with Vybrant DiD-labled purified LP-EVs as described above (3 x 10⁴ cells/well, MOI 10). After 5 h of incubation, cells were washed three-times with PBS and fixed in 4% PFA/PBS for 10 min at RT. After washing and neutralization in 50 mM NH₄/Citrate/PBS, 5 min at RT, the cells were permeabilized with 0.8% saponin/1% BSA/PBS for 30 min at RT and washed three-times with PBS. LAPMD were incubated with 0.1 mg/ml Hoechst 33342 (Life Technologies) for 15 min. After two additional washing steps in PBS, image acquisition of multiple fields per well was performed on an automated confocal microscope (OPERA Phenix, Perkin Elmer) using the 60× objective, excitation lasers at 405, 488, 561, and 640 nm, and emission filters at 450, 540, 600 and 690 nm. Images were transferred to the Columbus Image Data Storage and Analysis System version 2.9.1 (Perkin Elmer) for evaluation. High-Content Analyses (HCA) were performed by using in-house developed HCA scripts in Columbus, shared upon request. Briefly, Hoechst 33342 plus the Hb-stained cells were used to find the segment nuclei and cytoplasm in each cell. Then, DlD signal was used to find LP-EVs in the cytoplasm of LAPMDs, and the fluorescence of FISH probes was used to analyze which LP-EVs were positive for RsmY RNA.

LP-EV content release characterization: HIBit/LgbIT secretion assay. LP-EVs of bacteria expressing GroEL-HibIT (pMMB) were purified as described before. The Luciferase activity of the total content was quantified using the Nano-Glo (Promega) reagent and the solution of GroEL-HibIT following the manufactures instructions. LgbIT was cloned into the pCMV-Tag 2 vector (Agilent) and the vector was transfected into THP-1 cells using the SG Cell Line Kit (Lonza). After 24 h, transfected LgbIT expressing THP-1 cells were incubated with purified LP-EVs containing the His-BiTagged GroEL protein for 3 h, before the cells were washed and resuspended in a Nano-Glo (Promega) with a Synergy2 (BioTek) following the manufactures instructions. The values were normalized relative to the total input of GroEL-HibIT (Lytic...
Detection) in %. As negative control, Lp-EVs were purified from L. pneumophila without GroEL-HiBiT vector and comparable amounts of Lp-EVs were added in the assay. When indicated, 10 µM of cytochalasin D (Sigma) or 200 nM of Rafbomycin A1 (Sigma) respectively, were added to THP-1 cell cultures and incubated for 30 min before infection. All experiments were performed in n > 5 biological replicates.

**Live cell imaging and analysis.** U2OS-Sec61β cells were infected with VYbrant DiD-labeled L. pneumophila or incubated with Lp-EVs as described above. For mitochondrial or acidic organelle labelling, the cells were stained prior to the infection with MitoTracker Red FM or LysoTracker Red DND-99 (Life Technologies) for 30 min and then washed 3 times with assay medium; nuclear staining was performed with Hoechst 33342 (Life Technologies) for 15 min. Rab5 and Rab7 labelling via CellLight was performed according to the manufacturer’s instructions for at least 16 h. In the Lp-EV uptake experiments, Cytochalasin D (Sigma) or Dynasore (Sigma) was added to the cell culture medium at a final concentration of 1 and 80 µM, respectively. Live image acquisition of multiple labelling via CellLight was performed according to the manufacturer’s instructions. For Z-stack time-lapse 3D imaging, U2OS-Sec61β cells were plated in IBIDI dishes and imaged on an Ultraview VOX spinning-disk confocal microscope (Perkin Elmer) using the 60× objective, excitation lasers at 405, 488, 561, and 640 nm, and emission filters at 450, 540, 600, and 690 nm. For the analysis, the Columbus Image Data Storage and Analysis System (Perkin Elmer) was used. HCA was performed as described above. The DiD signal was used to detect Lp-EVs in the cytoplasm of U2OS-Sec61β and the fluorescence of MitoTracker, LysoTracker, Sec61β-GFP, Rab5, and Rab7-CellLight were measured inside each Lp-EV in order to analyze whether they co-localized with mitochondria, lysosomes, ER, Rab5, or Rab7 endosomes, respectively. For Z-stack time-lapse 3D videos, U2OS-Sec61β cells were plated in IBIDI dishes and imaged in an Ultraview VOX spinning-disk confocal microscope (Perkin Elmer) using a 60× objective, excitation lasers at 405, 488, 561, and 640 nm, and emission filters at 450, 540, 600, and 690 nm. Time-lapse 3D videos were analyzed and edited using Velocity software version 6.5 (Perkin Elmer).

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The sequence reads as well as the coverage files of the RNAseq libraries of the Lp-EV analyses have been deposited in the NCBI Gene Expression Omnibus (GEO) database. Accession number GSE190376. The RNAseq data from THP-1 cells have been deposited in the NCBI Gene Expression Omnibus (GEO) database. Accession number GSE190376. Flow cytometry data are submitted to flow-repository public database (https://flowrepository.org) accession numbers FR-FCM-Z2XL and FR-FCM-Z2XM. Source data are provided with this paper.

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Author contributions

T.S. and C.B. designed the experiments, T.S. and C.B. wrote the article, C.B. supervised the work and thanks the anonymous reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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Competing interests

The authors declare no competing interests.

Additional information

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