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Potentiation of epithelial innate host responses by
intercellular communication
Potentiation of Epithelial Innate Host Responses by Intercellular Communication

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Abstract

The epithelium efficiently attracts immune cells upon infection despite the low number of pathogenic microbes and moderate levels of secreted chemokines per cell. Here we examined whether horizontal intercellular communication between cells may contribute to a coordinated response of the epithelium. Listeria monocytogenes infection, transfection, and microinjection of individual cells within a polarized intestinal epithelial cell layer were performed and activation was determined at the single cell level by fluorescence microscopy and flow cytometry. Surprisingly, chemokine production after L. monocytogenes infection was primarily observed in non-infected epithelial cells despite invasion-dependent cell activation. Whereas horizontal communication was independent of gap junction formation, cytokine secretion, ion fluxes, or nitric oxide synthesis, NADPH oxidase (Nox) 4-dependent oxygen radical formation was required and sufficient to induce indirect epithelial cell activation. This is the first report to describe epithelial cell-cell communication in response to innate immune activation. Epithelial communication facilitates a coordinated infectious host defence at the very early stage of microbial infection.


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Introduction

Intestinal epithelial cells line the enteric mucosal surface and provide a physical barrier to maintain the integrity of this vulnerable body surface and prevent invasive infection by luminal microorganisms. Like professional immune cells, intestinal epithelial cells express receptors of the innate immune system such as Toll-like receptors (TLR) or nuclear oligomerization domain (NOD)-like receptors (NLR) [1,2]. Recognition of microbial structures leads to epithelial production of antimicrobial effector molecules and proinflammatory chemoattractive mediators. Thus, it facilitates an active role in the initiation of the mucosal host response [3,4,5]. The recruitment of professional immune cells to the site of infection occurs within hours and provides a highly efficient dynamic mechanism of the epithelial host defence. It remains unclear, however, how low number of pathogenic microorganisms as well as the limited spectrum and only moderate amount of chemokine secretion per epithelial cell facilitates stimulation of an effective host defence. We therefore hypothesized that a horizontal intercellular communication between intestinal epithelial cells might help to induce a coordinated epithelial response towards infectious challenge and thereby to amplify the epithelial innate host defence.

Listeria monocytogenes is an important human pathogen that causes meningitis, sepsis, and abortion in susceptible individuals. It is acquired with food such as unpasteurized milk and cheese and enters the body following penetration through the intestinal epithelial barrier. The microbial pathogenesis and the bacteria-host cell interaction of this facultative intracellular bacterium has been studied for many years [6]. L. monocytogenes induces its own internalization and subsequently lyses the endosomal membrane of its host cell by the secretion of listeriolysin O (LLO) and phospholipases, thus gaining access to the cytosolic space. Here, Listeria upregulates polar expression of ActA that recruits and polymerizes host actin filaments resulting in propulsive locomotion. Together with LLO and the phospholipases this allows to enter neighbouring cells and to spread within the epithelial cell layer. Importantly, recognition of Listeria by the epithelial innate immune system only occurs after internalization and lysis of the endosomal membrane through cytosolic innate immune receptors [7,8,9,10]. Since infection of individual cells can be traced using reporter gene technology, L. monocytogenes provides an excellent model to study cellular responses in respect to immune recognition at the single cell level.

In the present study, we analyzed innate immune recognition and epithelial responses at the single cell level using the model of Listeria infection of polarized intestinal epithelial cells in addition to transfection and microinjection. We present the surprising finding that non-infected epithelial cells were the main source of chemokine production during infection.
secretion in response to bacterial challenge. We identify oxygen radical species produced by NADPH oxidase (Nox) 4 in response to cytosolic bacteria to facilitate horizontal intercellular communication. Epithelial cells form a physical barrier to separate the underlying sterile tissue from the environment. In addition, epithelial cells actively sense bacterial and viral infection. The recognition of pathogenic microorganisms results in cell stimulation and the secretion of soluble mediators that attract professional immune cells to the site of infection. This first line host defense works very efficiently despite the often low number of pathogens and the limited amount of mediators secreted per epithelial cell. We therefore investigated whether infection of one individual epithelial cell would result in activation of other, non-infected cells within a confluent epithelial monolayer resulting in a more substantial host response. Indeed, using the model of the gut pathogen Listeria monocytogenes and monitoring infection and epithelial activation at a single cell level, we can clearly show that the epithelial response is mainly mediated by non-infected cells. Also, we identify oxygen radicals as potential mediators to facilitate horizontal epithelial host response upon microbial infection facilitated by horizontal epithelial communication.

Results

Invasion-dependent recognition but indirect epithelial cell activation after Listeria infection

Infection of a confluent monolayer of intestinal epithelial m-ICcl2 cells with wild-type (wt) L. monocytogenes induced rapid cellular activation illustrated by secretion of the proinflammatory chemokine Cxcl-2 (Fig. 1A). Strong epithelial activation was only observed using wt Listeria able to reach the cytosolic space (Fig. 1B) facilitating recognition by cytoplasmic innate immune receptor molecules (Fig. 1C) [7,8,9,10]. Bacterial mutants unable to lyse the endosomal membrane such as isogenic hly or hly/plcA/plcB triple mutants as well as heat inactivated bacteria exhibited a significantly reduced or even absent epithelial activation (Fig. 1B and D). Of note, lack of hly or hly, plcA, and plcB expression did not affect bacterial invasion or intracellular viability (Fig. SIA). Endosomal lysis-dependent stimulation of L. monocytogenes infected epithelial cells was also observed using flow cytometry. A time-dependent increase of the number of Cxcl-2+ and Cxcl-5+ epithelial cells was detected after infection with wt Listeria (Fig. 1E). In contrast, a strongly reduced number of epithelial cells stained positive for Cxcl-2 after infection with hly mutant Listeria (Fig. 1F). In accordance with the published literature, internalization-dependent activation was observed in epithelial cells, but not in macrophages (Fig. S1B). These results provide the first experimental evidence for a yet unknown mechanism of intercellular communication between epithelial cells in response to innate immune stimulation and thus significantly broaden our understanding of mucosal innate host defence.

Figure 1. Listeria-induced activation of intestinal epithelial cells is largely dependent on invasion and endosomal lysis. (A) m-ICcl2 cells were infected with wild-type (wt) Listeria monocytogenes. Cxcl-2 was determined after the indicated time in cell culture supernatant by ELISA. (B) m-ICcl2 cells were infected with wt, hly mutant or hly/plcA/plcB triple mutant L. monocytogenes. Cxcl-2 was determined after the indicated time in cell culture supernatant by ELISA. (C) m-ICcl2 cells were treated with small interfering RNA (siRNA) control or siRNA Tlr2, MyD88, Rip2, Nod2, Ipaf or Nalp3 and infected with actA mutant L. monocytogenes. Cxcl-2 was determined 4 h after infection in cell culture supernatant by ELISA. **, p<0.01. (D) m-ICcl2 cells were infected with viable or heat inactivated (h.i.) wt L. monocytogenes. Cxcl-2 was determined 6 h after infection in cell culture supernatant by ELISA. (E) m-ICcl2 cells were infected with wt L. monocytogenes expressing green fluorescence protein (GFP) under control of the actA promoter (PactA-gfp). The number of GFP+ (Listeria-infected, black square) or immunolabelled (EI) Cxcl-2+ or (EE) Cxcl-5+ (white square) cells was determined after the indicated time by flow cytometry. (F) m-ICcl2 cells were infected with hly mutant PactA-gfp L. monocytogenes. The number of GFP+ (Listeria-infected, black square) or immunolabelled Cxcl-2+ cells was determined after the indicated time by flow cytometry. All experiments were performed at a multiplicity of infection of 100:1. Results are representative for three independent experiments and are presented as mean ± SD (ELISA) or show one representative experiment. doi:10.1371/journal.ppat.1001194.g001
suggested that activation of epithelial cells occurred primarily in directly Listeria-infected cells.

To monitor Listeria infection and cellular activation simultaneously at the single cell level, bacteria transformed with a vector expressing green fluorescence protein (GFP) either under control of the inducible actA promoter [11] or the constitutive sod promoter [12] were used for subsequent experiments (for details see Table 1). Surprisingly, flow cytometry revealed that the vast majority of Cxcl-2⁺ epithelial cells (95%) were Listeria-negative. In addition, only a minor fraction of GFP-positive, Listeria-infected epithelial cells exhibited MIP-2 synthesis (Fig. 2A). Similar results were obtained using biotinylated Listeria (Fig. S2A). These results were confirmed by immunohistological staining. Cxcl-2 and Cxcl-5 synthesis was not restricted to GFP⁺ Listeria-infected cells but, in fact, predominantly detected in neighbouring non-infected epithelial cells (Fig. 2B and Fig S2B). Also flow cytometric cell sorting and quantitative RT-PCR analysis strongly supported this unexpected result. A marked upregulation of Cxcl-2 and Cxcl-5 mRNA expression was detected in GFP⁺ Listeria-infected cells but, in fact, predominantly detected in neighbouring non-infected epithelial cells (Fig. 2B and Fig S2B). Also flow cytometric cell sorting and quantitative RT-PCR analysis strongly supported this unexpected result. A marked upregulation of Cxcl-2 and Cxcl-5 mRNA expression was detected in GFP⁺ Listeria-infected cells but, in fact, predominantly detected in neighbouring non-infected epithelial cells (Fig. 2B and Fig S2B). Also flow cytometric cell sorting and quantitative RT-PCR analysis strongly supported this unexpected result. A marked upregulation of Cxcl-2 and Cxcl-5 mRNA expression was detected in GFP⁺ Listeria-infected cells but, in fact, predominantly detected in neighbouring non-infected epithelial cells (Fig. 2B and Fig S2B). Also flow cytometric cell sorting and quantitative RT-PCR analysis strongly supported this unexpected result.

Cell-to-cell spread, attachment-induced activation, or listeriolysin are not responsible for indirect cell activation

Several mechanisms might account for the observed activation of Listeria-negative epithelial cells. Activated epithelial cells might only appear to be Listeria-negative due to secondary bacterial escape facilitated by propulsion through ActA-induced actin polymerization in the cytosol and subsequent invasion of the neighbouring cell. Cells primarily infected but secondarily left by lateral spread might thereby appear Listeria-negative but in fact would have been previously in contact with cytosolic bacteria (and thus were, in fact, directly activated). To avoid lateral cell-to-cell spread and restrict intraepithelial bacteria to apically infected cells, a Listeria actA mutant strain was employed. ActA-deficient Listeria exhibited a moderately reduced epithelial invasion (Fig. S3A), a lower percentage of infected epithelial cells, and an enhanced number of bacteria per cell (Fig. 3A and 3B). Nevertheless, high numbers of Cxcl-2-producing epithelial cells (Fig. 3B) and a strong chemokine secretion (Fig. 3C) was observed. Also, the number of activated, Cxcl-2 producing epithelial cells remained significantly higher than the number of Listeria-infected cells reaching approximately 10-fold excess of Cxcl-2⁺ cells (Fig. 3B and Fig. S3B). Thus, indirect activation of epithelial cells was not due to escape from previously infected cells by ActA-driven secondary lateral spread.

Epithelial cell stimulation could also be induced by bacteria either attached to the plasma membrane or remaining intraendosomal and membrane enclosed. To exclude a significant role of attached or intraendosomal Listeria, Cxcl-2⁺ and GFP⁺ epithelial cells were quantified after infection with Listeria expressing GFP either constitutively under control of the superoxide dismutase promoter (P⁻gfp) or inducible under the control of the actA promoter (P⁺gfp) (Table 1). Whereas P⁺gfp carrying Listeria exhibited strong reporter expression after growth in bacterial culture medium, only a moderate fluorescence was detected in P⁻gfp - positive bacteria (Fig. S3C). In contrast, strong GFP expression was noted in P⁺gfp Listeria isolated from infected epithelial cells (Fig. S3D). Flow cytometric detection of infected epithelial cells was observed after wt, but not hly mutant P⁺gfp Listeria illustrating the endosomal lysis-dependent induction of the actA promoter-driven GFP reporter gene expression (Fig. S3E). Infection with P⁻gfp or P⁺gfp carrying wt Listeria resulted in a significant number of Listeria-infected epithelial cells. Importantly, a higher number of Cxcl-2⁺ activated cells as compared to Listeria-infected cells was observed by flow cytometry after infection with both reporter constructs and epithelial Cxcl-2 synthesis was similarly noted in Listeria-negative epithelial cells (Fig. 3D). These results suggest that indirect epithelial activation was not a result of attached or intraendosomal bacteria [13].

Finally, the activation of Listeria-negative epithelial cells might be due to the stimulatory effect of secreted bacterial molecules, such as the cytolytic listeriolysin O (LLO) [14,15]. Therefore, the membrane damaging as well as the stimulatory effect of recombinant listeriolysin (rLLO) on red blood cells (RBC) and epithelial mIC₃₂₂ cells was analysed. High concentrations of rLLO induced significant hemoglobin and detectable lactate dehydrogenase (LDH) release by RBCs and epithelial mIC₃₂₂ cells, respectively (Fig. S3F and Fig. S3G). Quantitation of epithelial cell activation in response to rLLO, however, revealed an only minor response as compared to epithelial Cxcl-2 secretion after viable wt L. monocytogenes infection (Fig. 3E). Similarly, no significant Cxcl-2 secretion by epithelial cells was noted in response to bacteria-free culture supernatant derived from Listeria cultures with bacterial counts precisely corresponding to the infection model described.

Table 1. Listeria strains used in this study.

<table>
<thead>
<tr>
<th>L. monocytogenes EGD</th>
<th>Plasmid</th>
<th>Characteristic</th>
<th>Ref.</th>
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<tr>
<td>wt</td>
<td>-</td>
<td>wild-type strain EGD</td>
<td>[60]</td>
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<tr>
<td>wt</td>
<td>P⁺gfp</td>
<td>wild-type; gfp expression mainly after endosomal lysis</td>
<td>this study</td>
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<tr>
<td>hly</td>
<td>-</td>
<td>LLO-deficient; strongly reduced endosomal lysis</td>
<td>[61]</td>
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<tr>
<td>hly</td>
<td>P⁺gfp</td>
<td>LLO-deficient; strongly reduced endosomal lysis; gfp expression mainly after endosomal lysis</td>
<td>this study</td>
</tr>
<tr>
<td>hly plcA plcB</td>
<td></td>
<td>LLO- and PLC-deficient; no endosomal lysis</td>
<td>Chakraborty T.</td>
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<tr>
<td>actA</td>
<td></td>
<td>deficient in polar actin accumulation; no lateral cell-to-cell spread</td>
<td>[60]</td>
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<tr>
<td>actA</td>
<td>P⁺gfp</td>
<td>deficient in polar actin accumulation; no lateral cell-to-cell spread, gfp expression mainly after endosomal lysis</td>
<td>this study</td>
</tr>
<tr>
<td>actA</td>
<td>P⁻gfp²</td>
<td>deficient in polar actin accumulation; no lateral cell-to-cell spread, constitutive GFP expression</td>
<td>this study</td>
</tr>
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P⁺gfp and P⁻gfp are listerial actA and sod gene promoters, respectively. Abbreviations: actA: actin assembly inducing protein precursor; gfp: green fluorescent protein; hly: listeriolysin O precursor; LLO: listeriolysin O; plc: phospholipase C; sod: superoxide dismutase.

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Innate Immune Activated Epithelial Communication

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above (Fig. 3F). Yet, culture supernatants derived from wild-type or ActA-deficient bacteria exhibited significant hemolytic activity, in contrast to supernatant from hly-deficient Listeria (Fig. S3H). No significant membrane damage was noted after infection of intestinal epithelial cells with wt, actA, or hly mutant Listeria (Fig. S3I).

Although a supportive effect of released bacterial factors cannot be excluded, these results suggest that bacterial mediators do not play a major role in the observed indirect epithelial activation. Thus, neither basolateral cell-to-cell spread nor membrane attachment, or the secretion of LLO in the cell culture supernatant appear to be responsible for indirect epithelial cell activation after L. monocytogenes infection. This suggests the presence of a previously unrecognized mechanism of epithelial intercellular communication in response to bacterial infection.

**Indirect epithelial activation is not induced by epithelial transcriptional activation per se**

To examine whether indirect epithelial stimulation by horizontal cell-to-cell communication might be a general effect of transcriptional activation of intestinal epithelial cells, a bicistronic expression vector encoding the NF-κB subunit RelA/p65 together with GFP under the control of a constitutive cytomegalovirus (CMV) promoter was employed (Fig. S4A). Transient overexpression of RelA/p65 alone or bicistronic expression of RelA/p65 and GFP readily induced epithelial activation as illustrated by NF-κB reporter gene upregulation (Fig. S4B). Although detectable Listeria in the sorted GFP+/low cell fraction was demonstrated by PCR amplification of the bacterial hly gene (sensitivity limit: 10^3–10^4 genome copies), all experiments were performed at a multiplicity of infection of 100:1. Results are representative for three independent experiments and are presented as mean ± SD (RT-PCR) or show one representative experiment.

doi:10.1371/journal.ppat.1001194.g002

**Figure 2. Analysis of bacterial infection and epithelial activation at the single cell level.** (A) m-iCcl2 cells were left uninfected (Ai, left) or infected with wt P<sub>actA-gfp</sub> L. monocytogenes (Ai, right). The number [%] of GFP<sup>+</sup> (Listeria-infected, green) or immunolabelled Cxcl-2<sup>+</sup> (red) cells was visualized 4 h after infection by flow cytometry. Single channel analysis (GFP: FL-1; Cxcl-2: FL-4) was depicted on the side of the axis. (Aii) The number of GFP<sup>+</sup> (Listeria-infected) cells among activated, Cxcl-2<sup>+</sup> cells and (Aiii) the proportion of Cxcl-2<sup>+</sup> cells among GFP<sup>+</sup> (Listeria-infected) cells was demonstrated gating on the respective population. (B) m-iCcl2 cells were infected with wt P<sub>actA-gfp</sub> L. monocytogenes. Intracellular Listeria (GFP, green) and immunolabelled Cxcl-2<sup>-</sup> (red) or Cxcl-5<sup>-</sup> (red) was visualized 4 h after infection by fluorescence microscopy. Magnification ×400, counterstaining with Dapi (blue). Scale bar, 5 μm. (C) m-iCcl2 cells were infected with actA mutant L. monocytogenes expressing constitutively GFP under control of the sod promoter (P<sub>sod-gfp</sub>). 4 h after infection the GFP<sup>-</sup> expressing (Listeria-negative) cell fraction was sorted by flow cytometry (see Fig. S2C) and analysed for Cxcl-2 (upper) or Cxcl-5 (lower) mRNA expression by RT-PCR. The absence of detectable Listeria in the sorted GFP<sup>-</sup> cell fraction was demonstrated by PCR amplification of the bacterial hly gene (sensitivity limit: 10^3–10^4 genome copies). All experiments were performed at a multiplicity of infection of 100:1. Results are representative for three independent experiments and are presented as mean ± SD (RT-PCR) or show one representative experiment.
Figure 3. Epithelial activation is not due to bacterial cell-to-cell spread, extracellular attachment, or secreted listeriolysin. (A) m-ICcl2 cells were infected with wt (left) or actA mutant (right) PactA-gfp L. monocytogenes. Intracellular Listeria (GFP+, green) was visualized 6 h after infection by fluorescence microscopy. Magnification ×400, counterstaining with phalloidin (red) and Dapi (blue). White arrows indicate the actin tail assembly by wt Listeria. Scale bar, 5 μm. (B) m-ICcl2 cells were infected with actA mutant PactA-gfp L. monocytogenes. The number of GFP+ (Listeria-infected, black square) or immunolabelled Cxcl-2+ (white square) cells was determined after the indicated time by flow cytometry. (C) m-ICcl2 cells were infected with wt (white) or actA mutant (black) L. monocytogenes. Cxcl-2 was determined after the indicated time in cell culture supernatant by ELISA. ns, not significant (mutant versus wt for the indicated time points). (D) m-ICcl2 cells were infected with actA mutant PactA-gfp or PactA-gfp L. monocytogenes. The number of GFP+ (Listeria-infected, black) or immunolabelled Cxcl-2+ (white) cells was determined after the indicated time by flow cytometry (lower panel). Additionally, intracellular Listeria (GFP+, green) and immunolabelled Cxcl-2 (red) was visualized 4 h after infection by fluorescence microscopy (upper panel). Magnification ×400, counterstaining with Dapi (blue). Scale bar, 5 μm. (E) m-ICcl2 cells were infected with wt L. monocytogenes or exposed to recombinant listeriolysin at lytic to sublytic concentrations or to the solvent control (DTT). Cxcl-2 was determined 4 h after infection in cell culture supernatant by ELISA. (F) m-ICcl2 cells were infected with wt L. monocytogenes or exposed to undiluted or diluted filtered culture supernatant. Conditioned cell culture supernatant. The number of GFP+ (Listeria-infected, black) or immunolabelled Cxcl-2+ (white) cells was determined after the indicated time by flow cytometry (lower panel). Additionally, intracellular Listeria (GFP+, green) and immunolabelled Cxcl-2 (red) was visualized 4 h after infection by fluorescence microscopy (upper panel). Magnification ×400, counterstaining with Dapi (blue). Scale bar, 5 μm. (E) m-ICcl2 cells were infected with wt L. monocytogenes or exposed to recombinant listeriolysin at lytic to sublytic concentrations or to the solvent control (DTT). Cxcl-2 was determined 4 h after infection in cell culture supernatant by ELISA. (F) m-ICcl2 cells were infected with wt L. monocytogenes or exposed to undiluted or diluted filtered culture supernatant. Conditioned cell culture supernatant. The number of GFP+ (Listeria-infected, black) or immunolabelled Cxcl-2+ (white) cells was determined after the indicated time by flow cytometry (lower panel). Additionally, intracellular Listeria (GFP+, green) and immunolabelled Cxcl-2 (red) was visualized 4 h after infection by fluorescence microscopy (upper panel). Magnification ×400, counterstaining with Dapi (blue). Scale bar, 5 μm. (E) m-ICcl2 cells were infected with wt L. monocytogenes or exposed to recombinant listeriolysin at lytic to sublytic concentrations or to the solvent control (DTT). Cxcl-2 was determined 4 h after infection in cell culture supernatant by ELISA. All infection experiments were performed at a multiplicity of infection of 100:1. Results are representative for three independent experiments and are presented as mean ± SD (ELISA) or show one representative experiment. doi:10.1371/journal.ppat.1001194.g003

Analysis of cytokine secretion, ion channel stimulation, and gap junction activity in horizontal cell-cell communication

Next we investigated the mechanism underlying horizontal cell-to-cell communication and coordinated epithelial chemokine upregulation in response to Listeria infection. Functional gap junctional transport was examined by microinjection of transferable Lucifer Yellow together with non-transferable high molecular weight dextran. Fluorescence imaging visualized transport of Lucifer Yellow from the microinjected cell to the surrounding neighbouring cells. Addition of inhibitors of gap junctional transport, effectively reduced lateral diffusion of Lucifer Yellow after microinjection (Fig. 4A and B). Inhibition of gap junctional intercellular communication, however, did not decrease the number of activated epithelial cells after Listeria infection as illustrated by the unaltered high ratio of activated (Cxcl-2+) to infected (GFP+) epithelial cells measured by flow cytometry (Fig. 4C). Although these results do not completely rule out transfer of very small signaling molecules by gap junctional transport channels, they do not support a major role in the process of horizontal communication.

Similarly, the potential role of a secreted protein messenger was examined. Intestinal epithelial m-ICcl2 cells were exposed to brefeldin A (BFA), an effective inhibitor of the secretion of newly synthesized proteins (Fig. S4F), prior and after infection with actA mutant L. monocytogenes. The number of Listeria-induced Cxcl-2+ cells, however, was not altered irrespective whether BFA was administered 30 min prior or 60 min after infection (Fig. 4D). Second, cell culture medium was obtained 10, 20, 30, 40, or 60 min after Listeria infection, centrifuged to remove bacteria, and immediately transferred to naïve uninfected epithelial cells. Yet no epithelial activation was observed after exposure to conditioned culture supernatant despite significant Cxcl-2 synthesis detected in the Listeria infected cell population (Fig. S4G). Of note, factors released by Listeria-infected cells might be unstable or immediately bound to neighbouring cells preventing their efficient release in the conditioned cell culture supernatant.

Finally, widely used pharmacological inhibitors of prostaglandin synthesis and known intestinal epithelial ion channels were employed. Indomethacin, an inhibitor of cyclooxygenase isoenzymes (COX1, COX2) involved in prostaglandin synthesis, thapsigargin, an inhibitor of the endoplasmic Ca2+ATPase, CFTR II, a selective apical Cl− ion channel inhibitor, and bumetanide, an inhibitor of a basolateral epithelial Na+K+Cl− cotransporter had no significant influence on the number of activated epithelial cells after Listeria infection illustrated as ratio of
L. monocytogenes fluorescence microscopy. Magnification acquiring Lucifer Yellow (LY) by gap junction mediated transport after microinjection in the absence or presence of gap junction inhibitors. A presence of gap junction inhibitors by flow cytometry. (immunolabelled Cxcl-2 microinjection induced Cxcl-2 synthesis in neighbouring cells liberating organic agent within the cell culture medium or by exposure of epithelial cells to cumene hydroperoxide, a ROI permissible Lucifer Yellow in the absence (Ai, Control) or presence (Aii) of the gap junction inhibitor oleamide (Ole, 0.1 mM), carbonoxolone (Carbo, 0.01 mM), or α-glycerethinic acid (AGA, 0.1 mM). The cellular spread of Lucifer Yellow was visualized 5 min after microinjection by live imaging fluorescence microscopy. Magnification ×400. Phase contrast images were added to visualize single cells. Scale bar, 5 μm. (B) Number of cells acquiring Lucifer Yellow (LY) by gap junction mediated transport after microinjection in the absence or presence of gap junction inhibitors. A minimum of 10 microinjected cells were analysed per experiment; **, p<0.01, ***, p<0.005. (C) m-iCδ2 cells were infected with actA mutant P actA:gfp L. monocytogenes. The ratio of immunolabelled Cxcl-2+ cells to GFP+ (Listeria-infected) cells was determined 4 h after infection in the absence or presence of gap junction inhibitors by flow cytometry. (D) m-iCδ2 cells were infected with actA mutant P actA:gfp L. monocytogenes. The number of immunolabelled Cxcl-2+ cells was determined after the indicated time by flow cytometry. Brefeldin A was added 60 min after (post, white bars) or 30 min prior to (pre, black bars) infection. All infection experiments were performed at a multiplicity of infection of 100:1. Results are representative for three independent experiments and are presented as mean ± SD (microinjection) or show one representative experiment. doi:10.1371/journal.ppat.1001194.g004

activated (Cxcl-2+) to infected (GFP+) cells (Fig. S4H). These results do not identify a significant role of gap junctional transport, secreted protein or prostaglandin mediators, or ion fluxes in the observed indirect activation of epithelial cells after L. monocytogenes infection.

**Listeria**-induced horizontal epithelial communication depends on oxygen radical synthesis

Since unstable and highly reactive host-derived factors were not excluded by the previous experiments, a possible involvement of oxygen or nitrogen radicals in horizontal epithelial cell-cell communication was subsequently evaluated. Expression of members of two enzyme families, NADPH oxidases and nitric oxide synthase (NOS), has been described in epithelial cells [16]. Indeed, addition of the NADPH oxidase inhibitor diphenylene iodonium (DPI) resulted in a significant reduction of *Listeria*-induced epithelial activation (Fig. 5A). DPI did not reduce *Listeria* survival in epithelial cells (Fig. S5B) and had no effect on LPS or PMA-induced epithelial activation (Fig. S5A). In contrast to DPI, the NOS inhibitor N (G)-nitro-L- arginine methyl ester (L-NAME) did not influence the number of activated epithelial cells (Fig. 5B). In accordance with an inhibitory effect of DPI, synthesis of reactive oxygen intermediates (ROI) after *Listeria* infection was observed (Fig. 5C). ROI was detected in focal areas of confluent epithelial cells surrounding *Listeria*-positive, infected cells in accordance with local production and lateral spread of ROI as early as 10 min after infection (Fig. 5D).

Innate immune receptor stimulation by *Listeria* infection of epithelial cells resulted in rapid activation of the mitogen-activated protein (MAP) kinase Erk in a ROI-dependent manner (Fig. 5E). Whereas impairment of the MAP kinase Erk had no significant effect on ROI production (Fig. 5F), *Listeria*-induced Cxcl-2 synthesis by intestinal epithelial cells was completely abrogated by Erk inhibition and partially also dependent on the MAP kinases p38 and JNK (Fig. 5G). Of note, Erk inhibition did not affect bacterial invasion and the viability of intracellular *Listeria* (Fig. S5B). Finally, exposure of epithelial cells to cumene hydroperoxide, a ROI liberating organic agent within the cell culture medium or by microinjection induced Cxcl-2 synthesis in neighbouring cells similar to *L. monocytogenes* infection (Fig. 5H). Thus, *Listeria*-infection induces significant epithelial ROI synthesis, which in turn mediates MAP kinase Erk activation and downstream Cxcl-2 production.

Oxygen radical synthesis is performed by an oligomeric protein complex involving a cell type-specific NADPH-oxidase (Nox) protein. Only significant expression of the Nox4 isoform was detected in primary small intestinal epithelial cells (Fig. 6A). Nox4 synthesis was restricted to intestinal epithelial cells as demonstrated by immunostaining with a paranuclear expression pattern in accordance with a previous report (Fig. 6B) [16]. Importantly, downregulation of Nox4 expression in epithelial cells by siRNA interference significantly reduced Cxcl-2 secretion (Fig. 6C) and ROI production upon *Listeria*-infection (Fig. 6D). In contrast, downregulation of Nox4 expression did not alter LPS- or PMA-induced chemokine secretion (Fig. S6). Thus, ROI production by Nox4 appears to be both necessary and sufficient to induce horizontal cell-cell communication in intestinal epithelial cells leading to chemokine secretion in neighbouring cells in response to *Listeria* infection.

**Discussion**

Communication between individual cells is a fundamental feature of multicellular organisms. For instance, it mediates a coordinated reaction of muscle cell contraction, and allows neuronal signal transmission or endocrinological regulatory circuits. Cell-cell communication is also characteristic for the complex regulatory networks of the adaptive immune system. Cytokines bridge anatomical distances to coordinate and amplify the host response against pathogens. In the present study we investigated whether cell-cell communication between neighbouring cells might also contribute to innate immune activation within a confluent epithelial cell layer to coordinate the antimicrobial host defence at an early stage of the infection. Although inhibition of the overall epithelial responses by interference significantly reduced Cxcl-2 secretion (Fig. 6C) and ROI production upon *Listeria*-infection (Fig. 6D). In contrast, downregulation of Nox4 expression did not alter LPS- or PMA-induced chemokine secretion (Fig. S6). Thus, ROI production by Nox4 appears to be both necessary and sufficient to induce horizontal cell-cell communication in intestinal epithelial cells leading to chemokine secretion in neighbouring cells in response to *Listeria* infection.
the first report to demonstrate epithelial horizontal cell-cell communication upon bacterial innate immune stimulation.

For three reasons, *Listeria* infection of confluent intestinal epithelial cells represents an ideal model to study epithelial cell-cell communication downstream of innate immune stimulation. First, similar to other pathogenic bacteria *Listeria monocytogenes* escapes from the endosomal vacuole and proliferates within the host cell cytosol [6]. Endosomal escape is associated with a dramatic change in bacterial gene expression. Although expressed at low levels also during in vitro culture, a very strong upregulation

Figure 5. Epithelial intercellular communication is dependent on *Listeria*-induced oxygen radical synthesis. (A) m-ICcl2 cells were infected with actA mutant *P. aeruginosa*-gfp *Listeria monocytogenes*. The number of immunolabelled Cxcl-2+ cells was determined 4 h after infection in the absence or presence of diphenylene iodonium (DPI, mM) by flow cytometry. (B) m-ICcl2 cells were infected with actA mutant *P. aeruginosa*-gfp *Listeria monocytogenes*. The ratio of immunolabelled Cxcl-2+ cells to GFP+ (*Listeria*-infected) cells was determined 4 h after infection in the absence or presence of N (G)-nitro-L-arginine methyl ester (L-NAME) or DPI (each at 0.1 mM) by flow cytometry. (C) m-ICcl2 cells were loaded with DCF-DA and infected with wt or actA mutant *L. monocytogenes*. DCF relative fluorescence, reflecting reactive oxygen intermediates (ROI) production was quantified 20 min after infection by fluorescence spectroscopy. H2O2 (1 mM) was used as positive control. (D) m-ICcl2 cells were loaded with DCF-DA and infected with immunolabelled wt *L. monocytogenes* (red). DCF fluorescence, reflecting ROI production was visualized at the indicated time after infection by fluorescence microscopy. Magnification ×400, counterstaining with Dapi (blue). Scale bar, 5 μm. (Ei) m-ICcl2 cells were treated with small interfering RNA (siRNA) control or siRNA Nox4, Rip2, Nod2, Ipaf or Nalp3 and infected with wt *L. monocytogenes*. p44/42 phosphorylation was determined 50 min after infection by immunoblotting. (Eii) m-ICcl2 cells were infected with wt *L. monocytogenes* in the absence or presence of DPI (0.1 mM) or exposed to H2O2 (1 mM) [left], or alternatively in the absence or presence of UO-126 (10 μM) [right]. p44/42 phosphorylation was determined after the indicated time by immunoblotting. (F) m-ICcl2 cells were infected with wt (black square) or actA mutant (gray square) *L. monocytogenes*. DCF relative fluorescence, reflecting ROI production was visualized 4 h after infection or stimulation by fluorescence microscopy. The microinjected cell was loaded with Texas Red-conjugated dextran (green). Magnification ×400, counterstaining with Dapi (blue). Scale bar, 5 μm. All experiments were performed at a multiplicity of infection of 100:1. Results are representative for three independent experiments and are presented as mean ± SD (ROI, ELISA) or show one representative experiment. *, p<0.05, **, p<0.01, ***, p<0.005, ns, not significant. doi:10.1371/journal.ppat.1001194.g005
Figure 6. Activation of the NADPH oxidase (Nox) 4 mediates indirect epithelial activation. (A) Expression of gp91^phox (Nox2), Nox1, 3, and 4 in isolated highly pure (≥98% E-cadherin”, CD45”) primary intestinal epithelial cells. (B) Immunostaining for Nox4 expression in sections of C57BL/6 mouse small intestinal tissue. Magnification ×200; counterstaining with phallolidin (green) and Dapi (blue). Scale bar, 50 μm. For demonstration of the subcellular localisation of Nox4 (red), the insert (white frame) was enlarged ×630 (right panel). White arrows highlight the subcellular localization of Nox4. Scale bar, 5 μm. (C) m-ICcl2 cells were treated with small interfering RNA (siRNA) control or siRNA Nox4 and left uninfected (white) or were infected (black) with actA mutant L. monocytogenes. Cxcl2-2 was determined 4 h after infection in cell culture supernatant by ELISA (upper panel). Nox4 downregulation was verified by immunoblotting (lower panel). (D) m-ICcl2 cells were treated with small interfering RNA (siRNA) control or siRNA MyD88, Trl2, or Nox4, loaded with DCF-DA and infected with actA mutant L. monocytogenes. DCF relative fluorescence, reflecting ROI production, was quantified 20 min after infection by fluorescence spectroscopy. All infection experiments were performed at a multiplicity of infection of 100:1. Results are representative for three independent experiments and are presented as mean ± SD (ELISA, ROI) or show one representative experiment. doi:10.1371/journal.ppat.1001194.g006

of the actin polymerizing protein ActA provides an excellent reporter for detection of cytosolic entry [22]. Second, bacteria lacking hly, plcA, or plcB mediating endosomal lysis only induce an only minor activation which might result from intraendosomal recognition or a so far unidentified minor mechanism of endosomal escape. Thus, in contrast to macrophages that recognize Listeria also at the plasma membrane, epithelial cell stimulation is mainly observed when bacteria reach the cytosol, facilitating contact with cytosolic innate immune receptors such as Ipaf, Nalp3, and Nod2 [9,10]. This finding excludes innate immune recognition and receptor-mediated initiation of signal transduction in non-infected, Listeria-negative cells. Third, one amino acid exchange between the mouse and human E-cadherin causes a strongly reduced infection rate in murine epithelial cells [23], leaving most cells of a confluent cell layer uninfected and accessible to the analysis of indirect cellular activation. Using reporter gene technology, intracellular chemokine staining and flow cytometric analysis, we were able to demonstrate that the chemokine secretion in response to Listeria infection is mainly derived from uninfected, indirectly activated epithelial cells. Of note, the commonly used quantification of cytokine secretion in the cell culture supernatant or immunoblotting of total cell lysate proteins would not have disclosed this surprising finding.

Epithelial stimulation on the transcriptional level by p63/RelA overexpression did not result in detectable indirect cell activation. Several possible mechanisms of horizontal cell-cell communication downstream of innate immune receptor signaling were therefore considered. In response to microbial stimulation, epithelial cells produce chemokines, prostaglandins, and cause local alterations of ion concentrations by regulating transmembrane ion channel activity. Also, gap junctional intercellular communication (GJIC) represents a direct cytosolic connection and might be used to forward the information of innate immune recognition within the epithelial cell layer [17,21]. Ca2+ fluxes via intercellular gap junctions have been shown to promote lung epithelial chemokine secretion [18] and intact gap junction formation has also been linked to innate immune stimulation and maintenance of the epithelial barrier [19]. On the other hand, connexin-26 hemichannel-mediated Ca2+ signaling has also been proposed to promote bacterial invasion and lateral spread [24]. Yet, neither protein secretion, nor ion channel activity or gap junction formation appeared to be involved in Listeria-induced indirect epithelial cell activation.

Instead, our results indicate an important role of reactive oxygen intermediates (ROI) in horizontal epithelial cell-cell communication. ROI represent reduction products of molecular oxygen such as the radical superoxide (O2·−) and hydroxyl (·OH), and the non-radical hydrogen-peroxide (H2O2). ROI production by professional phagocytes during oxidative burst provides significant bactericidal activity but synthesis is also observed in non-phagocytic cells [25]. ROI at subtoxic doses has been recognized as an important intracellular signal transducing molecule during the recent years [26,27,28,29]. In accordance with our results ROI-induced activation of MAP kinase activity has been reported [30,31,32,33]. In addition, an involvement of ROI in the cellular signaling leading to NF-κB activation [34], apoptosis [31,35], epidermal growth factor receptor signaling [36], regulation of
cellular proliferation [37], and antimicrobial peptide production [38,39] has been described. ROI was also shown to prime Drosophila melanogaster hematogenic progenitor cells for differentiation [40] and to play an important role in the fruit fly's intestinal immunity [41]. Whereas the half-life of oxygen radical hydroxyl (•OH) is extremely short (10^{-9}s) and the superoxide (O_2^-) is membrane impermeable [40], H_2O_2 is able to diffuse to neighbouring cells and induce cellular activation. Indeed, a tissue gradient of H_2O_2 was shown to induce rapid recruitment of leukocytes into the wound margin following endothelial hypoxia [42,43].

NADPH oxidase activation has previously been linked to innate immune mediated antimicrobial killing [25] as well as receptor signal transduction [44,45,46,47,48,49,50,51]. Here we for the first time report Nox4 expression by intestinal epithelial cells and demonstrate Nox4-mediated ROI production in response to bacterial infection. Although enhanced Nox4 mRNA expression was shown to result in increased ROI production [52], the initiation of Nox4-dependent ROI production upon Listeria infection was noted as early as 5–10 minutes after bacterial challenge. This excludes a significant role of transcriptional regulation of Nox4 in our model. Whereas the prototypical NADPH oxidase of phagocytes, gp91^phox (Nox2), requires cytosolic proteins such as p47^phox to form a functional NADPH oxidase complex, Nox4 functions independent of cytosolic accessory proteins. Interestingly, Nox expression has previously been linked to innate immune receptor signaling: Nox4 activation was shown to be involved in TLR4-mediated NF-κB activation in human epithelial kidney cells and monocytes [47,53]. In contrast, our results revealed activation of intestinal epithelial cells by Listeria infection in a Nod2-, Ipaf-, and Nalp3-dependent fashion which was followed by ROI production and subsequent MAP kinase signaling. Our data are therefore in accordance with previous reports on MAP kinase activation after Nox4-mediated ROI production [54,55]. A future analysis of the local paracellular concentration of the different species of oxygen radicals might help improve our understanding of the regulatory role of Nox4-mediated ROI production for epithelial cell-cell communication. Interestingly, reduced chemokine synthesis was noted in directly infected, Listeria-positive cells. These cells were also impaired to respond to secondary innate immune stimulation illustrating the immune evasive behaviour of L. monocytogenes (data not shown). Although the underlying mechanism is currently not resolved, high concentrations of ROI were previously associated with reduced susceptibility to immunostimulatory agents [56]. Yet other bacterial or host factors such as antioxidant enzymes might reduce local ROI concentrations and interfere with cellular activation and chemokine production in infected epithelial cells.

In conclusion, our data for the first time analyzed intestinal epithelial activation in response to bacterial infection at a single cell level. We could detect Nox4 expression by intestinal epithelial cells which facilitated rapid ROI production upon infection and paracrine activation of neighbouring cells (Fig. 7). Our findings thus identify horizontal cell-cell communication to allow a coordinated innate immune activation of the intestinal epithelium. The present work significantly broadens our knowledge on the complex processes that underlie mucosal innate immune stimulation and illustrates the specific role of epithelial cells for an efficient activation of the antimicrobial host defence.

Materials and Methods

Antibodies and reagents

Intracellular Cxcl-2 (MIP-2) and Cxcl-5 was detected using rabbit antibodies from Nordic Biosite (Täby, Sweden). The rabbit polyclonal anti-actin antiserum was from Sigma-Aldrich (Taufenkirchen, Germany). The rabbit-anti-mouse Nox4 antiserum was produced [54,55]. A future analysis of the local paracellular concentration of the different species of oxygen radicals might help improve our understanding of the regulatory role of Nox4-mediated ROI production for epithelial cell-cell communication. Interestingly, reduced chemokine synthesis was noted in directly infected, Listeria-positive cells. These cells were also impaired to respond to secondary innate immune stimulation illustrating the immune evasive behaviour of L. monocytogenes (data not shown). Although the underlying mechanism is currently not resolved, high concentrations of ROI were previously associated with reduced susceptibility to immunostimulatory agents [56]. Yet other bacterial or host factors such as antioxidant enzymes might reduce local ROI concentrations and interfere with cellular activation and chemokine production in infected epithelial cells.

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obtained by immunization with recombinant peptide. The rabbit anti-p-p44/42 (phospho-Erk) and the mouse anti-p-p44/42 (total-Erk) was from Cell Signaling Technology (Beverly, MA, USA). The rabbit anti-Listeria antibody was from Dunn Labortechnik GmbH (Asbach, Germany). Cy5-, Cy3-, HRPO-conjugated secondary antibodies were from Jackson Immunoresearch (West Grove, PA, USA) and the Alexa Fluor (AF) 488-, and AF 555-conjugated donkey anti-rabbit IgG (H+L) was from Invitrogen (Molecular Probes). The MFP590- and MFP488-labelled phalloidin were purchased from MoBiTec GmbH (Goettingen, Germany). The Sulfo NHS-LC-Biotin was obtained from Pierce, Thermo Scientific (Rockford, IL, USA). Escherichia coli K12 D31m4 LPS was ordered from List Biological Laboratories (Campbell, CA, USA). Recombinant LLO (rLLO) was expressed and purified exactly as described before [57]. rLLO was applied to cells in a serial dilution with 0.05 μg/mL as highest concentration. Cxcl-2 was quantified using an ELISA from Nordic Biosite or R&D Systems (Quantikine, R&D Systems GmbH, Wiesbaden, Germany). The NF-κB reporter construct pBIX-luciferase carrying two copies (2× NF-κB) of the κB sequences from the Igκ enhancer was provided by S. Ghosh (Yale University Medical School, New Haven, CT, USA). Luciferase activity was quantified with luciferin substrate (PKJ GmbH, Kleinblittersdorf, Germany). The biocistronic RelA/p65 expression plasmid was cloned by removing the nef gene from a pCG- nef-IRE5-GFP expression plasmid (provided by J. Muench, Institute of Virology, University Clinic of Ulm, Germany) by digestion with the restriction enzymes XbaI and MluI (Fermentas, St Leon-Rot, Germany) and replacing it in frame with the p65 encoding gene amplified from a p65 expression plasmid (obtained from by U. Pahl, University Clinic, Freiburg, Germany) using the forward: 5’- ACC TCT AGA CCT TGG ACG ATC TGT TTC C-3’ and reverse: 5’- ACC TCT ACG CCT CGT CCA CTT CAG GAG CTG ATC TGA-3’ primers and digested with XbaI/MluI prior to ligation. Plasmid DNA for transfection was prepared using the endotoxin-free plasmid kit from Qiagen (Hilden, Germany). Targeted siRNA probes (Th2, Rip2, Nox4, Card12, Gask1, control siRNA) were from Qiagen (Hilden, Germany), the Card15 siRNA was from Santa Cruz (Heidelberg, Germany). Lipofectamin 2000 (Invitrogen, Carlsbad, CA, USA) and INTERFERin (Polyplus Transfection, New York, NY, USA) were used for plasmid and siRNA transfection, respectively. The pharmacological inhibitors and radical donors oleamide, carbonoxolone, α-glycerethinic acid, brefeldin A, thapsigargin, CFTR inhibitor II (CFTF II), indomethacin, bumetanide, N(G)-nitro-L- arginine methyl ester (LNAME), UO-126, hydrogen peroxide (H2O2) and cumene hydroperoxide were purchased from Sigma Aldrich. The p38 inhibitor 3-O-Acetyl-beta-boswellic acid and the L-steroisomer JNK inhibitor 1 were from Enzo Life Sciences (Lorrach, Germany), and diphenylene iodonium (DPI) from Cayman Chemical (Hamburg, Germany). Defibrinated sheep red blood cells (SRBC) were purchased from Oxoid (Basingoke, UK). The LDH Cytotoxicity Assay Kit was from Cayman Chemical (Hamburg, Germany). Colorimetric (ELISA, LDH), luminescent (luciferase) and fluorescent (ROE) measurements were carried out using a Victor3 Multilabel Plate Reader (Perkin Elmer, Waltham, MA, USA). Cell culture reagents were purchased from Invitrogen. All other reagents were obtained from Sigma Aldrich (Taufkirchen, Germany) if not stated otherwise.

Cell culture and stimulation assays

The m-ICcl2 small intestinal epithelial cell line has previously been described [58]. Cells were cultured in a modified, hormonally defined medium with DMEM and F12 (vol 1:1) supplemented with 5% FCS, 2% glucose, 20 mM Hepes, 2 mM glutamin, 5 μg/mL insulin, 50 nM dexamethasone, 60 nM sodium selenium, 10 ng/mL epidermal growth factor, 5 μg/mL transferrin, and 1 mM 3′,5′-triiodo-L-thyronine sodium salt. Cells were 42-70% passaged. All cells were grown at 37°C in a 5% CO2 atmosphere on collagen-coated cell culture plates or chambers to reach a polarized, confluent monolayer. Rat tail collagen was ordered from Institut Jacques Boy (Reims, France). Specific targeted or control siRNA was transfected at a final concentration of 10 nM 30 hours prior to functional analysis. Stimulation with lipopolysaccharide (LPS) was performed at a final concentration of 10 ng/mL.

Bacterial strains and epithelial infection

Listeria monocytogenes EGD wild-type (wt), actA, hly deletion mutant strains and the hly/plcA/p6B triple mutant strain are described in Table 1. Fluorescent bacteria were generated by transformation [59] with GFP expression vectors under the control of the actA or sod promoter (PactA, Psod; Table 1) Bacteria were routinely grown in Brain Heart Infusion (BHI) broth, supplemented with antibiotics when required. Overnight cultures were diluted 1:50, grown to middle logarithmic phase (OD600) with mild agitation at 37°C, washed, and added in cell culture medium at the multiplicity of infection (m.o.i.) of 100:1 (if not stated otherwise) followed by centrifugation (1500 rpm, 5 min, 4°C). 60 minutes after addition of bacteria, epithelial monolayers were washed three times with PBS, and fresh medium containing 50 μg/mL gentamycin was added to the culture medium to restrict extracellular bacterial growth. Unless indicated otherwise, infections were completed after 4 h post infection. To quantify bacterial invasion, co-culture of 20, 40 or 60 min was followed by 1 h incubation in fresh cell culture medium supplemented with 50 μg/mL gentamycin. For 4 h and 6 h invasion, gentamycin was supplemented 60 minutes after addition of bacteria, and incubation was carried out for additional 3 h or 5 h. After washing, cells were lysed in 0.1% Triton/H2O and the number of intracellular bacteria was determined (CFU) by serial dilution and plating. Bacteria free conditioned medium were prepared by centrifugation or filtering of cell culture medium, and immediately applied on naïve, uninfected m-ICcl2 cells. The rabbit anti-Listeria antibody was used for immunolabelling of bacteria (1:500). For alternative intracellular detection of Listeria, bacteria were biotinylated prior to infection according to the manufacturers protocol. Pharmaceutical inhibitors were added 30 min prior to infection if not stated otherwise.

Immunofluorescence microscopy and flow cytometric analysis

For intracellular Cxcl-2 or Cxcl-5 visualization, brefeldin A (0.5 μg/mL) was added to the cell culture medium 1 h after stimulation. Cells were fixed in 3% PFA and incubated with anti-Cxcl-2 or Cxcl-5 antisera (1:100). Nox4 was detected in formalin-fixed sections of mouse small intestine by incubation with a rabbit anti-Nox4 antisera (1:100) for 1 h at room temperature, followed after washing by a TR-conjugated secondary antibody. Cells were mounted in Vectashield Mounting Medium with Dapi (Vector Laboratories, Eching, Germany) and visualized using a Leica DM IRB Inverted Research Microscope with a TCS SP2 AOBS scan head (Leica Microsystems GmbH, Wetzlar, Germany). For fluorescent detection, immunolabelled Listeria was additionally stained with AF 555-conjugated secondary antibody prior to infection, or biotinylated bacteria were labelled by streptavidin-conjugated Cy3. For flow cytometry cells were trypsinized and fixed in Cytofix (BD Biosciences). Cxcl-2 or Cxcl-5 was stained following permeabilization in 0.5% saponin/1% FCS/
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Pijl: Analysis was performed on a FACS Calibur apparatus (BD Biosciences). The data acquisition on GFP+ (recorded in channel Fl-1) and Cxcl-2+ or Cxcl-5+ cells (Cy5-conjugated, Fl-4) was restricted to a total number of 10,000 events. The data acquisition on GFP+ (recorded in channel Fl-1) bacteria was restricted to a total number of 100,000 events. Flow cytometry cell sorting was performed using a MoFlo (XD Upgrade, Beckman-Coulter) at the Cell Sorting Facility, Medical School, Hanover.

Immunoblotting

Cell were lysed in 3:1 WB/SB vol/vol (WB: 50 mM Tris, pH 7.4, 120 mM NaCl; SB: 250 mM Tris, pH 6.5, 8% SDS, 40% glycerol; supplemented with a protease inhibitor cocktail [Complete Mini, Roche Diagnostics]). Samples were sonified and the protein concentration was determined (DC Protein Assay; Bio-Rad Laboratories). Protein was separated on 11% acrylamide gels and blotted on nitrocellulose. Membranes were incubated overnight at 4°C with the primary antibody. Detection was performed using peroxidase-labelled goat anti-rabbit or goat antimouse secondary antibodies in combination with the ECL kit (GE Healthcare). Before rehydrating, membranes were stripped for 45 min at 50°C in 62.5 mM Tris HCl, pH 6.7, 100 mM β-mercaptoethanol and 2% SDS, followed by three 15-min washing steps.

PCR and quantitative RT-PCR analysis

Cells were divided after cell sorting. DNA extraction was performed following incubation in lysosome (10 mg/mL), proteinase K (10 mg/mL), and 5% SDS using TRIzol (Invitrogen) according to the manufacturer’s instruction. DNA was washed in sodium citrate (0.1 mM) and precipitated in 75% ethanol. Listeria genomic DNA was detected by PCR (Tag DNA polymerase from Invitrogen) using primers specific for the listerial hly gene (forward: 5’-ATG TAA CTT GCG CAA CT-3’, reverse: 5’-TCA TGT GTG TTA AGC GGT TT-3’, annealing temperature 59°C, cycles 32). A fragment encoding eukaryotic hypoxanthine phosphoribosyltransferase (Hprt) was amplified using oligonucleotides 5’-TGC TGA GCC GCC ACA CA-3’ and 5’-GTG GTT TTA AGC GGT TT-3’, annealing temperature 57°C, cycles 32). Amplification products were analysed on a 2% agarose gel and visualized with SYBR Safe (Invitrogen). Total RNA was extracted using the RNeasy Protect Cell Mini Kit (Qiagen) and stored at −80°C. The reverse transcription reaction was performed following incubation in lysosome (10 mg/mL), proteinase K (10 mg/mL), and 5% SDS using TRIzol (Invitrogen) according to the manufacturer’s instruction. DNA was washed in sodium citrate (0.1 mM) and precipitated in 75% ethanol. Listeria genomic DNA was detected by PCR (Tag DNA polymerase from Invitrogen) using primers specific for the listerial hly gene (forward: 5’-ATG TAA CTT GCG CAA CT-3’, reverse: 5’-TCA TGT GTG TTA AGC GGT TT-3’, annealing temperature 59°C, cycles 32). A fragment encoding eukaryotic hypoxanthine phosphoribosyltransferase (Hprt) was amplified using oligonucleotides 5’-TGC TGA GCC GCC ACA CA-3’ and 5’-GTG GTT TTA AGC GGT TT-3’, annealing temperature 57°C, cycles 32). The amplified products were transferred into microcentrifuge tubes, sedimented at 8,000 × g for 5 min at 4°C, and 100 μL aliquots were dispensed in 96-well plates in triplicate. The index of oxidation (DCF) was calculated as the ratio of fluorescence intensity as compared to an untreated control.

Microinjection

Cells were grown in collagen-coated 8-well chamber slides (Nunc, Rochester, NY) continuously bathed in cell culture medium. The 70 kDa high molecular weight gap junction impermeant fluorescent compound Texas Red Dextran (Molecular Probes, 10 mg/mL) was mixed with either the <1 kDa low molecular weight Lucifer Yellow (Molecular Probes, 10 mg/mL) or 0.5 kM cumene hydroperoxide in injection buffer (25 mM HEPES, 125 mM K-acetate, 5 mM Mg-acetate, pH 7.1). Fluorescent mixtures were loaded into individual Femtojet II injection capillars (Eppendorf, Hamburg, Germany). Cells were transferred to a LSM 510 META laser scanning confocal microscope equipped with an inverted Axiovert 200M stand (Carl Zeiss, Germany) and single cell microinjection was performed using an InjecMan NF2/Femtojet injector system at pI: 180 hPa, pII: 0.25; pIII: 25 hPa. A minimum of 10 microinjected cells were analyzed per experiment. To study gap junctional intercellular communication, cells were analysed by live imaging microscopy after 5 min incubation. For ROI donor cumene hydroperoxide stimulation, cells were incubated 1 h, washed, and incubated in prewarmed fresh cell culture medium for an additional 3 h in the presence of 0.5 μg/mL brefeldin A. Cells were fixed in 3% PFA and further analyzed by intracellular chemokine staining and fluorescence microscopy.

Statistical analysis

All experiments were performed at least three times and results are given as the mean ± standard deviation (SD) of one representative experiment. Statistical analyses were performed using the Student’s t test. A p value <0.05 (*) or <0.01 (**) was considered significant.

Supporting Information

**Figure S1 (A)** m-IC± cells were infected with wt (white square) or hly mutant (dark grey square) or hly/plcA/plcB triple mutant (light grey square) Listeria monocytogenes. The number of intracellular bacteria was determined after the indicated time by gentamycin-killing invasion assay. **(B)** Macrophage-like RAW 264.7 cells were infected at the indicated multiplicity of infection (m.o.i.) with viable (wt) or heat inactivated wt (h.i.), or hly mutant L. monocytogenes. The number of immunolabelled Cxcl-2+ cells was determined 4 h after infection by flow cytometry. All experiments were performed at a multiplicity of infection of 100:1, if not stated otherwise. Results are representative for three independent experiments and are presented as mean ± SD (invasion) or show one representative experiment.

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**Figure S2 (A)** m-IC± cells were left uninfected (Ai, left) or infected with biotinylated (Cy3) wt L. monocytogenes (Ai, right). The number [%] of Cy3+ (Listeria-infected, blue) or immunolabelled Cxcl-2+ (red) cells was visualized 4 h after infection by flow cytometry. Single channel analysis (Cy3: FL-2; Cxcl-2: FL-4) was depicted on the side of the axis. **(Ai)** The number of Cy3+ (Listeria-infected) cells among activated, Cxcl-2+ cells and **(Aii)** the proportion of Cxcl-2+ cells among Cy3+ (Listeria-infected) cells was demonstrated gating on the respective population. **(Aiv)** Flow cytometric analysis of Listeria by biotinylination and streptavidin–
conjugated Cy3 labelling after growth in culture medium. **(Bi)** m-lnc cells were infected with wt PaeGFP Listeria monocytogenes at a multiplicity of infection of 10:1. Intracellular Listeria (GFP+, green) and immunolabelled Cxcl-2-2**α** cells was visualized 4 h after infection by fluorescence microscopy. Scale bar, 30 μm. Inserts at loci of infection (1-6) were enlarged (scale bar, 5 μm). Magnification ×100, counterstaining with Dapi (blue). **(Bii)** m-lnc cells were left untreated or stimulated with lipopolysaccharide (LPS, 10 ng/mL). Cxcl-2 **(Bii)** were left untreated or stimulated with lipopolysaccharide (LPS, 10 ng/mL). Cxcl-2 **(Bii)** was visualized 4 h after stimulation by fluorescence microscopy. Magnification ×400, counterstaining with Dapi (blue). Scale bar, 5 μm. **(C)** Illustration of the flow cytometric characterization of uninfected (G), wt PaeGFP L. monocytogenes infected (Gii) m-lnc cells. Comparison of the gate setting (Ci)ii used for flow cytometric cell sorting of GFP**low** Listeria-negative epithelial cells infected with actA mutant PaeGFP L. monocytogenes. All infection experiments were performed at a multiplicity of infection of 100:1, if not stated otherwise. Results are representative for three independent experiments and show one representative experiment.

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**Figure S3** (A) m-lnc cells were infected with wt (white square) or actA mutant (green square) Listeria monocytogenes. The number of intracellular bacteria was determined after the indicated time by gentamicin-killing invasion assay. **(B)** m-lnc cells were infected with actA mutant PaeGFP L. monocytogenes. The ratio of immunolabelled Cxcl-2**α** cells to GFP**+** (Listeria-infected) cells was determined after the indicated time by flow cytometry. **(C)** Illustration of the expression of GFP in actA mutant L. monocytogenes carrying the actA PaeGFP or the PaeGFP reporter plasmid after growth in culture medium until mid-log phase. **(D)** Flow cytometric analysis of GFP expression by wt PaeGFP L. monocytogenes after growth in culture medium (Di), as compared to after isolation from infected intestinal epithelial cells (Di). Bacteria were isolated 4 h after infection, immunolabeled with anti-Listeria antibody, and analysed for GFP expression after gating. **(E)** m-lnc cells were infected with wt (black) or hly mutant (white) PaeGFP L. monocytogenes. The number of GFP**+** (Listeria-infected) cells was determined after the indicated time by flow cytometry. Hemolytic (F) and cytolytic (G) activity of recombinant LLO (rLLO) as determined by hemoglobin release by red blood cells (RBC) or lactate dehydrogenase (LDH) release by m-lnc cells, respectively. **(H)** Hemolytic activity in uninduced, sterile-filtered culture supernatant (bact free media, normalised for multiplicity of infection of 100:1) of wt, actA, and hly mutant L. monocytogenes grown in m-lnc cell culture medium. Hemoglobin release by red blood cells was measured by photometric spectroscopy. **(I)** m-lnc cells were infected with wt, actA, and hly mutant L. monocytogenes. Cytolytic activity was measured by LDH release. In **(F-G)** DTT was used as solvent control, and in **(F-I)** hypotonic lysis in H2O was used as positive control. All infection experiments were performed at a multiplicity of infection of 100:1. Results are representative for three independent experiments and are presented as mean ± SD (invasion, hemolysis, LDH) or show one representative experiment.

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**Figure S4** (A) Illustration of the bicistronic expression vector for simultaneous expression of the NF-kB subunit RelA/p65 and GFP. *ires*: internal ribosome entry site. **(B)** m-lnc cells were transfected with an NF-kB-luciferase reporter in combination with an empty control vector (pFlag), a RelA/p65 expression plasmid (p[p65]), or the bicistronic p65-ires-gfp expression plasmid (p[p65]-ires-gfp). Luciferase activity was determined 6 h after transfection in cell lysate by luminescence spectroscopy. **(C)** m-lnc cells were transfected with an empty control vector (pFlag), a RelA/p65 expression plasmid (p[p65]), or the bicistronic p65-ires-gfp expression plasmid (p[p65]-ires-gfp). Cxcl-2 was determined 26 h after transfection in cell culture supernatant by ELISA. **(D)** m-lnc cells were transfected with an empty control vector (pFlag, left panel) or the bicistronic p65-ires-gfp expression plasmid (p[p65]-ires-gfp, right panel). The number of GFP**+** cells or immunolabelled Cxcl-2**α** cells was visualized 26 h after transfection by flow cytometry. **(E)** m-lnc cells were transfected with the bicistronic p65-ires-gfp expression plasmid (p[p65]-ires-gfp). The number of GFP**+** (black square) or immunolabelled Cxcl-2**α** (white diamond) cells was determined after the indicated time by flow cytometry. **(F)** m-lnc cells were stimulated with LPS (10 ng/mL) in the absence or presence of brefeldin A (BFA, 0.5 μg/mL). The amount of Cxcl-2 secreted into the cell culture supernatant as well as found in the cell lysate was quantified by ELISA. **(G)** m-lnc actA cells were infected with actA mutant PaeGFP L. monocytogenes (white) or exposed to freshly prepared sterile cell culture supernatant from Listeria infected cells obtained at the indicated time points after infection (bact free supernatant, black). The number of immunolabelled Cxcl-2**α** cells to GFP**+** (Listeria-infected) cells was determined 4 h after infection in the absence or presence of the indicated inhibitors by flow cytometry. Used inhibitors: thapsigargin (1 μM), CFTR II (1 μM), indomethacin (0.1 mM) or bumetanide (0.1 mM). All infection experiments were performed at a multiplicity of infection of 100:1. Results are representative for three independent experiments and are presented as mean ± SD (luciferase, ELISA) or show one representative experiment.

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**Figure S5** (A) m-lnc cells were left untreated (white) or stimulated with 10 ng/mL LPS (black) or 10 μM PMA (grey). Cxcl-2 was quantified 4 h after stimulation in cell culture supernatant by ELISA. **(B)** m-lnc actA cells were infected with wt Listeria monocytogenes in the absence (black diamond) or presence of DPI (dark grey diamond, 0.1 μM) or UO-126 (light grey diamond, 10 μM). The number of intracellular bacteria was determined 4 h after infection by gentamicin-killing invasion assay. ns, not significant.

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**Figure S6** m-lnc cells were treated with control small interfering RNA (siRNA) or Nox4 siRNA and subsequently left untreated (white) or stimulated with 10 ng/mL LPS (black) or 10 μM PMA (grey). Cxcl-2 was determined 4 h after stimulation in cell culture supernatant by ELISA. ns, not significant.

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**Author Contributions**

Conceived and designed the experiments: TD AV MWH. Performed the experiments: TD CC SBM. Analyzed the data: TD CC AV MWH. Contributed reagents/materials/analysis tools: TMF SW AV. Wrote the paper: TMF SW AV MWH.
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