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Listeria monocytogenes induced Rac1-dependent signal transduction in endothelial cells

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ABSTRACT

Infection of endothelial cells by *Listeria monocytogenes* is an essential step in the pathogenesis of listeriosis. Small GTPases of the Rho family act as molecular switches in signal transduction. We tested the hypothesis that Rho GTPases contribute to the regulation of cytokine expression following *L. monocytogenes* infection.

L. monocytogenes induced release of distinct CC and CXC, as well as Th1 and Th2 cytokines and growth factors by endothelial cells and activated RhoA and Rac1. Inhibition of Rac1 by inhibitor Nsc23766 reduced cytokine expression, and slightly yet significantly the uptake of bacteria. Blocking of Rho proteins by *Clostridium difficile* toxin B-10463 (TcdB) reduced *Listeria*-dependent cytokine expression, whereas activating Rho proteins by *Escherichia coli* CNF1 increased it. We analyzed regulation of IL-8 expression in more detail: *Listeria*-induced IL-8 release was reduced by inhibition of RhoA, Rac1 and Cdc42 (TcdB) or Rac1 while blocking of RhoA/B/C by *Clostridium limosum* C3 fusion toxin (C3FT) or Rho kinase by Y27632 reduced cytokine expression only slightly. Activation of RhoA, Rac1 and Cdc42 (CNF1), but not of RhoA alone (CNF_Y), enhanced *Listeria*-dependent IL-8 release significantly. Furthermore, inhibition of RhoA, Rac1 and Cdc42 (TcdB) and Rac1 (Nsc23766), but not of RhoA (C3FT) reduced *Listeria*-related recruitment of NF-κB/p65 and RNA polymerase II to the *il8* promoter, as well as acetylation of histone H4 and Ser10/Lys14-phosphorylation/acetylation of histone H3 at the *il8* gene promoter in HUVEC.

In conclusion, Rac1 contributed to *L. monocytogenes*-induced cytokine expression by human endothelial cells.

1. Introduction

Listeria monocytogenes, a gram-positive facultative intracellular bacterium [1], is a well-established model organism [2] and causes sepsis and meningitis [2]. Together with *L. innocua* it forms one group within the six members of the genus *Listeria* [3]. *L. innocua* has not been found to cause disease in humans or animals and lacks the *Listeria* pathogenicity island 1, coding for

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genes responsible for invasion and the intracellular life cycle of the bacterium, e.g. listeriolysin and phosphatidylinositol-specific phospholipase C [4].

Invasion of endothelial cells is integral part of listeriosis [5]. *L. monocytogenes* was found to activate the transcription factor NF-κB and induce endothelial expression of cytokines [6,7], and adhesion molecules [5,6]. Subsequent recruitment of leukocytes and host cell activation have been shown to be essential for clearance of *Listeria* [2,8–11]. Cell activation involves listerial factors like phospholipases and listeriolysin [6,12], evasion from the vacuoles [13], and activation of intracellular nucleotide-binding oligomerization domain protein 1 (NOD1)-dependent signaling [14].

Histone modifications may serve as code for the transcriptional activity of genes by loosening the DNA–histone interaction and unmasking of transcription factor binding sites [15]. A wide range of specific modifications of N-terminal histone tails are decisive for gene repression or activation [16]: phosphorylation at Ser10 on H3 and acetylation at Lys14 of H4 seem to have a special impact on gene regulation [17], e.g. for LPS-induced p38 mitogen-activated protein kinase-dependent phosphorylation at Ser10 on H3 and acetylation at Lys14 on H4 specifically at *il8*-, but not at *tnfα*-promoters [18]. Recently, we presented data implying histone modifications in *Listeria*-induced activation of endothelial cells [19].

The small GTP-binding proteins RhoA, Rac1 and Cdc42 act as molecular switches in cytoskeletal rearrangement [20] and signal transduction [21,22]. They are active in GTP-bound form and inactive after hydrolysis of GTP to GDP. Many bacterial toxins either activate or inactivate Rho GTPases and, therefore, can be used as molecular tools to address Rho GTPase function [23]: *Clostridium difficile* toxin B-10463 (TcdB) inactivates Rho proteins by monoglucosylation on Thr37 (RhoA) and Thr35 (Rac1, Cdc42) [24,25]. C3 toxin of *Clostridium limosum* specifically inactivates RhoA/B/C by ADP-ribosylation at Asn41 [26]. It lacks any specific binding and translocation units [27]. For optimal cellular uptake, a C3 fusion toxin (C3FT) was developed, which enters cells by using the binary *Clostridium botulinum* C2 toxin as a carrier [28,29]. *Escherichia coli* cytotoxic necrotizing factor 1 (CNF1) activates Rho proteins by deamidation of RhoA at Gln63 (Gln61 of Rac1/Cdc42, respectively), thereby inhibiting their intrinsic and GAP-stimulated GTPase activities [30,31]. Activation of Rac1 is strong, and Cdc42 is only slightly activated [32]. In contrast, cytotoxic necrotizing factor Y (CNF_Y) from *Yersinia pseudotuberculosis* specifically activates RhoA by deamidation at Gln63 [32]. Recently, a Rac1 specific chemical inhibitor (Nsc23766) has been described [33].

Although many intracellular bacteria target Rho GTPases, their role in host cell activation by *L. monocytogenes* is widely unknown. We hypothesized that Rho GTPases are involved in bacteria-induced promoter activation and expression of pro-inflammatory cytokines in human endothelial cells. Here we show that *L. monocytogenes* Rac1-dependently induced cytokine expression in human endothelial cells. IL-8 expression by *Listeria*-infected cells was reduced by inactivation of Rac1, but only slightly by inhibition of RhoA/B/C or Rho kinase. Rac1, but not RhoA, was also necessary for *Listeria*-induced acetylation of histone H4 and phosphorylation/acetylation of H3 at the *il8* gene-promoter. Thus, GTPase-dependent histone modifications contributed to pro-inflammatory cytokine expression in *Listeria*-infected human endothelial cells.

2. Material and methods

2.1. Materials

Antibiotics were obtained from Life Technologies (Karlsruhe, Germany) and Nsc23766 from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, Bethesda, MA. The C2IN-C3 fusion toxin [29], the C2II binding component [29], CNF1 and CNFy [32] were purified as recombinant glutathione S-transferase (GST) fusion proteins as described. Toxin B-10463 from *C. difficile* was purified as described [25]. All other chemicals used were of analytical grade and obtained from commercial sources.

2.2. Cell culture

Human umbilical cord vein endothelial cells (HUVEC) were isolated from umbilical cord veins and identified as described previously [21,34,35]. Studies performed were done using confluent endothelial cell monolayers in their second passage [5,12,14,19].

2.3. Bacterial strains

The wild-type *L. monocytogenes* serotype 1/2a strain EGD, and the *L. innocua* serotype 6b strain ATCC 33090 (INN) were grown in brain heart infusion broth (Difco Laboratories, Detroit, MI) at 37 °C and were used in the logarithmic growth phase as described [5,12,14,19].

2.4. IL-8 ELISA

Confluent HUVECs were stimulated for 15 h as indicated in a humidified atmosphere. After incubation supernatants were collected and processed for IL-8 quantification by sandwich-ELISA as described previously [14,19,36].

2.5. Bioplex protein array system

Confluent HUVECs were stimulated for 15 h as indicated in a humidified atmosphere. After incubation, supernatants were collected and cytokines were analyzed by the Bioplex Protein Array system (BioRad, Hercules, CA) using specific beads, according to the manufacturers instructions [19].

2.6. RhoA and Rac1 activation assay

RhoA/Rac1 activation in infected cells was determined with the RhoA/Rac1 activation kit (Chemicon International, Temecula, CA) according to the manufacturers instructions. Briefly, HUVEC were grown to 80–90% confluence (approx. 10^7 cells per sample lysate), serum-starved for 3 h, and infected with *L. monocytogenes* (10^7 cfu/ml) for 30–120 min. HUVEC lysates were mixed with the RBD or PAK-1 PBD agarose slurry, respectively, and beads were collected by centrifugation, washed with the

assay buffer and resuspended in SDS sample buffer. Activated RhoA/Rac1 was visualized by Western blot using an anti-RhoA/Rac1 monoclonal antibody and a Cy5.5-labeled anti-mouse secondary antibody using an Odyssey infrared imaging system (LI-COR Inc., Lincoln, NE).

2.7. Chromatin immunoprecipitation (ChIP)

Briefly, HUVECs were stimulated and ChIP was performed as described previously [19,36] with antibodies from Upstate, Waltham, MA (Ac-H4 and P-Ser10/Ac-Lys14-H3), and Santa Cruz Biotechnology, Santa Cruz, CA (p65, Polymerase II). Equal amounts of input DNA was controlled by gel electrophoresis [19,36].

The following promoter-specific primers were used: IL-8 sense 5′-AAG AAA ACT TTC GTC ATA CTC CG-3′, antisense 5′-TGG CTT TTT ATA TCA TCA CCC TAC-3′.

2.8. Confocal microscopy

HUVEC monolayers were infected as described and fixed with PFA. Subsequently, DAPI staining for cellular and bacterial DNA was performed as described [14,19]. For visualization of intracellular *Listeria*, additional F-actin staining was carried out with Phalloidin Alexa488 (Molecular Probes, Leiden, The Netherlands). Intracellular bacteria were quantified by averaging the number of 30 different visual fields per experiment ($n = 3$) (Axioskop 2 mot; objective: PlanNeoFluar 40×, NA 1.3; Zeiss, Jena, Germany). Controls were set 100% and compared to NSC pretreated probes.

2.9. Statistical methods

Data are shown as means ± S.E.M. of at least three independent experiments. An one-way ANOVA was used for data of Fig. 1, 2A/B, 3, and 5. Main effects were then compared by a Newman–Keuls' post-test. A $p < 0.05$ was considered to be significant and is indicated by asterisks or double-crosses.

3. Results

3.1. *L. monocytogenes* induced Rho GTPase-dependent cytokine release by human endothelial cells

HUVEC infected with *L. monocytogenes* (10^7 cfu/ml, 15 h) released significant amounts of G-CSF, IFNγ, IL-1β, IL-4, IL-5, IL-6, IL-7, IL-8, IL-13, MIP-1β and TNFα (Fig. 1). No significant increase in secretion of GM-CSF, IL-2, IL-10, IL-12 (p70) and IL-17 could be detected in wild-type *L. monocytogenes*-infected

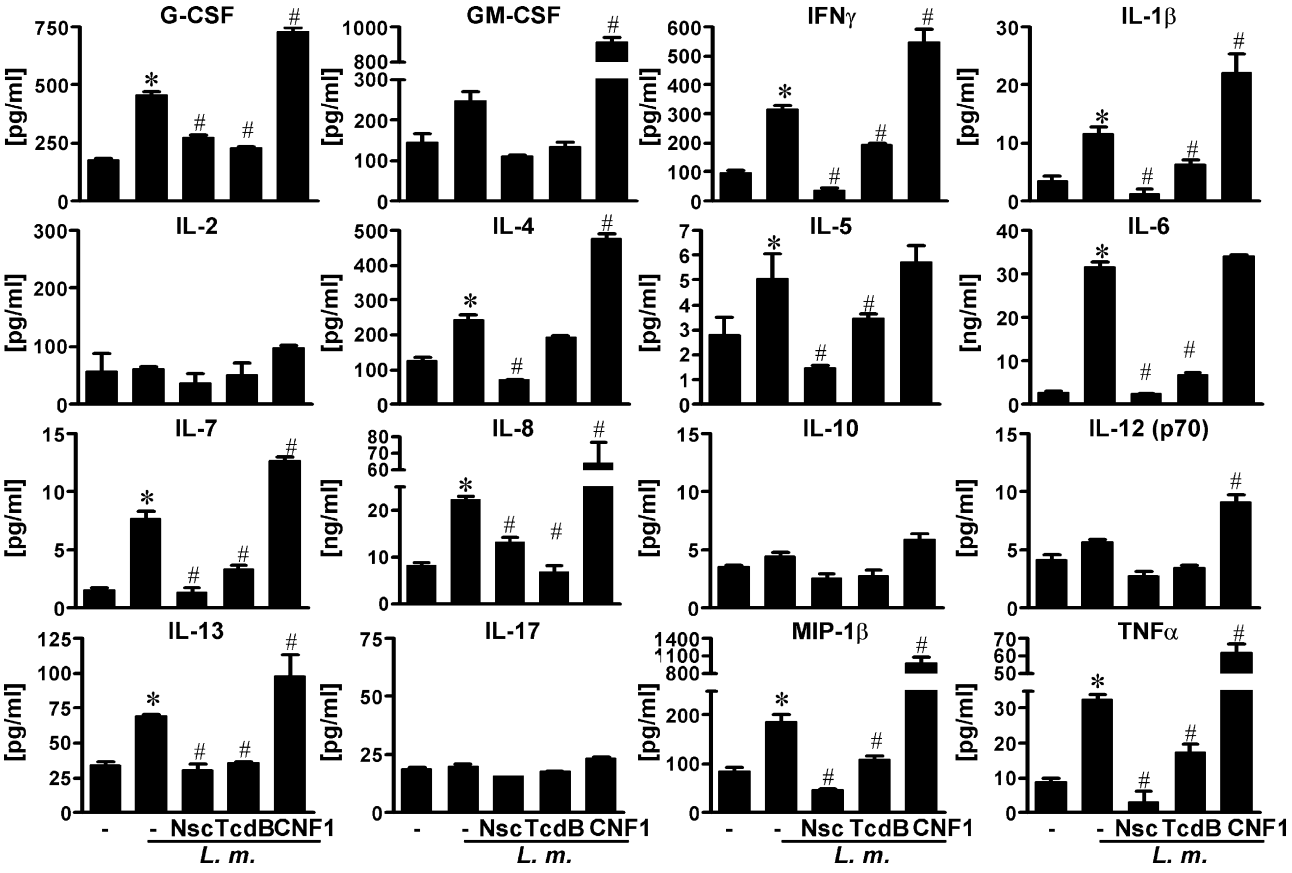

Fig. 1 – *L. monocytogenes* induced Rho protein dependent release of cytokines by human endothelial cells. HUVEC were pre-incubated for 24 h with Rac1 inhibitor Nsc (Nsc23766, 200 μM), and 2 h with Rho protein inhibitor TcdB (10 ng/ml) or Rho protein activator CNF1 (400 ng/ml) and infected with 10^7 cfu/ml *L. monocytogenes* for 15 h. Cytokine release was measured in the supernatant by Bioplex assay. Data are shown as means ± S.E.M. of at least three independent experiments. $^{*}p < 0.05$ compared to uninfected cells, $^{\#}p < 0.05$ compared to infected cells without inhibitors/toxins.

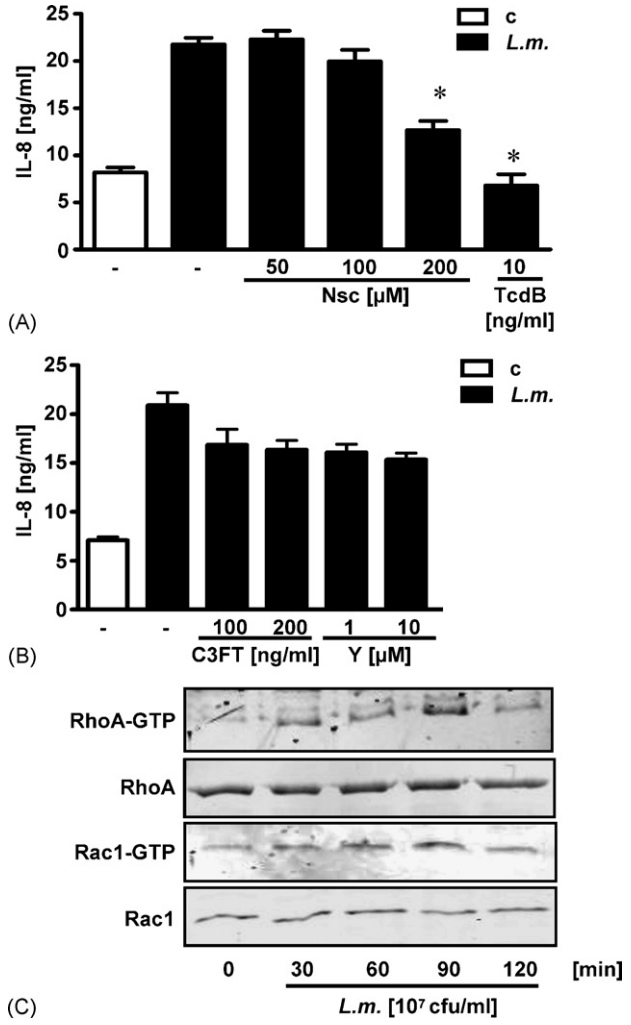

Fig. 2 – *L. monocytogenes* Rac1 dependently induced IL-8 release by human endothelial cells. HUVEC were pre-incubated with the indicated concentrations of the Rac1 inhibitor Nsc23766 (Nsc) for 24 h and the Rho protein inhibitor TcdB (A), or the RhoA/B/C inhibitor C3FT, or the Rho kinase inhibitor Y27632 (B) for 2 h, respectively. Subsequently, cells were infected with *L. monocytogenes* (10^7 cfu/ml) for 15 h. IL-8 release was measured in the supernatant by ELISA. Data are shown as means ± S.E.M. of at least three independent experiments. $^*p < 0.05$ from *L. monocytogenes*-infected cells without inhibitor. HUVEC were infected with *L. monocytogenes* (10^7 cfu/ml) for the indicated time periods and GTP-loading of RhoA and Rac1 was detected by GTP-binding assay (C). One representative blot of three independent experiments is shown.

endothelial cells. In contrast, extracellular *L. innocua* (10^7 cfu/ml, 15 h) did not induce any significant cytokine release within the time frame tested (data not shown).

We tested the hypothesis that Rho GTPases contributed to *Listeria*-related cytokine release. Inhibition of RhoA, Rac1, Cdc42 by TcdB, or Rac1 by Nsc23766 reduced *Listeria*-dependent release of G-CSF, IFNγ, IL-1β, IL-5, IL-6, IL-7, IL-8, IL-13, MIP-1β and TNFα (Fig. 1). Rac1-activation by CNF1 enhanced the release of G-CSF, GM-CSF, IFNγ, IL-1β, IL-4, IL-7, IL-8, IL-13, MIP-1β, TNFα. In addition, CNF1 promoted stimulation of IL-12 (p70), which was not induced by *Listeria* infection alone (Fig. 1). The same CNF1 dose (400 ng/ml) used alone induced no cytokine release of endothelial cells within the time frame tested (data not shown).

3.2. *L. monocytogenes*-induced IL-8-release was dependent on Rac1

Focusing on the important chemoattractant IL-8 as a model cytokine for *Listeria*-induced endothelial activation, we analyzed the role of Rho GTPases in more detail. Endothelial cells infected with *L. monocytogenes* (15 h) released IL-8 in a linearly dose-dependent manner between 10^6 and 10^7 cfu/ml, while extracellular *L. innocua* (10^5–10^7 cfu/ml, 15 h) had no effect on IL-8 secretion within the dose range and time frame tested (data not shown and Ref. [19]). Inhibition of Rho GTPases by TcdB or of Rac1 by Nsc23766 reduced *L. monocytogenes*-induced IL-8 expression (Fig. 2A). In contrast, blocking of RhoA/B/C by C3FT or inhibition of Rho kinase by Y27632 reduced IL-8 expression only slightly yet not significantly (Fig. 2B). Activity of C3FT on endothelial cells was confirmed by the observation of typical microfilament alterations in immunofluorescence (data not shown). In addition, infection of HUVEC by *L. monocytogenes* (10^7 cfu/ml) induced GTP-binding of RhoA and Rac1 after 30 min with a peak after 90 min as shown by pulldown assays (Fig. 2C). To analyze whether activation of Rho-GTPases synergizes with *L. monocytogenes* infection on IL-8 release, we infected endothelial cells with lower concentrations of *L. monocytogenes* and simultaneously activated GTPases with molecular tools: CNF1, which activates predominantly Rac1, enhanced *L. monocytogenes*-induced IL-8 release in a dose-dependent manner while RhoA activation by CNF_Y induced only a mild, not significant enhancement (Fig. 3). Both toxins did not induce IL-8 release when given individually to endothelial cells in the doses tested and no synergism but only mild increases could be seen when cells

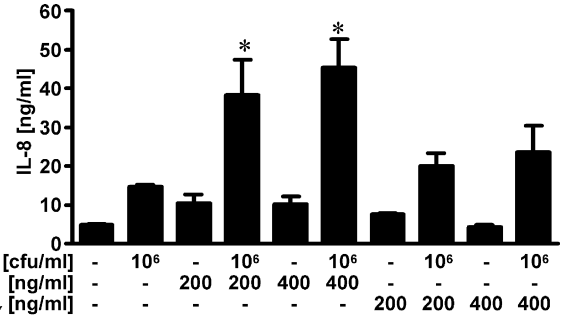

Fig. 3 – *L. monocytogenes* induced IL-8 release synergistically with activation of Rho proteins. HUVEC were pre-incubated (2 h) with the indicated concentrations CNF1, an activator of RhoA, Rac1 and Cdc42, or CNF_Y, an activator of RhoA alone, and then infected with *L. monocytogenes* (10^6 cfu/ml) for 15 h. IL-8-release was measured in the supernatant by ELISA. Data are shown as means ± S.E.M. of at least three independent experiments. $^*p < 0.05$ from *L. monocytogenes*-infected cells without CNF1 or CNF_Y.

were infected with higher concentrations of bacteria (data not shown).

3.3. *Listeria*-induced histone modifications were Rac1-dependent at the il8 promoter

We next analyzed the impact of Rho proteins on *Listeria*-induced histone modifications and promoter activation. HUVEC were pre-incubated with TcdB or Nsc23766, infected with *L. monocytogenes* for 2 h, and histone modifications at the *il8* promotor were detected by ChIP (Fig. 4A). Specific phosphorylation/acetylation of H3 (Ser-10/Lys-14) and acetylation of H4 at the *il8* gene promoter were reduced by inhibition of Rac1, and by TcdB-related inactivation of RhoA, Rac1, and Cdc42. In accordance with the IL-8 protein data obtained by ELISA, both, blocking of Rho proteins (TcdB), as well as specific Rac1 inhibition (Nsc23766) reduced the binding of NF-κB subunit RelA as well as the recruitment of Pol II at the *il8* gene promoter and hence subsequent gene transcription

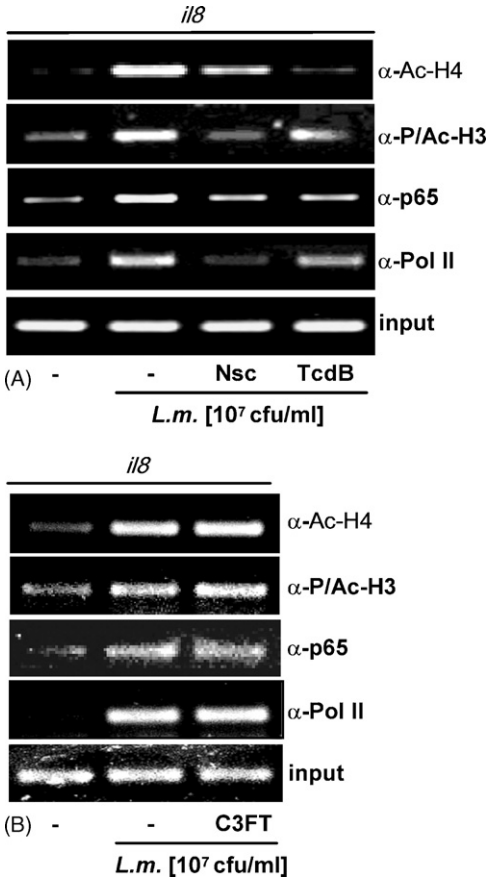

Fig. 4 – *L. monocytogenes*-induced histone modifications at the *il8* promoter were Rac1 dependent. HUVEC were pre-incubated with (A) the Rac1 inhibitor Nsc (Nsc23766, 200 μM) or the Rho protein inhibitor TcdB (10 ng/ml) or (B) C3FT (200 ng/ml) and infected with *L. monocytogenes* (10^7 cfu/ml) for 2 h. Histone modifications (acetylation of H4, phosphorylation/acetylation at Ser10/Lys14 H3) and RNA polymerase II were detected at the *il8* promoter by ChIP. Representative gels of three independent experiments were shown.

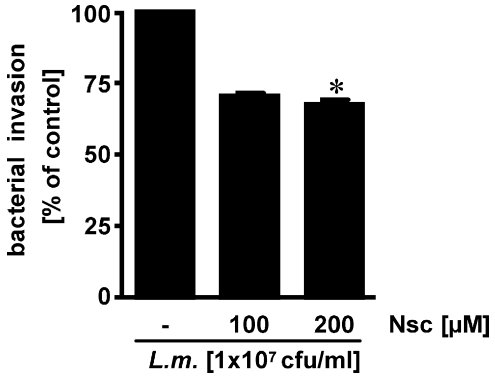

Fig. 5 – Rac1 inhibition reduced *L. monocytogenes* invasion of human endothelial cells. HUVEC were pre-incubated with the indicated concentrations of Rac1 inhibitor Nsc (Nsc23766) and infected with *L. monocytogenes* (10^7 cfu/ml) for 24 h. Cells were visualized with DAPI and Phalloidin Alexa488 and number of intracellular bacteria was determined. Data represent three independent experiments. $^*p < 0.05$ in comparison to cells without inhibitor.

(Fig. 4A). In contrast, after inhibition of RhoA/B/C by C3FT we observed no reduction of *Listeria*-related histone modifications, p65 or Pol II recruitment at the *il8* promotor (Fig. 4B).

3.4. Rac1-inhibition reduced *L. monocytogenes* uptake by endothelial cells

Listeria utilize the eukaryotic cytoskeleton during their uptake, for intracellular motility and during cell-to-cell spread [37,38] and the role of Rho proteins as central regulators of the cytoskeleton is well established [20,39]. We therefore tested whether Rac1-inhibition affected *Listeria* uptake. HUVEC were pre-incubated with Nsc23766 for 24 h, infected for 2 h with *L. monocytogenes* (10^7 cfu/ml), i.e. in a dose linearly inducing IL-8 release. Intracellular localization was detected and quantified using confocal microscopy (Fig. 5). Preincubation of HUVEC with 200 μM Nsc23766 reduced bacterial uptake by 33.8%.

4. Discussion

The data presented indicate that Rac1 contributes to the promoter activation and expression of pro-inflammatory cytokines in endothelial cells infected with intracellular bacteria. *L. monocytogenes* activated RhoA and Rac1 and induced Rho protein dependent cytokine expression in human endothelial cells. IL-8 expression by *Listeria*-infected cells was blocked by inactivation of Rac1, but only slightly reduced by inhibition of RhoA/B/C or Rho kinase. Rac1, but not RhoA/B/C activity was also necessary for *Listeria*-induced acetylation of histone H4 and phosphorylation/acetylation of histone H3, NF-κB/RelA and RNA polymerase II recruitment at the *il8* gene promoter. Thus, Rac1 contributed to *Listeria*-induced regulation of cytokine expression in human endothelial cells.

Clearance of *L. monocytogenes* in murine infection models depended on cytokine-induced chemotaxis and leukocyte

activation [8,11,40,41]. *L. monocytogenes*-infected human endothelial cells released chemoattractants (IL-8, MCP-1), Th1 cytokines (TNFα, IFNγ), Th2 cytokines (IL-4, IL-5, IL-6, IL-13), myeloid growth factors (G-CSF, IL-7) and the pro-inflammatory cytokine IL-1β, highlighting the important role of endothelial cells in the regulation of immune response.

Rho GTPases act as molecular switches in important signaling pathways and regulate cytoskeleton organization [20,39]. Rho proteins, in particular Rac1, have been shown to participate in IL-1β [42], TNFα [34], toll-like receptor (TLR)-2 [22] as well as TLR4 [21] signaling. Since Rho protein function was associated with inflammatory activation of endothelial cells [21,34,35], we tested the hypothesis that Rho GTPases regulated pro-inflammatory cytokine expression in *Listeria*-infected human endothelial cells. Blocking of Rac1 (Nsc23766) or RhoA, Rac1, and Cdc42 (TcdB) both reduced and activation of Rho GTPases by CNF1 enhanced release of distinct cytokines in *Listeria*-stimulated endothelial cells. In addition, liberation of IL-12 (p70), which was not induced by *Listeria*-infection alone, was noted in co-stimulated cells. These data indicate that Rho proteins, in particular Rac1, participated in *L. monocytogenes*-related activation of endothelial cells. CNF1-treatment by itself induced remarkable alterations of the endothelial microfilament system (data not shown, [30]). In contrast to data from Munro et al. [43], we observed only low and not significant cytokine release after CNF1 exposure of cells within the time- and dose-frame tested implicating that additional (*Listeria*-dependent) factors were needed to induce cytokine release in our model.

To gain insight into the role of Rho proteins in *Listeria*-dependent endothelial activation, we assessed the production of the model cytokine IL-8 in more detail. Blocking of Rac1, but not of RhoA/B/C or Rho kinase, reduced IL-8 secretion in *Listeria*-infected cells significantly. However, IL-8 release was not blocked completely by Rac1 inhibition. The remaining 1/3 of IL-8 increase might be due to incomplete Rac1 inhibition or the stimulation of additional, Rac1 independent signalling pathways. RhoA activation by CNF_Y increases *L. moncytogenes*-dependent IL-8 release mildly but not significantly. Since Rac1 was found to also regulate *Yersinia*-induced NF-κB activation and IL-8 release by HeLa cells [44], Rac1 has to be considered as a central molecular switch in the regulation of IL-8 expression in bacteria-infected cells.

The *il8* gene promoter has been previously described as being regulated by acetylation and phosphorylation of histone tails [19,45,46]. These modifications loosens DNA–histone binding, thereby facilitating binding of transcription factors and the basal transcription machinery [47,48]. Here we show that *L. monocytogenes*-induced acetylation of histone H4 and phosphorylation/acetylation (Ser10/Lys14) of histone H3 at the *il8* promoter in human endothelial cells was dependent on Rho GTPases, especially on Rac1. Expression of IL-8 depended upon the activation of the transcription factor NF-κB [49], and Rac1-related phosphorylation of NF-κB was implicated in (IL-8) gene transcription [22]. We noted reduced recruitment of NF-κB p65/RelA as well as of RNA polymerase II to the *il8* promoter in cells with inhibited Rac1 thus blocking IL-8 gene transcription. Athough *L. monocytogenes* activated RhoA, we observed no significant effect of C3FT on IL-8 protein expression or histone modifications, p65, and Pol II recruitment at the *il8* promotor. Thus, Rac1-related signaling and subsequent histone modifications may prepare endothelial cell genes for subsequent transcriptional activation. It is presently unclear how Rac1 is involved in signaling pathways such as p38- and ERK-MAPK pathway, which are thought to contribute to *Listeria*-related histone modifications [19]. Circumstantial evidence from TLR4 signaling towards NF-κB and IL-8 in human endothelial cells suggest a parallel rather than a sequential organization in cell signaling [21].

Since extracellular *Listeria* (INN) did not induce cytokine release (this study, [14,19]) and the cytosolic pathogen recognition receptor NOD1 is critical for *Listeria*-related endothelial activation [14], it is reasonable to assume Rac1-activation by cytosolic *Listeria*. Nevertheless, we observed only moderate yet significant reduction (33.8%) of *Listeria* uptake in a dose range linearly inducing IL-8 release, and Ebel et al. reported that Rho protein inhibition neither abrogated invasion of epithelial cells by *Listeria* nor affected actin-based motility of this pathogen [50]. Therefore, reduction of listerial uptake may not account alone for reduction of IL-8-release (70%) or histone modification (50–90%). Further studies are also required to address the role of individual listerial effector molecules such as phopholipases [12] in this activation process.

In conclusion, we report that Rho GTPase Rac1 controls release of important chemokines in *L. monocytogenes*-infected human endothelial cells. Rac1-dependent chromosomal regulation seems to contribute to the regulation of *Listeria*-dependent gene expression as shown for IL-8. This observation provides evidence for yet another facet of bacterial pathogenesis i.e. modifying host cell signaling pathways and their subsequent responses by the action of Rho GTPases.

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