Novel Src-dependent tyrosine phosphorylation of non-muscle Myosin Heavy Chain IIA restricts *Listeria monocytogenes* cellular infection

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Running title: Src phosphorylates Myosin IIA upon infection.
Abstract

Pathogens often interfere with host tyrosine phosphorylation cascades to cause infection. Given the role of tyrosine phosphorylation events in different human infections and our previous results showing the activation of the tyrosine kinase Src upon incubation of cells with *Listeria monocytogenes* (*Lm*), we searched for novel host proteins undergoing tyrosine phosphorylation upon *Lm* infection. We identify the heavy chain of the non-muscle myosin IIA (MIIA) and find that various bacterial pathogens trigger its tyrosine phosphorylation and accumulation at the vicinity of cell-associated bacteria. We show that, upon infection, Src phosphorylates MIIA in a previously uncharacterized tyrosine residue (Y158). We demonstrate that MIIA activity restricts *Lm* cellular infection and its phosphorylation on Y158 is important in that function. Our data suggest a novel mechanism of MIIA activity regulation relying on the phosphorylation of Y158 by Src.
Author Summary

Protein phosphorylation is one of the most common mechanisms to regulate protein function. Phosphorylation consists in the addition of phosphate groups to serine, threonine and tyrosine residues on target proteins by protein kinases, which can either directly modulate enzymatic activity or modify protein folding, cellular localization and molecular partners. Here, we describe and characterize for the first time the tyrosine phosphorylation of the human protein Myosin IIA in response to the infection by *Listeria monocytogenes*. Myosin IIA belongs to the family of myosin motor proteins best known for their role in cell division, migration and morphogenesis. We show that, upon bacterial challenge, the host kinase Src phosphorylates Myosin IIA on a specific tyrosine residue thereby restricting *Listeria* infection. This constitutes a previously unknown mechanism to modulate the function of myosin IIA and defend host cells against *Listeria* infection. Importantly, we demonstrate that tyrosine phosphorylation of Myosin IIA is a regulatory event triggered by different bacterial pathogens. Thus, given the central role of Myosin IIA in fundamental eukaryotic processes, our work not only provides new insights on the regulation of host cytoskeletal proteins in response to infection but has also implications in the study of cellular processes requiring tightly controlled Myosin IIA activity.
Introduction

*Listeria monocytogenes (Lm)* is an intracellular foodborne bacterial pathogen that causes disease in immunocompromised individuals. In the host it finds suitable replication niches in the liver and spleen, disseminates and can reach the central nervous system. In pregnant women, *Lm* targets the fetus, eliciting fetal infection and abortions [1]. The aptitude of *Lm* to cause disease relies on its capacity to invade nonphagocytic cells, replicate therein and spread to the entire organism overcoming the intestinal, blood-brain and fetoplacental barriers [2]. Through the expression of bacterial factors *Lm* establishes a crosstalk with host cells favoring the progression of the cellular infection [3]. In epithelial cells, *Lm* invasion is mainly driven by the bacterial surface proteins InlA and InlB that bind E-cadherin and c-Met, respectively, at the surface of host cells [4,5]. The engagement of host cell receptors triggers tyrosine phosphorylation-mediated signalling, resulting in the local activation of the Arp2/3 complex that initiates actin polymerization at the site of *Lm* attachment [6,7], causing membrane invagination that supports bacterial entry. InlB interaction with the receptor tyrosine kinase c-Met, stimulates its auto-phosphorylation, induces the tyrosine phosphorylation and recruitment of the adaptor proteins Cbl, Shc and Gab1, and the activation of phosphoinositide 3-kinase (PI3K) [5,8,9]. Phosphatidylinositol (3,4,5)-triphosphate generated by PI3K accumulates at the cell membrane during *Lm* infection [8] and plays a crucial role in the recruitment of molecules controlling the actin polymerization, such as Rac1 and WAVE2 [6,10,11,12]. InlA binding to E-cadherin induces the activation of Src tyrosine kinase that subsequently phosphorylates cortactin, E-cadherin and the clathrin heavy chain [7,13,14]. While cortactin and clathrin tyrosine phosphorylations are critical events for actin polymerization and recruitment at the *Lm* entry site [7,13], E-cadherin phosphorylation leads to its ubiquitination, internalization and further degradation [14]. In this study we aimed to identify new cellular proteins tyrosine phosphorylated in response to *Lm* infection and address whether tyrosine phosphorylation of such proteins would regulate cellular infection. Protein mass spectrometry analysis of proteins tyrosine phosphorylated in
response to *Lm* infection of epithelial cells, allowed us to identify the non-muscle myosin heavy chain IIA (hereafter MIIA) as being tyrosine phosphorylated during infection. MIIA is an actin-binding protein with motor and contractile properties, involved in cellular processes requiring force generation, cell movement and membrane reshaping [15]. In infection, MIIA is critical for viral entry [16,17] and supports bacterial invasion [18] and dissemination [19]. While the serine/threonine phosphorylation of the regulatory light chain is a well-known mechanism to regulate the non-muscle myosin IIA activity [15], the tyrosine phosphorylation of MIIA has never been characterized. Here we show that the MIIA undergoes tyrosine phosphorylation in response to bacterial pathogens. Our data indicate that upon *Lm* cellular infection MIIA is phosphorylated in tyrosine residue 158 by the Src kinase. We also found that MIIA restricts the intracellular levels of *Lm* and this function is impaired if tyrosine phosphorylation of MIIA is prevented. To our knowledge our findings describe a novel post-translational modification of MIIA with major consequences regarding bacterial infection. In addition our results suggest that phosphorylation of MIIA in tyrosine 158 could affect its activity and reveal a new mechanism used by pathogens to usurp the cellular machinery.
Results

*MIIA is tyrosine-phosphorylated in response to bacterial infection*

To identify new host proteins tyrosine phosphorylated in response to *Lm* and which could affect *Lm* internalization, we compared the tyrosine phosphorylation profiles of *Lm*-infected and non-infected (NI) HeLa cells. Cell extracts were collected at different time points post inoculation and subjected to immunoprecipitation (IP) using anti-phosphotyrosine antibodies (anti-pTyr). The IP fractions were resolved by SDS-PAGE followed by silver staining. Bands showing variable intensities in *Lm*-infected versus NI cells, were excised and processed for mass spectrometry identification. A band corresponding to a ≈250 kDa protein and displaying increased intensity throughout the infection (Fig. 1A) was identified as being the human non-muscle myosin heavy chain IIA (MIIA) (Figs. S1A, B).

To validate this result, we analyzed the tyrosine phosphorylation profile of MIIA in response to *Lm* using a MIIA-specific antibody. HeLa and Caco-2 cells were incubated with *Lm* for time periods ranging from 2 to 60 minutes. Whole cell lysates (WCL) were used for anti-pTyr IP assays and the presence of MIIA in IP fractions was assessed by immunoblot. We detected an increase of MIIA in anti-pTyr IP fractions from both *Lm*-infected HeLa and Caco-2 cells (Fig. 1B), suggesting that MIIA is tyrosine-phosphorylated in response to *Lm*. Total levels of MIIA in WCL remained unchanged upon infection (Fig. 1B) showing that increased levels of MIIA observed in the immunoprecipitated samples corresponded to differences in tyrosine phosphorylation rather than in MIIA expression. These data were further strengthened by a reverse IP assay in which we immunoprecipitated endogenous MIIA from NI and *Lm*-infected (Inf) HeLa cells and detected tyrosine-phosphorylated proteins in IP fractions. While the levels of immunoprecipitated MIIA were similar in NI and *Lm*-infected conditions, a band at the molecular weight of MIIA showing increased intensity in the infected sample was revealed by an anti-pTyr antibody (Fig. 1C). Altogether, these results indicate that *Lm* infection triggers the tyrosine phosphorylation of MIIA.

To evaluate whether the MIIA tyrosine phosphorylation (MIIA-pTyr) was a specific response
to Lm or a broader cellular response to extracellular stimuli, we assessed the MIIA-pTyr profile in response to the closely related non-pathogenic species Listeria innocua (Li), E. coli DH5α and latex beads. We observed that 60 minutes post-inoculation, Li only induces a residual MIIA-pTyr as compared to Lm (Fig. 1D). Total levels of MIIA and actin remained the same. In addition, MIIA-pTyr was barely detected upon stimulation with E. coli DH5α and latex beads (Fig. 1D). These results indicate that MIIA-pTyr is associated to the pathogenic features of Lm.

We then investigated whether the MIIA-pTyr could be induced in response to other human pathogens. Experiments were performed on HeLa cells using an invasive E. coli K12 strain expressing the Yersinia pseudotuberculosis (Yp) invasin (K12-inv) [20], an infection model allowing the study of signalling pathways triggered specifically downstream the interaction invasin-integrin, and the extracellular pathogenic E. coli EPEC and EHEC. MIIA-pTyr was assessed in NI and infected cells. Strikingly, K12-inv induced a massive MIIA-pTyr that was not observed in response to K12-Δinv, in which the invasin-encoding gene is disrupted (Fig. 1E), thus indicating that MIIA-pTyr observed upon K12-inv infection is driven by invasin. Cells were also incubated with EPEC and EHEC for actin pedestal formation (4 h) and a slight increase in MIIA-pTyr was detected (Fig. 1E). EPEC and EHEC mutants affected in their capacity to induce key signalling events during infection were also used. We observed that EPEC-Tir(Y474F), which is defective for the actin pedestal formation [21], induces MIIA-pTyr at similar levels to those induced by the wild type EPEC (Fig. S2), suggesting that MIIA-pTyr is independent from EPEC-induced pedestal formation. Interestingly, EHECΔdam, a strain showing increased adherence [22], induces MIIA-pTyr more efficiently than the wild type EHEC (Fig. S2). This increased MIIA-pTyr is partially dependent on EHEC type III translocation (EHECΔdamΔescN) and on EspFu (EHECΔdamΔespFu), a bacterial effector important for actin polymerization beneath the EHEC attachment site [22] (Fig. S2).

These results indicate that the tyrosine phosphorylation of MIIA is a common event triggered by different pathogens and prompted us to address the role of MIIA in cellular infection.
**MIIA accumulates at the vicinity of cell-associated bacteria**

To further reinforce our findings and explore the possible role of MIIA in the cellular infectious process of different human pathogens, we performed MIIA immunolocalization studies in infected cells. HeLa and Caco-2 cells were incubated with *Lm*, *Yp*, EPEC or EHEC and analyzed by immunofluorescence for MIIA detection. We observed that MIIA labeling is enriched at sites of bacterial interaction with HeLa (Fig. 2A) and Caco-2 cells (Fig. 2B). Every tested pathogen was able to induce the recruitment of MIIA to the vicinity of F-actin-rich structures. *Lm* and *Yp* induced a discrete MIIA recruitment that is probably related to the weak actin cytoskeleton remodeling that they trigger at the entry site, which is typical to the zipper-like mechanism of cellular invasion. In contrast, we observed stronger MIIA accumulation to the actin pedestal-like structures induced by EPEC and EHEC. Interestingly, MIIA was also reported to accumulate at the *Salmonella* entry site [18]. These observations support the results described above and point to the potential role of MIIA at the interface of pathogens with the host cell membrane.

**MIIA restricts *Lm* intracellular levels**

To assess the role of MIIA in *Lm* infection, we measured the intracellular levels of *Lm* following chemical inhibition of MIIA activity and siRNA-mediated silencing of MIIA expression (MIIA-si). Blebbistatin (Blebb), a myosin II ATPase-specific inhibitor [23], was added (10 µM and 100 µM) to HeLa and Caco-2 cells and the *Lm* infection efficiency was quantified by gentamicin protection assays. *Lm* intracellular levels were 2- to 8-fold increased in a dose-dependent manner, in both cell lines, following treatment with the active enantiomer of blebbistatin [(-)Blebb] as compared to the inactive enantiomer [(+)Blebb] (Fig. 3A). Our data are in agreement with a previous report showing that Blebb treatment of L2 cells increases *Lm*-host cell association and invasion [24]. In contrast, ML-7 and ML-9, indirect and non-specific inhibitors of MIIA activity that act on myosin light chain kinase (MLCK), strongly impaired *Lm* infection (Fig. S3), suggesting that MLCK inhibitors could be
affecting other cellular pathways required for \( Lm \) invasion. Recruitment of MIIA and formation of actin foci at \( Lm \) entry sites were both detected in (-)Blebb-treated HeLa cells (Fig. 3B). In addition, the number of \( Lm \)-actin foci per cell and the associated MIIA recruitment were similar in (-)Blebb- and DMSO-treated cells (data not shown). However, we found a 2-fold increase in the percentage of HeLa cells with actin foci following (-)Blebb incubation (Fig. 3C), which correlates with the increase observed in \( Lm \) invasion rates and suggests that MIIA activity plays a restrictive role in \( Lm \) entry. To further address the role of MIIA in bacterial uptake we quantified \( Lm \) invasion in MIIA-depleted HeLa cells, using two siRNAs (si#1 and si#2). In accordance with results obtained in (-)Blebb-treated cells, the levels of \( Lm \) uptake were 2-fold increased in MIIA-depleted cells as compared to cells transfected with a control siRNA (Ctr) (Fig. 3D). Efficiency of MIIA depletion was assessed by immunoblot. Whereas si#1 depleted 85% of the MIIA, si#2 diminished MIIA expression by 55% (Fig. 3D). Immunofluorescence analysis of \( Lm \)-infected MIIA-depleted HeLa cells showed a 3-fold increase in the number of cell-associated bacteria as compared to control cells (Figs. 3E, F). Quantifications of the number of actin foci per cell showed the same trend observed after (-)Blebb treatment. However, this slight increase was not significant (Fig. 3F). Expression levels of the isoform B of myosin II (MIIB) remained the same in MIIA-depleted and control cells (Fig. S4A), thus discarding the hypothesis of a possible overexpression of MIIB in MIIA-depleted cells that could account for an increase in \( Lm \) uptake. In addition, we found that \( Lm \) uptake decreases 2.5-fold in MIIB-depleted cells (Figs. S4B, C), suggesting that MIIA and MIIB play opposite roles in \( Lm \) uptake and thus undermining the possibility of their mutual functional replacement in the invasion process. To reinforce our findings and exclude potential uncontrolled off-target effects, we performed invasion assays following gene rescue experiments. We created a siRNA-resistant GFP-tagged MIIA construct (MIIA-siRes) by introducing silent point mutations within the si#2 target sequence. Whereas depletion of MIIA resulted in an increased rate of \( Lm \) uptake, levels of intracellular \( Lm \) in MIIA-depleted cells expressing MIIA-siRes dropped to those observed in control cells (NT) (Fig. 3G). In contrast, levels of intracellular \( Lm \) in MIIA-depleted cells expressing a wild type GFP-tagged MIIA
(MIIA-WT) remained similar to those observed in MIIA-depleted cells. Immunoblot analysis showed that the expression of endogenous MIIA was diminished in the presence of si#2 and exogenous MIIA was only detected in MIIA-siRes-transfected cells (Fig. 3G). In cells without si#2, similar expression of MIIA-siRes and MIIA-WT was observed (data not shown). These results confirm that the increase in Lm intracellular levels observed in MIIA-depleted cells is specifically due to MIIA depletion.

To analyze whether the role of MIIA on bacterial internalization was specific for Lm or could be broadened to other bacteria, we performed gentamicin protection assays using Li, Li expressing InlB (Li-inlB), the major internalin driving Lm entry in HeLa cells [25]; K12-inv and Yp. For Li and Li-inlB, the numbers of intracellular bacteria were not significantly different in MIIA-depleted cells as compared to Ctr (Fig. 3H). In contrast, levels of intracellular K12-inv and Yp were significantly lower in MIIA-depleted cells (Fig. S5A). Our data indicate that MIIA is specifically triggered by pathogenic Lm and does not respond to non-pathogenic extracellular stimuli, such as Li. In addition, the role of MIIA in Lm invasion appears independent of InlB-mediated uptake. In contrast to Lm, our data show that Invasin-mediated uptake requires MIIA. Interestingly, MIIA and MIIB are required for SopB-mediated invasion of Salmonella [18]. Our findings, together with published reports, reveal that MIIA plays opposite roles in different infection models: while its function is required for an utmost Yp and Salmonella internalization, it has a restrictive role in Lm invasion.

**Src kinase phosphorylates MIIA limiting Lm infection**

Considering our previous findings revealing the key role of the tyrosine kinase Src during Lm invasion [7], we addressed the role of Src kinase in MIIA-pTyr in the context of Lm infection. Prior to Lm incubation, HeLa cells were treated with PP1, an inhibitor of Src family kinases, or with Y-27632, an inhibitor of the serine/threonine kinase ROCK that regulates MIIA activity and is also involved in Lm internalization [24]. Cell lysates were subjected to anti-pTyr IP assay and the presence of MIIA in IP fractions was assessed by immunoblot. MIIA-pTyr induced by Lm was abolished in PP1-treated cells while the Y-27632 treatment did not affect
the MIIA-pTyr triggered by Lm (Fig. 4A). These data suggest that MIIA-pTyr is dependent on Src kinase activity. To confirm these results, we interfered with Src activity by overexpressing a Src kinase-dead variant (Src-KD) [26]. Levels of MIIA-pTyr were assessed in NI and Lm-infected (Inf) HeLa cells overexpressing Src-KD. In contrast to NT cells, the MIIA-pTyr was almost undetectable in Src-KD-overexpressing cells and did not increase upon Lm incubation (Fig. 4B), thus confirming the crucial role of Src in MIIA-pTyr upon infection.

To investigate the role of MIIA-pTyr in the MIIA restrictive role in Lm infection, we evaluated the levels of intracellular bacteria in conditions where MIIA-pTyr does not occur. Levels of intracellular Lm showed a 2.5-fold increase in PP1-treated HeLa cells as compared to control DMSO-treated cells (Fig. 4C). In agreement, intracellular Lm increased 1.5-fold in cells expressing Src-KD (Fig. 4D). Inversely, intracellular levels of K12-inv decreased 2-fold in PP1-treated cells as compared to control cells (Fig. S5B), as previously reported [27]. The increased levels of intracellular Lm observed in conditions where Src is inactivated and thus MIIA is not tyrosine-phosphorylated, correlates with our results showing increased levels of Lm in cells with reduced levels or inactivated MIIA. Altogether, these results indicate that Src kinase phosphorylates MIIA limiting Lm infection.

Lm infection induces MIIA phosphorylation in tyrosine 158

The MIIA amino acid sequence includes 34 tyrosine residues, most of which are located in the myosin motor domain (Fig. S6A). To identify the MIIA tyrosine residues phosphorylated by Src in response to Lm infection, we used combined in silico approaches (NetPhos 2.0 and NetPhosK). Among the 34 tyrosine residues, 9 were predicted to be potentially phosphorylated. However, the tyrosine in position 158 (Y158) was the only predicted to be a Src kinase substrate (Fig. S6B). To assess in silico predictions we determined whether tyrosine phosphorylation of MIIA still occurs in infected cells expressing either GFP-tagged MIIA-Y158F (in which Y158 residue was replaced by a phenylalanine), MIIA-Y190F (harboring the same amino acid substitution in position 190, randomly selected and unrelated to in silico predictions) or MIIA-WT. While Lm infection generated an increase in MIIA-pTyr in
NT, MIIA-WT- and MIIA-Y190F-expressing cells, the amino acid substitution in residue Y158 abrogated MIIA-pTyr in infected cells (Figs. 5A, B). Exogenous GFP-MIIA-Y190F and GFP-MIIA-WT were both tyrosine-phosphorylated upon Lm infection (Fig. 5B). The levels of endogenous MIIA were similar in the different conditions and GFP fusion proteins were expressed similarly in NI and infected cells (Fig. 5A, B). These results point out the central role of Y158 in the phosphorylation of MIIA upon infection. To further support our data, we probed total lysates from NI and Lm-infected cells overexpressing MIIA-WT with an antibody raised against a peptide comprising phosphorylated Y158 residue of MIIA (pY158). We observed a 1.5-fold increase in the levels of MIIA-pTyr in Lm-infected cells (Fig. 5C). These results corroborate in silico predictions and shows that Y158 of MIIA is phosphorylated in response to Lm infection.

To confirm that Src kinase is able to phosphorylate MIIA specifically on Y158, we performed an in vitro kinase assay. GFP-MIIA-WT or MIIA-Y158F ectopically expressed in HEK293 cells were immunoprecipitated using an anti-GFP antibody and incubated with purified Src kinase and \[^{32}\text{P}\text{-ATP}]. As positive control we used a synthetic peptide commonly used as Src substrate. In the absence of kinase, the control peptide and IP fractions of MIIA-WT and MIIA-Y158F exhibit residual levels of incorporated radiolabeled ATP. In the presence of Src the MIIA-WT IP fraction and the control peptide (Ctr) became radiolabeled while the radioactivity incorporated by the sample MIIA-Y158F remained at the basal level (Fig. 5D). Altogether these results confirm that Y158 of MIIA is a substrate for Src kinase, becoming phosphorylated in response to Lm infection, and strongly suggest a role for this event as a cell defense mechanism to infection.

**Tyrosine 158 phosphorylation of MIIA restrains Lm infection**

To address the role of MIIA Y158 phosphorylation in the Lm cellular infection, we evaluated the intracellular levels of Lm in cells expressing either the GFP-MIIA-WT (WT) or the non-phosphorylable variant GFP-MIIA-Y158F (Y158F). We used HeLa cells that express endogenous MIIA and COS-7 cells that lack MIIA expression, therefore allowing to overcome
the effects from endogenous MIIA. *Lm* intracellular rates were determined by gentamicin protection assays (Fig. 6A) and immunofluorescence scoring in COS-7 cells (Fig. 6B). In agreement with the restrictive role of MIIA in *Lm* uptake, intracellular levels of *Lm* were reduced in HeLa and COS-7 cells expressing MIIA-WT as compared to NT cells. However, *Lm* infection was not affected by the expression of MIIA-Y158F (Fig. 6A). In accordance, immunofluorescence scoring revealed a 2-fold increase in the number of intracellular bacteria in COS-7 cells expressing MIIA-Y158F as compared to MIIA-WT (Fig. 6B). Furthermore, both GFP-MIIA-WT and GFP-MIIA-Y158F showed the same localization and accumulate at the site of *Lm* entry in HeLa cells (Fig. 6C). These results indicate that, whereas MIIA subcellular localization and recruitment to the site of bacterial uptake are unrelated to Y158, the phosphorylation of this specific MIIA tyrosine plays a key role in restraining *Lm* infection.
Discussion

Pathogens interfere with host phosphorylation cascades to foster adhesion, invasion and intracellular survival. Here, we searched for new host proteins undergoing tyrosine phosphorylation upon \textit{Lm} infection and showed that the host protein MIIA is tyrosine phosphorylated in response to human pathogens such as \textit{Lm}, EPEC, EHEC and the K12-\textit{inv}. This constitutes a previously unknown MIIA-pTyr event, triggered by Src kinase and involving residue Y158 of MIIA, that limits \textit{Lm} intracellular levels.

Previous studies showed that the unconventional Myosin VI and Myosin VIIa are both required for \textit{Lm} invasion of cells \cite{13,28}. \textit{Lm} cellular infection is thus a dynamic process requiring the concerted action of actin polymerization/depolymerization and myosin activity. Furthermore, myosin II isoforms were recently involved in viral and bacterial infections either promoting or limiting pathogen progression. Yet their role in such processes is still mainly descriptive. MIIA is required for KSHV and HSV1 entry into cells \cite{16,17,29}, facilitates \textit{Salmonella} invasion and regulates its intracellular growth \cite{18,30} and promotes \textit{Chlamydia} dissemination \cite{19}. Conversely, myosin II limits bacterial cell-to-cell spread by restraining \textit{Lm} protrusion formation \cite{31} and participating in the formation of \textit{Shigella}-associated septin cages \cite{32}. MIIB is involved in the formation of actin-rich structures that accumulate near the \textit{Salmonella}-containing vacuole and restrain bacterial intracellular multiplication \cite{33}. Altogether, these data suggest that the different outcomes associated with myosin II during infection are probably related to the cellular receptors or intracellular machinery engaged in the different infectious processes. Our results indicate that MIIA activity is required to constrain \textit{Lm} infection. MIIA-depleted or inactivated cells were reported to lose cytoplasm cohesion and show increased membrane activity and plasticity \cite{34,35}. These phenotypes could thus suggest that the increased numbers of intracellular \textit{Lm} in such cells would be greatly due to the disruption of cellular mechanics and membrane rigidity. However, if this was the case, cells displaying low MIIA activity should be more permissive to any extracellular pathogen, which was not observed in KSHV \cite{17}, HSV1 \cite{16} and \textit{Salmonella}.
infections. In addition, we show here that MIIA sustains invasin-mediated Yp infection. Our data also show that the intracellular levels of the non-invasive bacteria Li and the invasive Li expressing InlB are not significantly affected by MIIA-depletion, thus excluding a non-specific cell invasion mechanism.

Although the cellular invasion of Lm and Yp share similarities [36] and both trigger MIIA-pTyr, different receptors are engaged and thus MIIA could serve differently in both processes. To enter epithelial cells, Lm interacts with E-cadherin (E-cad) [4] and/or c-Met [5]. Invasion is mediated by E-cad in Caco-2 cells and by c-Met in HeLa cells. Our data thus shows that MIIA restrains Lm infection dependent on both routes of cellular invasion. In intercellular junctions, MIIA is critical for the E-cad apical localization [37] and Src activation is required for actin polymerization at cell-cell contacts [38], as it is during E-cad-mediated Lm invasion [7]. Interestingly, Src activation and recruitment of c-Cbl are key events to control c-Met signalling [39]. Here we show that Src activity restricts c-Met-mediated uptake of Lm (HeLa cells) raising the hypothesis that Src is acting through the tyrosine phosphorylation of MIIA to inhibit entry. Remarkably, in KSHV infection, which also depends on integrin and Src activation [40], MIIA interacts with the ubiquitin ligase c-Cbl [17]. The complex c-Cbl-MIIA associates with the receptor tyrosine kinase EphA2 that amplifies Src signaling to promote viral macropinocytosis [29]. It is thus possible that c-Cbl, which is required for Lm infection [41], associates with MIIA and c-Met to modulate Lm infection through tyrosine phosphorylation events. To invade cells, Yp binds β1-integrin [42], which via its cytoplasmic tail interacts with MIIA to regulate cell migration [43]. As in adhesion and cell migration processes [44], during Yp infection the engagement of β1-integrin leads to the activation of Src kinase [45] that could also act on MIIA and trigger its tyrosine phosphorylation at the site of bacterial attachment thereby promoting Yp invasion.

To our knowledge this is the first report showing and characterizing the MIIA-pTyr. We propose that the phosphorylation of MIIA Y158 modulates the outcome of MIIA activity either by affecting its subcellular localization, determining its molecular partners or directly
influencing MIIA activity. The residue Y158 locates in the motor domain of MIIA nearby the ATP-binding pocket (Fig. S6A) and is highly conserved among species ranging from *Saccharomyces cerevisiae* to *Homo sapiens* (Fig. S6C). This strongly suggests a role for this tyrosine in the regulation of MIIA functions and raises the hypothesis that MIIA-pTyr in Y158 could be triggered by specific physiological conditions engaging MIIA activity. Interestingly, an *in silico* study suggested that the residue Y163 of the muscle myosin heavy chain (matching Y158 in MIIA) could be phosphorylated [46]. The analysis of the crystal structure of myosin motor domain [47] showed that Y158 is exposed at the surface of the protein and is thus accessible for phosphorylation. The outcomes of such modification are now critical to elucidate.

Our data suggest that, upon infection, only a small pool of MIIA becomes phosphorylated in Y158, probably concentrated in a restricted subcellular localization of MIIA where it would impact infection. Yet, we observed that both MIIA-WT and MIIA-Y158F concentrated around bacteria at the entry site. Nevertheless, phosphorylation of Y158 should somehow affect MIIA cellular localization and/or define interactions with specific binding partners. We also found that phosphorylation of Y158 does not affect the phosphorylation of the myosin regulatory light chain (our unpublished data), that is achieved by MLCK and is required for activation of MII motor activity [15]. Interestingly, Src was previously shown recruited to membrane blebs where it associates with MLCK and myosin II [48,49]. In response to cell swelling, Src and MLCK form a complex in which Src activates MLCK and both regulate a compensatory membrane retrieval that requires myosin II [49]. It is thus conceivable that Src and MLCK could work together to fine-tune the activity of myosin II in the context of infection.

Our data open new perspectives in the regulatory mechanisms governing MIIA functions in infection and physiological cellular processes. Further work should reveal whether MIIA-pTyr affects its motor activity, binding partners and cellular localization.
Material and Methods

Bacterial strains and cell lines

Listeria and E. coli strains were grown aerobically at 37 °C, with shaking, in brain-heart infusion (BHI, Difco) and Luria Bertani (LB, Difco) media, respectively. Yersinia was grown aerobically at 26 °C, with shaking, in LB media. When required, ampicillin (Amp, 100 µg/ml), kanamycin (Kan, 50 µg/ml), or chloramphenicol (Cm, 20 µg/ml) were added to growth media. Details are provided in Table S1. Caco-2 cells (ATCC HTB-37) were cultivated in MEM with L-glutamine, supplemented with non-essential amino acids, sodium pyruvate, and 20% fetal bovine serum (FBS). HeLa (ATCC CCL-2), HEK293 (ATCC CRL-1573) and COS-7 (ATCC CRL-1651) cells were cultivated in DMEM with glucose (4.5 g/L) and L-glutamine, supplemented with 10% FBS. Cells were maintained at 37 °C in a 5% CO₂-enriched atmosphere. Cell culture media and supplements were purchased from Lonza.

Plasmids, antibodies and reagents

Plasmids used are listed in Table S2. Plasmids GFP-MIIA-Y158F and GFP-MIIA-Y190F were generated using GFP-MIIA-WT from Addgene [50] and the QuickChange II XL site-directed mutagenesis kit (Agilent Technologies). For MIIA rescue assays, a plasmid encoding siRNA-resistant GFP-MIIA-WT transcripts was generated. Oligonucleotide sequences are provided in Table S3. Antibodies are listed in Table S4. F-actin was labeled with Alexa Fluor 647- or 555-conjugated phalloidin (Invitrogen). Chemical inhibitors ML-7, ML-9 and Y-26732 (Sigma-Aldrich), Blebbistatin and PP1 (Calbiochem) were handled as recommended. FluoSpheres-carboxylate modified microspheres were from Invitrogen (F-8814).

Invasion assays

Invasion assays were performed as described [51]. When indicated cells were incubated with serum-free medium containing ML-7, ML-9, blebbistatin, PP1 or DMSO. Cells were challenged with Lm at a multiplicity of infection (MOI) of 50 for 1 h, treated with 20 µg/ml
gentamicin for 90 min, washed in PBS, lysed with 0.2% Triton X-100 and serial dilutions were plated for CFU counting. For immunofluorescence scoring, cells infected with EPEC, EHECΔdam and Yp (MOI 10) or Lm (MOI 50) were treated with 100 µg/ml gentamicin for 10 min, and washed with 20 µg/ml gentamicin prior fixation.

**Immunoprecipitation assays**

HeLa or Caco-2 cells grown until confluence were washed twice with warm phosphate-buffered saline (PBS), serum-starved (5 h) and left non-infected (NI) or incubated with Lm as described [51] at MOI 200 for different periods of time, or with *E. coli* (EPEC, EHEC or K12-inv strains) at MOI 200 for 4 h as described [22]. When indicated, cells were treated with 10 µM PP1 or 50 µM Y-27632. After washing twice with ice-cold PBS, cells were lysed in 1 ml of lysis buffer [1% Igepal CA-630 (Sigma-Aldrich), 50 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM AEBSF (Interchim), PhosSTOP and cComplete Protease Inhibitor Cocktail (Roche Pharmaceuticals)] and lysates recovered after centrifugation (15,000 × g, 10 min, 4 ºC). Cell lysates (500 µg) were incubated overnight (4 ºC) with 1 µg of anti-phosphotyrosine 4G10 or 5 µg of anti-MI1A antibodies. Immune complexes were captured with 50 µl of PureProteome Protein A magnetic beads (Millipore) or Protein G Sepharose 4 Fast Flow (50% slurry GE Healthcare), respectively. Immunoprecipitated fractions were resolved by SDS-PAGE and gels were silver-stained using the ProteoSilver™ Plus Silver Staining Kit (Sigma-Aldrich) or processed for immunoblotting. For kinase assay, HEK293 lysates were harvested 24 h post-transfection, GFP fusion proteins were immunoprecipitated with anti-GFP conjugated agarose beads (sc-9996 AC, Santa Cruz Biotechnology) and eluted in 0.2 M glycine, pH 2.5.

**Protein identification by mass spectrometry (MS)**

Protein identification was performed by MALDI TOF/TOF mass spectrometry as described [52]. Protein bands were excised from the silver-stained SDS-PAGE gels, reduced with
dithiothreitol, alkylated with iodacetamide and in gel digested with trypsin. Peptides were extracted, desalted, concentrated using ZipTips (Millipore), crystallized onto a MALDI sample plate and analyzed using a 4700 Proteomics Analyzer MALDI-TOF/TOF (Applied Biosystems). Peptidic mass spectra were acquired in reflector positive mode at a 700 - 4000 m/z mass window and the unknown protein identified by Peptide Mass Fingerprint approach using the Mascot software (Matrix Science, UK) integrated in the GPS Explorer software (ABSCIEX, CA) and searched against the SwissProt/UniProt Homo sapiens protein sequence database. The maximum error tolerance was 35 ppm and up to two protein missed cleavages were allowed.

**Immunoblotting**

Proteins were resolved in SDS-PAGE gels and transferred onto Nitrocellulose membranes (Hybond ECL, GE Healthcare Life Sciences). Membranes were blocked with 5% skimmed milk in buffer A (150 mM NaCl, 20 mM Tris-HCl pH 7.4 and 0.1% Triton X-100) for 1 h at room temperature or overnight at 4ºC. Primary and secondary antibodies were diluted in 2.5% skimmed milk in buffer A. Membranes used for anti-phosphotyrosine detection were blocked with Western Blocker solution (Sigma Aldrich) and the primary and secondary antibodies were diluted in the same solution as 4G10 or PY20 antibodies.

**Immunofluorescence analysis**

Cells were fixed in 3% paraformaldehyde for 15 min, quenched with 20 mM NH₄Cl for 1 h, permeabilized with 0.1% Triton X-100 for 5 min and blocked with 1% BSA in PBS for 30 min. Antibodies were diluted in PBS containing 1% BSA. Coverslips were incubated 1 h with primary antibodies washed three times in PBS and incubated with secondary antibodies and Phalloidin Alexa-555 or 647. DNA was counterstained with DAPI (Sigma-Aldrich). Coverslips were mounted onto microscope slides with Aqua-Poly/Mount (18606, Polysciences). Images were collected with a confocal laser-scanning microscope (Zeiss Axiovert LSM 510 or Leica SP2 AOBSE SE) and processed using Adobe Photoshop software.
Transfection of siRNA duplexes and plasmid DNA

HeLa cells seeded in 24- or 6-well plates were transfected with 60 nM of control siRNA-D (sc-44232 Santa Cruz Biotechnology) or specific siRNAs for MIIA or MIIB depletion, using Lipofectamine RNAiMax (Invitrogen) following manufacturer's instructions. Assays were performed 48 h later. Sequences of siRNAs are provided in Table S3. For transient protein expression, HeLa cells were seeded in 24-well plates (1x10^5 cells/well), 6-well plates (4x10^5 cells/well), or 10 cm dishes (3x10^6 cells/dish), and transfected at 90% confluency with 500 ng, 2.5 µg or 15 µg of plasmid DNA, respectively, using Lipofectamine 2000 (Invitrogen). Assays were performed 24 h later. For rescue assays, HeLa cells were transfected with MIIA-si#2 and 24 h later transfected with plasmids encoding siRNA-resistant GFP-MIIA-WT or GFP-MIIA-Y158F transcripts.

Kinase Assay

Kinase assays were performed using the Src Assay Kit (17-131, Millipore), following manufacturer's instructions. Anti-GFP-immunoprecipitated fractions from HEK293 cells expressing GFP-MIIA variants were incubated (10 min, 30 °C) with 10 units of recombinant Src (14-117, Millipore), in 30 µl of kinase reaction buffer supplemented with 9 µl of Manganese/ATP Cocktail and 10 µCi γ^{32}P-ATP (PerkinElmer). Reactions including a Src-specific substrate or lacking Src were used as positive and negative controls. Reactions were precipitated with 40% TCA and spotted onto P81 phosphocellulose paper squares, washed three times with 0.75% phosphoric acid, once with acetone and transferred to microtubes containing UniverSol liquid scintillation cocktail (MP Biomedicals). Incorporation of 32P was determined in a Wallac 1450 MicroBeta TriLux liquid scintillation counter (PerkinElmer), as counts per minute (cpm). Radioactivity measurements were performed in duplicate in two independent assays.
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References


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**Figure legends**

**Figure 1.** MIIA is tyrosine-phosphorylated in response to human bacterial pathogens. (A) Silver-stained acrylamide gel showing the tyrosine phosphorylation profiles of non-infected (NI) and *Lm*-infected HeLa cells, for the indicated periods of time. Total tyrosine-phosphorylated proteins were immunoprecipitated with an anti-pTyr antibody. Molecular weight standards are indicated. Arrow indicates a protein band showing increased intensity over the time of infection and identified by mass spectrometry analysis. (B) HeLa and Caco-2 cells were left non-infected (NI) or incubated with *Lm* and harvested at indicated time points post-infection (p.i.). Tyrosine-phosphorylated proteins were immunoprecipitated (IP: anti-pTyr) from whole cell lysates (WCL) and MIIA was detected by immunoblot (MIIA) in IP fractions and WCL. Detection of actin protein levels served as loading control. (C) MIIA was immunoprecipitated (IP: anti-MIIA) from WCL of NI and *Lm*-infected (Inf, 60 minutes) HeLa cells. Tyrosine-phosphorylated proteins (pTyr) and MIIA were revealed in immunoprecipitates. As control, MIIA and actin proteins were detected in WCL. (D) HeLa cells were left NI or incubated with either *Lm*, *L. innocua* (*Li*) (left panel), *E. coli* DH5α (*Ec*) or latex beads (right panel). Tyrosine-phosphorylated proteins were immunoprecipitated from the WCL recovered at different time points. MIIA was revealed on IP fractions and WCL. (E) HeLa cells were left NI or incubated for 4 h with *E. coli* K12 expressing a functional (*inv*) or mutated variant (*Δinv*) of *Y. pseudotuberculosis* invasin, and pathogenic *E. coli* (EPEC and EHEC). Tyrosine-phosphorylated proteins were immunoprecipitated and MIIA revealed by immunoblot in IP fractions and WCL.

**Figure 2.** MIIA is recruited to bacterial-plasma membrane foci. Single confocal sections of (A) HeLa and (B) Caco-2 cells incubated with *Lm* for 1 h, *Yp* for 20 min, EPEC and EHEC-*Δdam* for 4 h. Infected cells were immunolabelled for MIIA (green) and stained for actin (phalloidin, red) and DNA (DAPI, blue) (False color; scale bar 10 µm).
**Figure 3.** MIIA restricts *Lm* cellular infection. (A) Levels of intracellular *Lm* assessed by gentamicin protection assay and CFU counting, in HeLa and Caco-2 cells treated with 10 or 100 µM blebbistatin (Blebb). Values are the ratio of number of intracellular bacteria in cells treated with (-)Blebb (active enantiomer) and (+)Blebb (inactive enantiomer). (B) Single confocal sections of HeLa cells infected with *Lm* in the presence of DMSO (control) or 50 µM (-)Blebb. Infected cells were immunolabelled for MIIA (green) and *Lm* (blue) and stained for actin (red). (C) Percentage of cells showing *Lm*-associated actin foci. (D) Intracellular levels of *Lm* assessed by gentamicin protection assay in HeLa cells non-transfected (NT) or transfected with either control siRNA (Ctr) or MIIA-specific siRNAs (MIIA-si#1 and #2). Efficiency of MIIA knockdown was assessed by immunoblot. MIIA expression levels in siRNA-transfected cells were quantified and values are relative to actin and MIIA expression levels in NT cells. (E) Single confocal sections of Ctr or MIIA-si#1 HeLa cells incubated with *Lm* for 1 h. Infected cells were immunolabelled for MIIA (green), actin (red) and *Lm* (blue). (F) Number of bacteria and actin foci per cell. (G) Expression of MIIA was restored by a siRNA-resistant GFP-MIIA expression vector (MIIA-siRes). Intracellular levels of *Lm* assessed by gentamicin protection assay in HeLa cells expressing different levels of MIIA. Non-treated and MIIA-depleted cells expressing a wild type GFP-MIIA (MIIA-WT) were used as controls. Endogenous MIIA silencing and GFP-MIIA expression was evaluated by immunoblot. Detection of actin levels served as loading control. In panels D and G, the number of intracellular *Lm* in NT cells was normalized to 100% and those in siRNA-transfected cells were expressed as relative values. (H) Intracellular levels of *L. innocua* (*Li*) and *L. innocua* expressing *inlB* (*Li-inlB*) were assessed by gentamicin protection assay in HeLa cells transfected with either control siRNA (Ctr) or MIIA-specific siRNA (MIIA-si#1). The numbers of intracellular bacteria were normalized to 100% in Ctr and are expressed as relative values in MIIA-si#1 cells. Results shown in panels A, C, D, F, G and H are the mean of at least three independent experiments, each done in triplicate. Error bars represent SEM. Statistically significant differences are indicated: * p<0.05; *** p<0.001.
Figure 4. Src kinase phosphorylates MIIA upon *Lm* cellular infection. (A) HeLa cells were left NI or incubated with *Lm* for 1 h (Inf) in the presence of 10 µM PP1 or 50 µM Y-27632. (B) Control (NT) or Src kinase dead (Src-KD)-expressing HeLa cells were left NI or incubated with *Lm* for 1 h (Inf). In A and B, total tyrosine-phosphorylated proteins were immunoprecipitated and MIIA was detected by immunoblot in IP fractions and WCL. Detection of actin levels served as loading control. Protein levels of Src-KD were confirmed by immunoblot. (C and D) Intracellular levels of *Lm* assessed by gentamicin protection assays in the presence of 10 µM PP1 (C) or in Src-KD-expressing HeLa cells (D). The numbers of intracellular bacteria were normalized to 100% in control DMSO-treated cells and in NT and were expressed as relative values in presence of PP1 and in Src-KD cells. Results shown in panels C and D are means of three independent experiments, each done in triplicate. Error bars represent SEM. Statistically significant differences are indicated: *p*<0.05; **p*<0.01.

Figure 5. Tyrosine residue in position 158 of MIIA is phosphorylated in response to *Lm* infection. (A) HeLa cells expressing the wild type GFP-MIIA (WT) or the mutant MIIA-Y158F (Y158F) were left NI or incubated with *Lm* for 1 h (Inf). MIIA was detected by immunoblot in anti-pTyr immunoprecipitates and WCL. (B) Anti-pTyr immunoprecipitations performed on cell extracts of HeLa cells NT or expressing either GFP-MIIA-Y190F (Y190F) or GFP-MIIA-WT (WT), in NI or *Lm*-infected (Inf) conditions. Endogenous MIIA, GFP-MIIA-Y190F and GFP-MIIA-WT (GFP) were detected by immunoblot in IP fractions and WCL. Detection of actin levels served as loading control. (C) HeLa cells expressing GFP-MIIA-WT were left NI or incubated with *Lm* for 1 h (Inf). Cell extracts were used in immunoblot using an antibody raised against MIIA-pY158. MIIA-pY158 signal intensity was normalized to the actin signal and the value for *Lm*-infected is shown relative to NI sample normalized to 100%. (D) Anti-GFP IP fractions obtained from WCL of HEK293 cells expressing either GFP-MIIA-WT (WT) or GFP-MIIA-Y158F (Y158F) were used in *in vitro* Src kinase assays. A synthetic peptide
was used as positive control (Ctr). Incorporation of radiolabeled \([\gamma-^{32}\textrm{P}-\textrm{ATP}]\) was measured in CPM (counts per minute) for each condition. Results are representative of two independent experiments.

**Figure 6.** MIIA phosphorylation in tyrosine 158 is required to limit *Lm* cellular infection. (A) HeLa and COS-7 cells were NT or transfected to express either GFP-MIIA-WT (WT) or GFP-MIIA-Y158F (Y158F). Intracellular levels of *Lm* were assessed by gentamicin protection assays. Results are means of three independent experiments, each done in triplicate. (B) Intracellular levels of *Lm* assessed by immunofluorescence scoring in COS-7 cells ectopically expressing WT or Y158F variants. At least 50 cells were counted for each condition. Results are representative of two independent experiments. Error bars represent SEM. Statistically significant differences are indicated: * p<0.05; *** p<0.001. (C) Single confocal section of COS-7 cells ectopically expressing either WT or Y158F GFP-MIIA variants incubated with *Lm* for 1 h and stained for actin (phalloidin, red) and DNA (DAPI, blue) (scale bar 10 um).
Figure 2

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Figure 4
Figure 5
Figure 6

(A) Graph showing intracellular bacteria levels (%). The results indicate that the Y158F mutation decreases intracellular bacteria levels compared to WT in both HeLa and COS-7 cells.

(B) Graph showing bacteria per cell. The Y158F mutation results in a higher number of bacteria per cell compared to WT.

(C) Confocal microscopy images showing DAPI/Lm, Actin, GFP-MIIA, and Merge for WT and Y158F. The images show the distribution of intracellular bacteria under different conditions.

Figure 6