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CELLULAR & MOLECULAR BIOLOGY LETTERS
http://www.cmbl.org.pl

Received: 30 December 2009
Final form accepted: 28 May 2010
Published online: 04 June 2010

Volume 15 (2010) pp 496-506
DOI: 10.2478/s11658-010-0019-z


Short communication

# INTEGRIN RECEPTORS PLAY A ROLE IN THE INTERNALIN B-DEPENDENT ENTRY OF *Listeria monocytogenes* INTO HOST CELLS

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**Abstract:** *Listeria monocytogenes* enters non-phagocytic cells by binding its surface proteins *inlA* (internalin) and *inlB* to the host's E-cadherin and Met, respectively. The two internalins play either separate or cooperative roles in the colonization of infected tissues. Here, we studied bacterial uptake into HeLa cells using an *L. monocytogenes* mutant strain (*ΔinlA*) carrying a deletion in the gene coding for *inlA*. The *ΔinlA* mutant strain showed the capability to invade HeLa cells. The monoclonal anti-$\beta_3$- and anti-$\beta_1$-integrin subunit antibodies prevented bacterial uptake into the cells, while the anti-$\beta_2$- and anti-$\beta_4$-integrin subunit antibodies failed to affect *L. monocytogenes* entry into HeLa cells. Three structurally distinct disintegrins (kistrin, echistatin and flavoridin) also inhibited bacterial uptake, showing different potencies correlated to their selective affinity for the $\beta_3$- and $\beta_1$-integrin subunits. In addition to inducing Met phosphorylation, infection of cells by the *L. monocytogenes ΔinlA* mutant strain promoted the tyrosine phosphorylation of the focal adhesion-associated proteins FAK and paxillin. Our findings provide the first evidence that $\beta_3$- and $\beta_1$-integrin receptors play a role in the *inlB*-dependent internalization of *L. monocytogenes* into host cells.

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Abbreviations used: BHI – brain-heart infusion; BSA – bovine serum albumin; CFU – colony-forming unit; DMEM – Dulbecco's modified Eagle's medium; FAK – focal adhesion kinase; FBS – bovine fetal serum; InlA – internalin A; InlB – internalin B; MOI – multiplicity of infection; PBS – phosphate-buffered saline; SDS – sodium dodecyl sulphate

**Key words:** *Listeria monocytogenes*, Internalin B, Integrins, FAK, Paxillin

## INTRODUCTION

*Listeria monocytogenes* is a gram-positive facultative intracellular food-borne bacterial pathogen of humans [1]. It is the causative agent of listeriosis, which affects immunocompromised individuals and pregnant women, respectively causing meningo-encephalitis and abortions. *L. monocytogenes* can be engulfed by professional phagocytes such as macrophages or neutrophils, and by non-phagocytic cells, including intestinal epithelial cells, hepatocytes, endothelial cells and syncytiotrophoblasts [2].

Two major pathways mediate *L. monocytogenes* entry into non-phagocytic mammalian cells. They are initiated by the binding of the bacterial surface proteins internalin A (*inlA*) and internalin B (*inlB*) to their receptors on the cell surface [3]. While *inlA* interacts with E-cadherin, a cell receptor involved in the formation of adherent junctions in polarized epithelial cells [4, 5], *inlB* interacts mainly with the tyrosine kinase receptor of the hepatocyte growth factor Met [1, 6]. *InlA* and *inlB* may play either separate or cooperative roles in the colonization of infected tissues. Both routes of bacterial internalization require reorganization of the host cell F-actin cytoskeleton, although the specific signal transduction pathways that promote cytoskeletal remodelling downstream of E-cadherin or Met differ [7]. While *inlA*-mediated invasion is apparently restricted to the intestinal epithelium, *inlB* is essential for *L. monocytogenes* uptake by most non-phagocytic cell types. The activation of Met is required for *inlB*-mediated entry [8]. Upon *inlB* binding, Met becomes phosphorylated, thus triggering the signals needed for the cytoskeleton re-arrangement which allows bacterial engulfment.

Compelling evidence has been provided that Met coordinates with integrins to transmit signals [9-11]. Integrins, cell surface receptors that fulfill multiple functions, are heterodimeric receptors formed by the non-covalent association of α- and β-subunits [12]. In humans, there are 19 distinct α-subunits and eight β-subunits, which are combined in 24 different receptors. Integrins bind extracellular matrix components containing the RGD sequence. Ligands induce integrin clustering on the cell surface. Both ligand occupancy and integrin clustering lead to the formation of "focal adhesions", where integrins link intracellular complexes and bundles of actin filaments [13]. By linking actin dynamics to extracellular matrix components, integrins are involved in a wide range of cellular processes that are associated with or require cytoskeletal remodelling and cell-shape changes [14, 15]. Integrin engagement is commonly exploited by many microbial pathogens as a route for invading host cells [16-18]. Some integrins and growth factor receptors appear to be normally in relatively close proximity and to share many common elements in their signalling pathways. Although the mechanisms of *L. monocytogenes* entry into

host cells have been extensively investigated, it remains unknown whether integrin receptors play a role in the *inlB* interaction with Met.

In this study, we investigated the potential involvement of host surface integrin receptors in the *inlB*-dependent *L. monocytogenes* internalization into host cells. For this purpose, we analyzed the ability of a *L. monocytogenes* mutant strain carrying a deletion in the gene coding for *inlA* (*ΔinlA*) to infect cultured HeLa cells [19]. Monoclonal anti-integrin antibodies and structurally distinct disintegrins (echistatin, flavoridin and kistrin) with selective binding affinity for integrin receptor subunits [20] were exploited to characterize the integrin subunits involved in the bacterial entry into the host cells. We also investigated the activation of signalling pathways that are common to both integrin and growth factor receptors, such as the tyrosine phosphorylation of the focal adhesion-associated protein FAK, as a consequence of bacterial infection of cells.

## MATERIALS AND METHODS

### Antibodies and chemicals

The monoclonal antibody anti-phosphotyrosine (PT66) IgG (P-3300), goat anti-mouse IgG and goat anti-rabbit IgG conjugated to peroxidase, echistatin, flavoridin, kistrin, Dulbecco's modified Eagle's medium (DMEM) and bovine serum albumin (BSA) were purchased from Sigma Aldrich (St Louis, MO, USA). Bovine fetal serum (FBS) was from Hyclone Laboratories (Logan, UT, USA), polyclonal antibody anti-Met (sc-10) from Santa Cruz Biotecnology (Heidelberg, Germany), rabbit polyclonal anti-FAK antibody (06-543) from Upstate Biotechnology (Lake Placid, NY, USA), and monoclonal anti-paxillin antibody (P-13520) from Transduction Laboratories (Lexington, KY, USA). Mouse monoclonal anti-β1 (Mab1987, clone P4C10) and anti-β3 (Mab2023Z, clone B3A) integrin antibodies and mouse IgG1 (CBL600, clone DD7) were from Chemicon International Inc. (Temecula, CA, USA), and mouse monoclonal anti-β4 (G-7, sc-13127) and anti-β2 (CTB104, sc-8420) integrin subunit antibodies from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

### Cell and bacterial cultures

The human cervical epithelial cell line HeLa was cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, and 1% non-essential amino acids at 37ºC in 10% $CO_2$. Cells were detached with 0.25% trypsin/0.02% EDTA in phosphate-buffered saline (PBS), pH 7.2, washed with DMEM, and resuspended in DMEM for propagation or uptake studies. The *L. monocytogenes* mutant strain A76 (*ΔinlA*), provided by Prof. W. Goebel and Dr. B. Bergmann (University of Würzburg, Würzburg, Germany), was grown for 24 h at 37ºC in Difco brain-heart infusion (BHI) from Becton-Dickinson (Milan, Italy). Overnight cultures were diluted in BHI broth, incubated for 3 h at 37ºC, collected by centrifugation at 200 x g for 1 min, and washed with PBS. The

bacterial concentration was measured by assessing the optical density at 600 nm. Before infection, the bacteria were diluted to a concentration of $10^7$ bacteria per ml.

**Bacterial uptake assay**

Cells ($10^5$ cells per well), plated onto 24-multiwell plastic dishes overnight at 37ºC, were washed with DMEM and infected with the bacterial suspension at a multiplicity of infection (MOI) of 50 for 1 h at 37ºC. For the uptake inhibition assay, cells were pre-treated with different doses of a specific antibody (0.25-10 μg/ml) or a disintegrin (0.05-1 μg/ml) for 15 min at 37ºC. At the end of the incubation time, the bacteria that had not entered the cells were removed by sequential washings with DMEM, and the cells were treated with 100 μg/ml gentamycin for 2 h at room temperature. Then, the cells were washed three times with DMEM, and lysed with Triton X-100 (0.3% final concentration). The number of bacteria internalized by the cells was counted on agar plates using the colony-forming unit (CFU) method [21]. The percentage of bacterial uptake into the cells was determined by calculating the number of bacteria internalized into infected cells with respect to the total number of bacteria used for the infection.

**Protein immunoprecipitation and Western blot**

Freshly resuspended cells (1 x $10^5$) were plated onto tissue culture plastic dishes and allowed to adhere at 37ºC for 3 h. The cells were washed twice with DMEM and then were infected with the bacteria at an MOI of 50 for different time intervals (0, 5, 10, 20, 30 and 60 min). At the end of the incubation time, the cells were washed three times with DMEM and treated with 100 μg/ml gentamycin for 2 h at room temperature. The cells were washed with DMEM and lysed in ice-cold RIPA buffer containing 1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.1% sodium dodecyl sulphate (SDS), 0.5% deoxycholic acid, 10 mM NaF, 1 mM Na3VO4, 1 mM phenylmethyl-sulphonyl fluoride, 10 μg/ml leupeptin and 0.1 U/ml aprotinin for 30 min on ice. The lysates were clarified by centrifugation at 14000 rpm for 10 min at 4ºC. The amount of proteins in the samples was determined by a Bio-Rad DC protein assay (Bio-Rad Laboratories, Segrate, MI, Italy). Lysates containing an equal amount of proteins were incubated with swollen protein A- or G-agarose for 2 h at 4ºC. After centrifugation at 3000 rpm for 10 min at 4ºC, the supernatant fractions were incubated with anti-Met or anti-FAK, or anti-paxillin antibody at a concentration of 1 μg/100 μg total proteins for 6 h at 4ºC. Protein A- or G-agarose was then added to the samples, which were incubated overnight at 4ºC. Proteins were re-suspended in Laemmli buffer, boiled for 5 min and run on 10% SDS-PAGE, and the samples were transferred to a nitrocellulose membrane using a Mini Trans-Blot apparatus (Bio-Rad Laboratories), according to the manufacturer's instructions. The blots were incubated for 1 h at 42ºC in 5% BSA in TBS-T (150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.3% Tween-20), and after the incubation time, washed with TBS-T. To probe for phosphotyrosine-containing proteins, the filters were incubated overnight with the PT66 antibody

diluted 1:10000 in 1% BSA in TBS. The blots, washed with TBS-T, were incubated for 1 h at room temperature with an anti-IgG peroxidase-conjugated secondary antibody diluted 1:3000 in 1% BSA in TBS, and washed with TBS-T. The proteins were visualized using an ECL chemiluminescence kit (Amersham, Milan, Italy). The same blots were stripped and re-probed with anti-Met, anti-FAK or anti-paxillin antibody to confirm that there was an equal amount of immunoprecipitated protein in all of the lanes.

**Statistical analysis**

The data is expressed as the average and standard deviation (SD). Student's t test was used for the statistical comparisons when appropriate, and differences were considered significant at $P < 0.05$.

## RESULTS AND DISCUSSION

First, the ability of the *L. monocytogenes* mutant strain *ΔinlA* to be incorporated by HeLa cells was evaluated. The mutant strain was internalized by the cells, with a bacterial uptake percentage ranging from 0.5 to 1% depending on the experiment (data not shown). Thus, the *ΔinlA* strain retains the capacity to enter HeLa cells, confirming previous evidence that bacterial entry into non-phagocytic cells may efficiently occur either through the *inlA*- or *inlB*-mediated pathway or through both [1, 2]. However, besides *inlA* and *inlB*, additional internalins may also play important roles in the infectious process of *L. monocytogenes* [3].

In order to evaluate the involvement of host surface integrin receptors in *L. monocytogenes* entry into HeLa cells, uptake inhibition studies were carried out using monoclonal antibodies directed against specific integrin subunits. HeLa cells were pre-incubated with increasing doses of monoclonal anti-$\beta_1$, -$\beta_2$, -$\beta_3$, and -$\beta_4$ subunit antibodies before the infection of the cells. Control experiments were included by pre-treating the cell monolayer with a mouse IgG1 negative control prior to bacterial infection. In the control experiments, no inhibition of *ΔinlA* strain uptake into HeLa cells was detected (data not shown). The anti-$\beta_1$- and -$\beta_3$-integrin antibodies inhibited the uptake of the *L. monocytogenes ΔinlA* mutant strain into HeLa cells (Fig. 1, Panel A), indicating that the anti-$\beta_3$ antibody is more effective than the anti-$\beta_1$ antibody ($P < 0.05$). At 10 μg/ml, the anti-$\beta_3$ and anti-$\beta_1$ antibodies inhibited the uptake of *ΔinlA* strain by 50% and 40%, respectively. Under our experimental conditions, the anti-$\beta_2$ and -$\beta_4$ subunit antibodies failed to affect the uptake of *ΔinlA* mutant strain into HeLa cells. Whether these results depend on different expression levels of the tested integrin subunits in the HeLa cells remains to be established. Thus, $\beta_3$-integrin subunits and, to a lesser extent, $\beta_1$-integrin subunits appear to be involved in the *inlB*-mediated bacterial internalization into host cells.

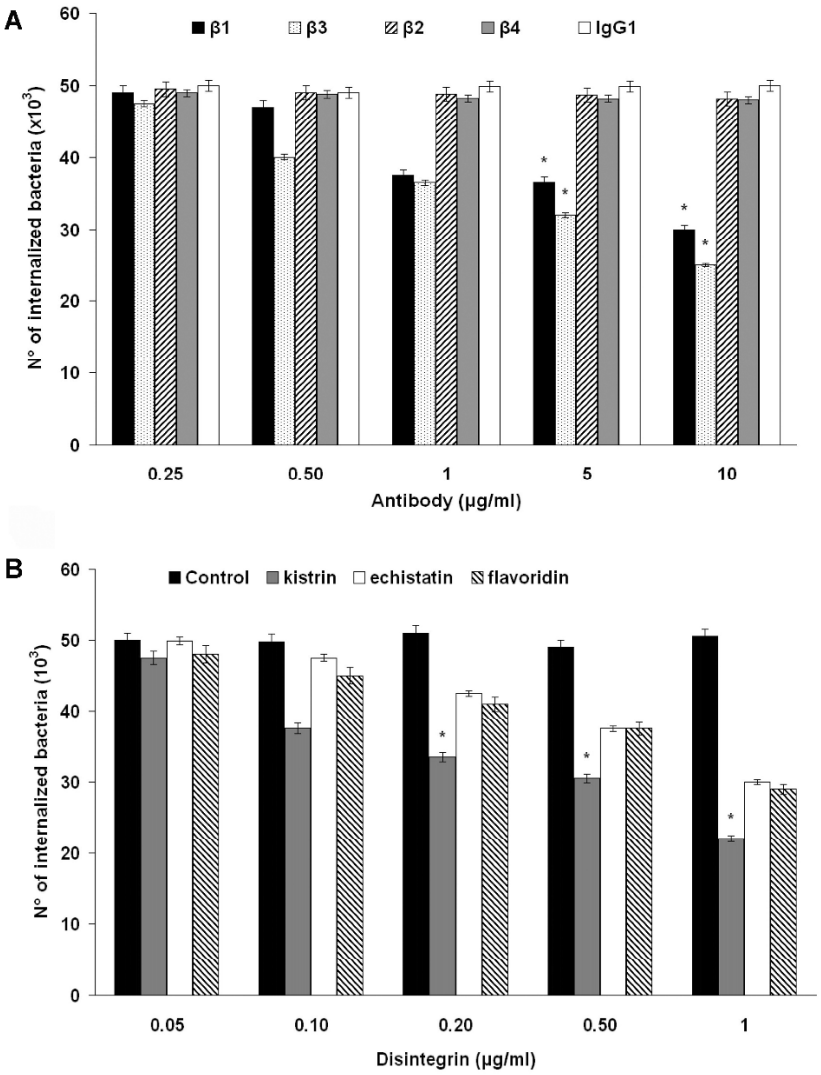


Fig. 1. Inhibition of *L. monocytogenes* uptake into HeLa cells by anti-integrin subunit antibodies and disintegrins. A – HeLa cells were incubated for 15 min with increasing amounts (0.25-10 µg/ml) of anti-$\beta_1$, anti-β3, anti-$\beta_2$ or anti-$\beta_4$ integrin antibodies, or IgG1 serum. B – HeLa cells were incubated for 15 min with increasing amounts (0.05-1 µg/ml) of kistrin, echistatin, flavoridin or medium. After the incubation with the antibodies or disintegrins, the cells were infected with the *L. monocytogenes* mutant strain *ΔinlA* for 60 min. The bacteria that had not entered the cells were removed by treatment with gentamycin for 2 h at room temperature. The number of bacteria internalized into infected cells pre-treated with antibodies or disintegrins was determined using the CFU method. The data is the average values of six determinations performed in triplicate, with standard deviations indicated by the vertical bars. *$P < 0.05$.

The role of the $\beta_3$- and $\beta_1$-integrin subunits in the *inlB*-pathway of *L. monocytogenes* entry into cells was further investigated by uptake inhibition studies using three structurally distinct disintegrins: kistrin, flavoridin and echistatin. Due to structural motifs other than the RGD sequence present in their molecules, disintegrins show a high selectivity in their binding to different integrin receptors, and are thus a useful tool for studying integrin-mediated cell functions and bacterial pathogenesis [21]. Kistrin binds $\alpha_v\beta_3$ with high affinity, and it is recognized by $\alpha_5\beta_1$, albeit with a lower affinity than flavoridin and echistatin [20]. Echistatin structural motifs in addition to the RGD sequence support its affinity binding to $\alpha_5\beta_1$ and $\alpha_v\beta_3$ [22], while the flavoridin NMR structure resolution has shown that the C-terminal of this disintegrin acts as a secondary binding site for the highly specific interaction of flavoridin with $\alpha_5\beta_1$ integrin [23].

Before evaluating the ability of kistrin, echistatin and flavoridin to inhibit *L. monocytogenes* uptake into HeLa cells, we determined their cytotoxicity by exposing the cells to increasing concentrations of each disintegrin (0-100 μg/ml) for different time intervals (10 min to 24 h). The viability of the cells, as measured uding the trypan blue exclusion method [21], was higher than 90% after 24 h of cell exposure to 100 μg/ml of each disintegrin (data not shown). However, for the uptake inhibition studies, echistatin, flavoridin and kistrin were used at the low doses of 0.05-1 μg/ml. The HeLa cells were pre-incubated with increasing doses of each disintegrin before infection. The three disintegrins showed different potencies in inhibiting the uptake of the *L. monocytogenes ΔinlA* strain into HeLa cells (Fig. 1, Panel B), with kistrin being the most active ($P < 0.05$). Echistatin and flavoridin showed an almost similar inhibitory activity. In particular, at the dose of 1 μg/ml, kistrin, flavoridin and echistatin inhibited the uptake of *ΔinlA* strain by 57, 47 and 41%, respectively. These results correlate with the respective affinities of the three disintegrins for $\beta_3$- and $\beta_1$-integrin receptors [20] and confirm the results obtained in inhibition studies using anti-integrin subunit antibodies, indicating a major role for $\beta_3$- and, to a lesser extent, for $\beta_1$-integrins in the *inlB*-dependent *L. monocytogenes* entry into host cells.

Protein tyrosine phosphorylation of the focal adhesion-associated protein FAK is one of the earliest signalling events occurring in response to integrin engagements in most cells [24]. FAK is implicated in the tyrosine phosphorylation of several substrates. Paxillin is a substrate of FAK or of Src kinase activated by FAK. Changes in the phosphorylation levels of these components of focal adhesions correlate with their disorganization and reduced cell adhesion [25]. Focal adhesion disassembly is concurrent with the disorganization of actin microfilaments [26]. Evidence exists that FAK and paxillin play a key role in the actin cytoskeletal remodelling required for phagocytosis of some bacteria [16, 18]. In order to establish whether, in addition to promoting Met phosphorylation, *L. monocytogenes* entry into host cells also

activates integrin-dependent signalling pathways, the tyrosine phosphorylation of the focal adhesion-associated proteins FAK and paxillin was evaluated. Uninfected (control) cells and cells infected with the *L. monocytogenes ΔinlA* strain for different time intervals were lysed and subjected to immunoprecipitation experiments, using anti-Met, anti-FAK or anti-paxillin antibody. The immunoprecipitated proteins were subjected to SDS-PAGE electrophoresis and Western blotting using the anti-phosphotyrosine antibody PT-66. While the three proteins appeared to be only sparcely phosphorylated in uninfected cells (Fig. 2, lanes 1, upper blots), Met, and FAK and paxillin were highly phosphorylated in the lysates of HeLa cells infected for 10 min by the *ΔinlA* strain (Fig. 2, lanes 2, upper blots). The phosphorylation levels of the three proteins did not change when the time of exposure to the bacteria was prolonged up to 60 min (data not shown). The basal levels of FAK and paxillin tyrosine phosphorylation observed in the control (uninfected) cells are probably due to the activation of integrin signalling by cell adhesion [27]. When the upper blots were stripped and re-probed with anti-Met, anti-FAK and anti-paxillin antibodies, the same amount of immunoprecipitated Met, FAK and paxillin protein was observed in all lanes (Fig. 2, lower blots).

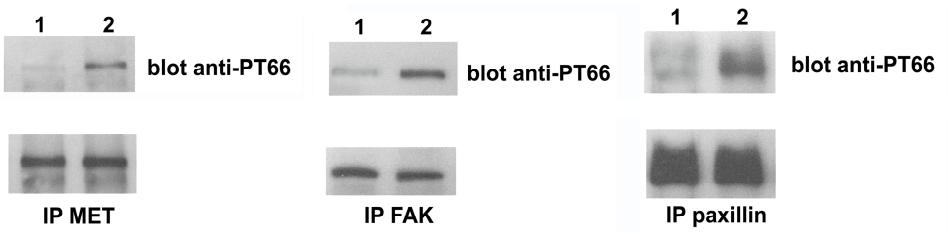


Fig. 2. *L. monocytogenes* infection of HeLa cells induces tyrosine phosphorylation of Met, pp125$^{FAK}$ and paxillin. Freshly resuspended cells were plated onto plastic dishes and allowed to adhere for 3 h at 37ºC. After the incubation, the cells were treated with serum-free medium (lanes 1) or exposed to *L. monocytogenes* mutant strain *ΔinlA* (lanes 2) for 10 min. The cells were then lysed and subjected to immunoprecipitation using anti-Met, anti-FAK or anti-paxillin antibodies. The immunoprecipitated proteins were run on a 10% SDS/polyacrylamide gel, transferred to nitrocellulose, and blotted with an anti-phosphotyrosine antibody (PT66; upper blots). The same blots were stripped and re-probed with the anti-Met, anti-FAK and anti-paxillin antibodies to ensure that the same amount of protein was immunoprecipitated in all of the samples (lower blots). Similar results were obtained from five separate experiments of identical design.

Thus, the bacterial infection of HeLa cells by the *L. monocytogenes ΔinlA* mutant strain induces an increase in the tyrosine phosphorylation levels of Met, FAK and paxillin as compared to those of the uninfected cells. Whether FAK phosphorylation is due to the activation of integrin signalling or Met activation consequent to *inlB* engagement remains to be established. However, our findings fit well with evidence demonstrating that FAK is a crucial molecule in integrating signals from integrins and growth factor receptors in processes such

as cell survival, proliferation and motility, and invasion [28]. Our results strongly suggest that the $\beta_3$ and/or $\beta_1$ integrins cooperate with the Met receptor in promoting *inlB*-mediated *L. monocytogenes* internalization into host cells.
Multiple pieces of evidence show that integrins co-operate with other types of receptor to control diverse aspects of cell fate [11]. In particular, an interplay occurs between integrins and growth factor receptors: $\beta_3$- and $\beta_1$-integrins work together with growth factors to control angiogenesis [9]; the crosstalk between c-Met and the $\alpha_2\beta_1$ integrin contributes to mast-cell activation in autoimmune and inflammatory disorders [29]; and integrin-dependent adhesion triggers ligand-independent epidermal growth factor receptor activation to transduce downstream signalling [30]. Although further studies are needed to establish the precise role of integrins in the *inlB*-mediated *L. monocytogenes* internalization into host cells, our findings provide evidence suggesting the occurrence of an interplay between integrins and Met in the pathogenesis of the *L. monocytogenes*.

**Acknowledgements.** This work is dedicated to the memory of Dr. Antonio Scibelli. The research study was supported by a grant from the Ministero della Sanità, Rome, Italy.

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