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Listeria monocytogenes encodes a functional ESX-1 secretion system whose expression is detrimental to in vivo infection

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ABSTRACT

Bacterial pathogenicity deeply depends on the ability to secrete virulence factors that bind specific targets on host cells and manipulate host responses. The Gram-positive bacterium Listeria monocytogenes is a human foodborne pathogen that remains a serious public health concern. To transport proteins across its cell envelope, this facultative intracellular pathogen engages a set of specialized secretion systems. Here we show that L. monocytogenes EGDe uses a specialized secretion system, named ESX-1, to secrete EsxA, a homolog of the virulence determinants ESAT-6 and EsxA of Mycobacterium tuberculosis and Staphylococcus aureus, respectively. Our data show that the L. monocytogenes ESX-1 secretion system and its substrates are dispensable for bacterial invasion and intracellular multiplication in eukaryotic cell lines. Surprisingly, we found that the EssC-dependent secretion of EsxA has a detrimental effect on L. monocytogenes in vivo infection.

Introduction

The capacity to secrete proteins is crucial for the pathogenesis of many bacteria. In Gram-positive bacteria, proteins are delivered by highly specialized secretion systems across cell envelope to reach specific targets.[1] A portion of these proteins, including virulence factors, are often secreted by Sec-independent systems. ESX-1, also called WXG100, is a Sec-independent secretion system first described in Mycobacterium tuberculosis.[2-4] This system allows the secretion of ≈100 amino-acid-long proteins that lack the classical signal peptide but contain a Trp-X-Gly motif (WXG100 proteins). M. tuberculosis ESAT-6 and CFP-10 are prototypes of WXG100 proteins, both encoded by the region of difference 1 (RD1), involved in virulence and described as highly immunogenic proteins.[5] Deletion of the esx-1 locus abrogates ESX-1-dependent secretion and strongly attenuates the virulence of M. tuberculosis.[6] Apart from mycobacterial species, ESX-1 systems are also found in Firmicutes, among which the S. aureus ESX-1 is one of the best-characterized.[7,8] It comprises genes encoding the canonical

ESX-1 substrates (EsxA, EsxB), a membrane-anchored FtsK/SpoIIIE-like ATPase (EssC) essential for the secretion machinery, genes coding for membrane-embedded proteins (EssA, EssB, EssD) required for secretion of ESX-1 substrates, as well as staphylococci-specific ESX-1 substrates (EsxC, EsxD), and modulators of ESX-1 activity (EsaA, EsaB).[7-10] S. aureus EsxA and EsxB share features with the M. tuberculosis ESAT-6 and CFP-10, including the presence of a WXG motif and the co-dependent secretion. However, unlike ESAT-6 and CFP-10, EsxA and EsxB do not interact. EsxA dimerizes with itself or associates with EsxC, while EsxB interacts with EsxD.[8] Disruption of key components of the S. aureus ESX-1 secretion machinery (EssC) or deletion of esxA and esxB causes a significant reduction in the ability of S. aureus to establish kidney or liver abscesses.[7,9,11] Moreover, the S. aureus ESX-1 secretion system is required for nasal colonization and virulence in a murine lung pneumonia model.[12] EsxA was also shown to interfere with host cell apoptotic pathways, affecting bacterial survival and mediating S. aureus release from

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host cells.[13] Other ESX-1 substrates, such as EsaC, although dispensable for the establishment of acute infections, are required for the formation of persistent infection.[11] Functional ESX-1 secretion systems were also characterized in *Bacillus anthracis*, *Bacillus subtilis*, *Actinobacterium* and *Streptomyces coelicolor*.[14-17]

Listeria monocytogenes (*Lm*) is a ubiquitous Gram-positive bacterium responsible for listeriosis, a severe opportunistic foodborne disease occurring mainly in immunocompromised individuals, newborn, elderly and pregnant women. Listeriosis is the most frequent cause of hospitalization and death due to the consumption of contaminated food in Europe, and involves high illness costs and quality life losses.[18] Clinical features of listeriosis includes septicemia, meningitis, meningoencephalitis and abortions. This facultative intracellular pathogen has evolved multiple strategies to survive inside phagocytic cells, invade non-phagocytic cells and spread from cell to cell.[19] Each step of its cell infection cycle depends on specific virulence determinants that play specific roles, most of them being surface or secreted proteins.[20,21] Genes encoding a potential ESX-1 secretion system were identified in *Lm*[22] and the *Lm* EsxA homolog was previously shown dispensable for *Lm* mouse infection.[23] However, although ESX-1 was postulated to represent a broad Gram-positive secretion system,[1] the functionality of this apparatus and its role in infection were never previously investigated in *Listeria*.

Results

Lm *encodes a putative ESX-1 secretion system*

In agreement with the model proposed for the ESX-1 secretory apparatus[1,22] and following bioinformatic analyses (BLAST, TopPred2, ProDom) we determined that the *Lm* ESX-1 locus contains genes coding for: the 2 canonical WXG100 substrate paralogs EsxA (Lmo0056) and EsxB (Lmo0063), the integral membrane FtsK/SpoIIIE-type ATPase EssC (Lmo0061); EsaA (Lmo0057), a polytopic membrane protein with 5 predicted transmembrane helices; EssA (Lmo0058) and EssB (Lmo0060), 2 predicted membrane proteins with respectively one and 2 transmembrane domains; and EsaB (Lmo0059) and Lmo0062, 2 putative cytoplasmic proteins (Fig. 1A). Membrane topology or soluble character of proteins encoded by the *Lm* ESX-1 locus was predicted and compared to *M. tuberculosis* and *S. aureus* ESX-1 secretion systems (Fig. 1B). This revealed large similarities with the *S. aureus* ESX-1 systems. However, the 2 ESX-1 substrates (EsxC and EsxD) and the EssD transmembrane protein described in *S. aureus* are absent in *Lm*. Analysis of complete genome sequences available for different *Listeria* species revealed the high level of conservation of the ESX-1 locus within the *Listeria* genus (Fig. 1A). Of note, *esxB* and *lmo0062* are absent from 3 non-pathogenic species (*L. welshimeri*, *L. seeligeri* and *L. grayi*).

Lm *ESX-1 secretion system is functional although weakly expressed*

To evaluate the expression of the *Lm esx-1* locus, we analyzed by RT-PCR the transcription of genes encoding the 2 major ESX-1 effectors (*esxA* and *esxB*), and 2 integral membrane proteins essential for the secretion machinery (*essB* and *essC*).[7-10] RNAs extracted from bacteria in exponential growth phase in BHI at 37°C were processed for analysis and results showed that all the genes selected are transcribed in these conditions (Fig. 2A). However, *esxB, essC* and *essB* appeared to be weakly expressed as compared to *esxA* and control genes (*inlA, actA, iap*) encoding known *Lm* virulence factors. We also observed that the expression of *esxA* appeared to decrease upon entry into stationary growth phase, which was confirmed by qRT-PCR (Fig. 2B). We also attempted to assess by qRT-PCR the expression of *esxB* and *essC* during growth in exponential or stationary phases. However, transcript levels appeared insufficient to obtain quantifiable amplification, in particular during the stationary growth phase, thus underlining the weak level of expression of these genes. *esxA* was previously shown to be negatively regulated by SigB in *S. aureus*.[24] SigB being the major regulator of the stationary growth phase, we assessed if the decreased expression of *esxA* upon entry into this phase would be due to SigB regulation. We analyzed by qRT-PCR the expression of *esxA* in a *sigB* deletion mutant as compared to the WT strain during growth in exponential and stationary phases, and showed that in both growth phases *esxA* expression appeared independent of the presence of *sigB* (Fig. 2C). *bsh,* which encodes a bile salt hydrolase, was used as a SigB-dependent control gene.[25]

To analyze whether the putative *Lm* ESX-1 system was functional, we constructed a deletion mutant strain for *essC* (ΔessC), which encodes a structural protein essential for *S. aureus* ESX-1 functionality,[7] as well as the corresponding complemented strain (ΔessC+essC). Both strains were confirmed by PCR and sequencing, and their growth rate in BHI at 37°C was comparable to that of the WT (Fig. S1A-B). To test the functionality of the ESX-1 secretion system in *Lm*, we expressed a myc-tagged EsxA protein (EsxA-myc) in WT bacteria and analyzed its secretion during bacterial growth. Western blot of bacterial culture supernatants showed a band of 11 kDa, the expected size for EsxA-myc, with a higher intensity in the exponential growth phase (Fig. 2D left panel). These results indicate that *Lm* EsxA was secreted in these
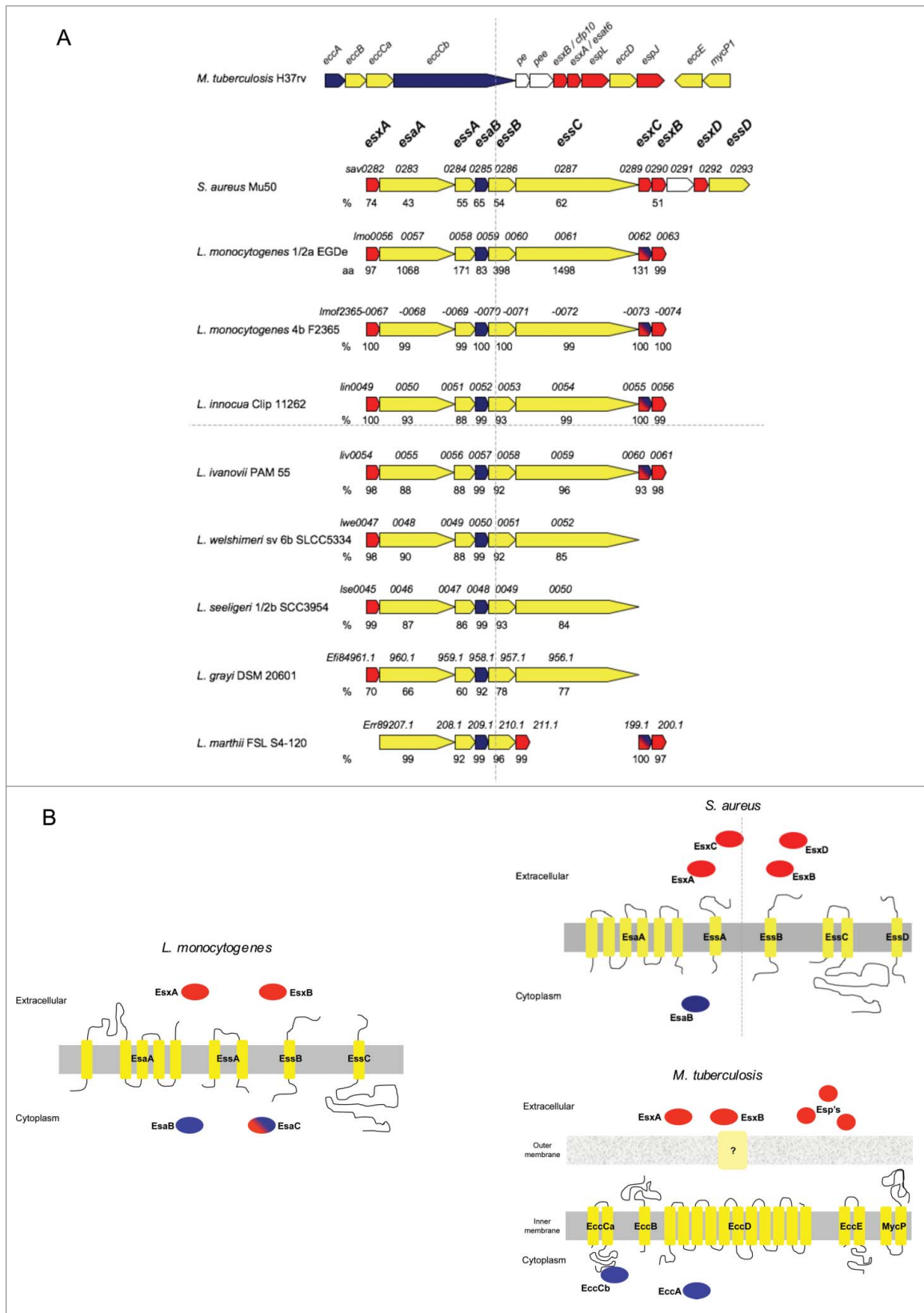
Figure 1. The ESX-1 locus. (A) Comparison of ESX-1 loci of *M. tuberculosis, S. aureus, L. monocytogenes* EGDe, and other *Listeria* species as indicated. Protein homology percentages relative to *L. monocytogenes* EGDe are indicated under each corresponding encoding gene. (B) Schematic representation showing membrane topology or soluble character of proteins encoded by the *L. monocytogenes, S. aureus* and *M. tuberculosis* ESX-1 locus. (A and B) Genes and proteins are colored following the same code: red corresponds to WXG100 encoding genes or proteins predicted to be secreted to the extracellular medium; blue indicates genes predicted as encoding soluble cytoplasmic proteins; yellow is related to genes or proteins predicted as transmembrane proteins.
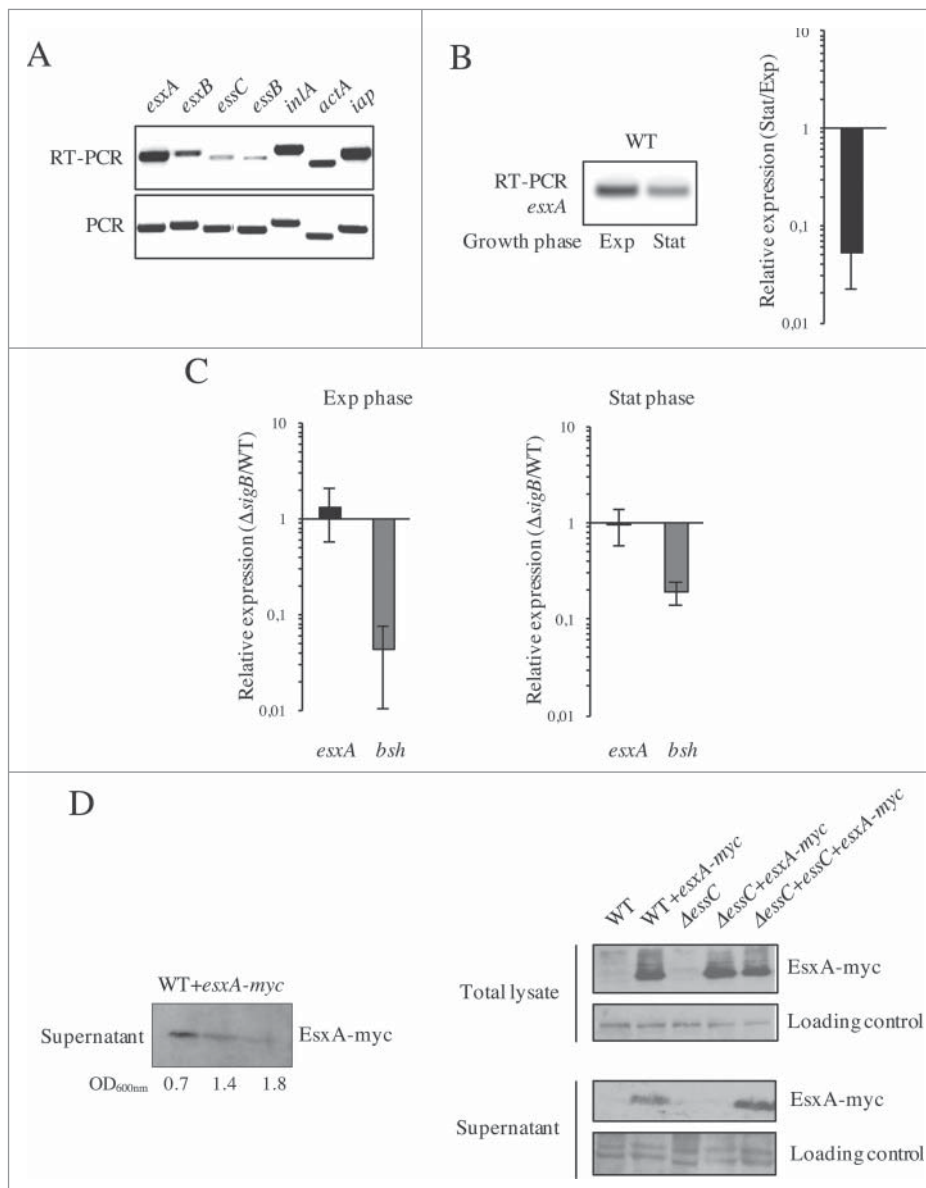
Figure 2. *Lm* ESX-1 system is weakly expressed but functional. (A) Expression of ESX-1 genes in standard growth conditions. The expression of *esxA, esxB, essC* and *essB* was analyzed by RT-PCR on total RNAs extracted from logarithmic cultures grown in BHI at 37°C. *inlA, actA* and *iap* were used as control genes. (B) Expression of *esxA* at exponential (Exp) and stationary (Stat) phase of growth measured by RT-PCR (left panel) and qRT-PCR (right panel). Expression value in stationary phase is expressed relative to the value obtained in exponential growth phase. (C) SigB-independent expression of *esxA*. qRT-PCRs performed on total RNAs extracted from WT and ΔsigB strains at the exponential (left panel) and stationary (right panel) phase of growth in BHI at 37°C. *bsh was* used as control gene whose expression is SigB-dependent. Gene expression levels in the ΔsigB mutant were normalized to those in the WT. (B and C) Values are mean ± SD (n = 3). (D) Secretion of EsxA is dependent on EssC. Detection of myc-tagged EsxA protein (EsxA-myc) in supernatants of *Lm* EGDe+esxA-myc (WT+esxA-myc) at different stages of growth (OD_{600nm} = 0.7, 1.4 and 1.8) (Left panel), and in total bacterial lysates and supernatants from WT, WT+esxA-myc, ΔessC, ΔessC+esxA-myc and ΔessC+essC+esxA-myc strains in exponential growth phase (Right panel).

conditions. We then expressed EsxA-myc in the ΔessC and ΔessC+essC strains and performed the same western blot analysis on proteins from the WT, WT+esxA-myc, ΔessC, ΔessC+esxA-myc and ΔessC+essC+esxA-myc total bacterial lysates and culture supernatants in exponential growth phase. In the WT+esxA-myc strain, EsxA-myc was detected both in the total lysates and culture

supernatants, indicating that the protein is produced and secreted (Fig. 2D right panel). In the ΔessC+esxA-myc strain, EsxA-myc was absent from the supernatant and retained in total lysates, demonstrating that EsxA is secreted in an EssC-dependent manner. This was confirmed by the complementation of the ΔessC+esxA-myc mutant that restored the secretion of EsxA-myc

($\Delta essC+essC+esxA-myc$ line). Altogether, these results show that Lm expresses a functional ESX-1 secretion system, albeit at low levels, and that EsxA secretion requires the putative membrane ATPase EssC.

Lm *ESX-1 is dispensable for host cell invasion and intracellular multiplication*

To investigate the role of the ESX-1 system in Lm cell invasion and intracellular multiplication, we constructed deletion mutants for $esxA$, that we showed to encode a substrate of Lm ESX-1, and $esxB$ that encodes another putative ESX-1 substrate.[7] Mutants were confirmed by PCR and sequencing, and their growth rates observed in BHI at 37°C were comparable to that of the WT (Fig. S1A-B).

The WT, $\Delta esxA$, $\Delta esxB$ and $\Delta essC$ strains were tested for their capacity to invade epithelial cell lines in which Lm entry is mainly mediated by internalin A (InlA) (Caco-2) or InlB (Vero). No significant difference in invasion was observed between mutant and WT bacteria in both cell lines (Fig. 3A), suggesting that the ESX-1 system is not required for Lm invasion of epithelial cells.

To analyze the role of ESX-1 in Lm intracellular multiplication, the behavior of the WT, $\Delta esxA$, $\Delta esxB$ and $\Delta essC$ strains was studied after internalization in J774 murine macrophage-like cells. All strains grew with similar multiplication rates after uptake (Fig. 3B), indicating that none of these genes is required for Lm intracellular replication in macrophage-like cells. Altogether these results indicate that the ESX-1 secretion system is dispensable for Lm cell invasion and intracellular multiplication.

ESX-1 activity impairs Lm *infection*

To analyze the involvement of the ESX-1 system in Lm infection *in vivo*, we monitored the number of bacteria in the liver and spleen of mice infected intravenously with $\Delta esxA$, $\Delta esxB$, $\Delta essC$ or WT bacteria. Unexpectedly, 72h post-infection, all the mutant strains showed a slight increase in bacterial counts in both organs as compared to WT, which was statistically significant for $\Delta esxB$ (Fig. 4A), implying that ESX-1 activity might have a negative impact on Lm infection.

To investigate the potential role of ESX-1 in the gastrointestinal phase of the infectious process, we performed oral inoculation of mice with the WT, $\Delta esxA$, $\Delta esxB$ and $\Delta essC$ strains. Three days post-inoculation, mutant strains appeared again to be slightly more virulent than the WT. This increased infection was statistically significant for $\Delta esxA$ in mouse livers (Fig. 4B).

These data indicate that neither the ESX-1 apparatus nor its substrates are crucial for Lm infection in the mouse model. Conversely, they suggest that the expression/function of this secretion system causes an adverse effect in Lm pathogenicity.

Overexpression of esxA *in the context of a functional ESX-1 system is detrimental to* Lm *infection*

To further investigate if the ESX-1 function could have a negative effect on Lm infection, we performed intravenous infection of mice with Lm overexpressing $esxA$ ($+esxA$), together with the WT and $\Delta esxA$ strains. The $esxA$ overexpression in the $+esxA$ strain was first confirmed by qRT-PCR (Fig. S2A). Growth rate of the $+esxA$ strain in BHI or minimal medium at 37°C, as well as its cell adhesion and infection capacity were comparable to that of the WT strain (Fig. S2B-C), indicating that $esxA$ overexpression has no significant impact on Lm growth and cellular infectious properties. Three days post infection the $\Delta esxA$ mutant appeared slightly more virulent as compared to the WT, as already observed (Fig. 4A), whereas $+esxA$ bacteria showed a significant number decrease in both mouse organs (Fig. 5A). Inversely, $esxA$ overexpression had no effect on the phenotype of a $\Delta essC$ mutant that, similarly to the $\Delta esxA$ mutant, also appeared to colonize more efficiently mouse organs than the WT strain (Fig. 5B). These results demonstrate that the detrimental effect of ESX-1 on Lm pathogenicity is due to EsxA secretion and depends on a functional ESX-1 machinery.

The production of IFN-γ and TNF-α by immune cells promotes bacterial clearance and is critical in controlling primary *L. monocytogenes* infections.[26] To investigate if the adverse effect of $esxA$ overexpression on Lm infection is related with higher levels of host IFN-γ and/or TNF-α, we analyzed by qRT-PCR levels of IFN-γ and TNF-α transcripts in the liver of WT or $+esxA$-infected mice. No significant difference was observed regarding expression levels of IFN-γ and TNF-α (Fig. 5C). In addition, to discard any role of IFN-γ in the increased resistance of mice to $esxA$ overexpressing Lm, WT and IFN-γ knockout mice (IFN-$\gamma^{-/-}$) were intravenously infected with WT or $+esxA$ bacteria. Three days post-infection, bacterial loads were overall higher in the organs of IFN-$\gamma^{-/-}$ than in WT mice (Fig. 5D). These data indicated that IFN-γ deficient mice are more susceptible to Lm infection and confirmed the role of IFN-γ in the immune response against Lm.[27] In line with data observed in BALB/c mice (Fig. 5A), the $+esxA$ strain showed significant infection attenuation in both organs of WT mice as compared to Lm WT bacteria (Fig. 5D). However, this infection defect was similar in IFN-γ deficient animals,
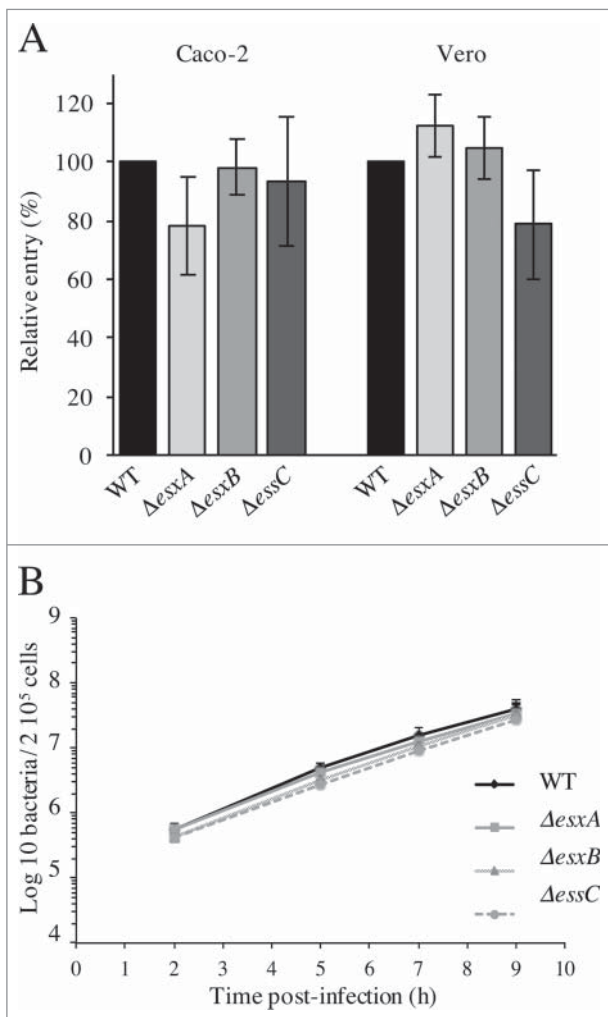
Figure 3. The *Lm* ESX-1 secretion system is dispensable for epithelial cell invasion and intracellular multiplication in macrophages. (A) Entry of the WT, ΔesxA, ΔesxB and ΔessC into Caco-2 and Vero cell lines. Values are expressed relative to WT values arbitrarily fixed to 100%. (B) Intracellular replication behavior of the WT, ΔesxA, ΔesxB and ΔessC strains in J774 cells. Values are mean ± SD (n = 3).

Figure 4. *Lm* ESX-1 secretion system is detrimental for *in vivo* infection. Bacterial counts for the WT, ΔesxA, ΔesxB and ΔessC strains, in spleens and livers of BALB/c mice (n = 5), 72h after (A) intravenous infection with 10^4 bacteria or (B) oral infection with 10^9 bacteria. Data are presented as scatter plots, which each animal represented by a dot and the mean is indicated by a horizontal line. *, $p \leq 0.05$.

suggesting that IFN-γ is not involved in the increased resistance of mice to *Lm* overexpressing *esxA*.

Discussion

The analysis of the bacterial ability to secrete proteins to or beyond their surface is crucial in the understanding of bacterial pathogenesis. In *M. tuberculosis* and *S. aureus,* ESX-1 and its substrates were shown to play an important role in virulence.[5,7] ESX-1 appears to be very conserved in *Listeria*, in particular among pathogenic species, and only partially present in the majority of non-pathogenic *Listeria* species.
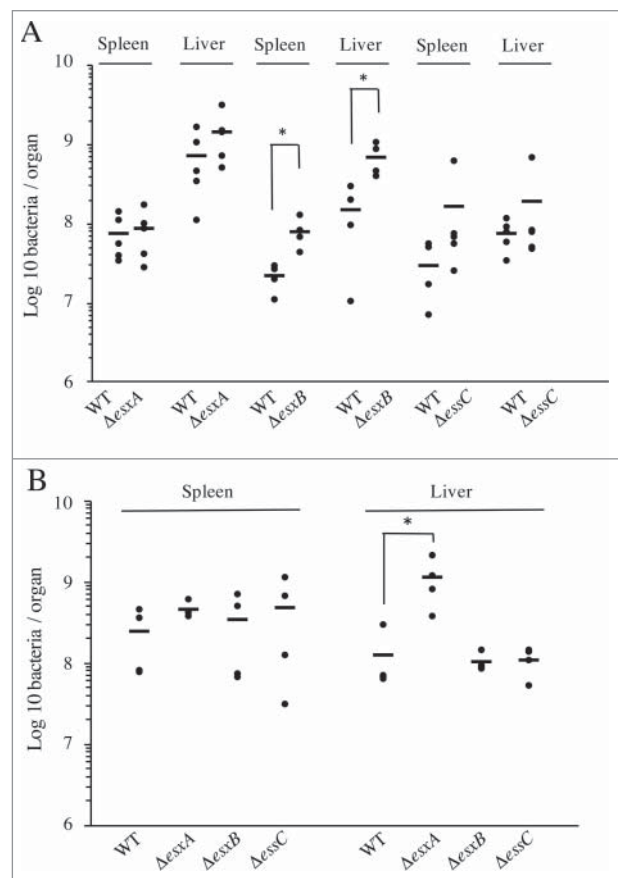
We showed that the ESX-1 locus of *L. monocytogenes* EGDe is expressed in standard growth conditions, with *esxA* appearing more expressed than *esxB*, *essB* and *essC*. These results are in agreement with previous transcriptional analyses[28] and are consistent with the presence of a transcription terminator between *esxA* and *esaA*. This could suggest a different transcriptional regulation between *esxA* and the other ESX-1 genes. Regarding the expression of ESX-1 genes in different conditions, no change was observed when bacteria were grown at 37°C, 25°C or 7°C,[29,30] nor in presence of 6% NaCl,[29] nor when grown at pH 5,[31] nor when grown in culture media supplemented with glucose, cellobiose or glycerol.[32,33] In addition, as compared to bacteria grown in BHI at 37°C, the expression of the ESX-1 locus appears also unchanged in *Listeria* recovered from infected murine macrophages,[34,35] or from mouse intestinal lumen[36] and spleens.[37] Interestingly, the entire ESX-1 locus was shown as up-regulated after incubation of *Lm* in human blood,[36]
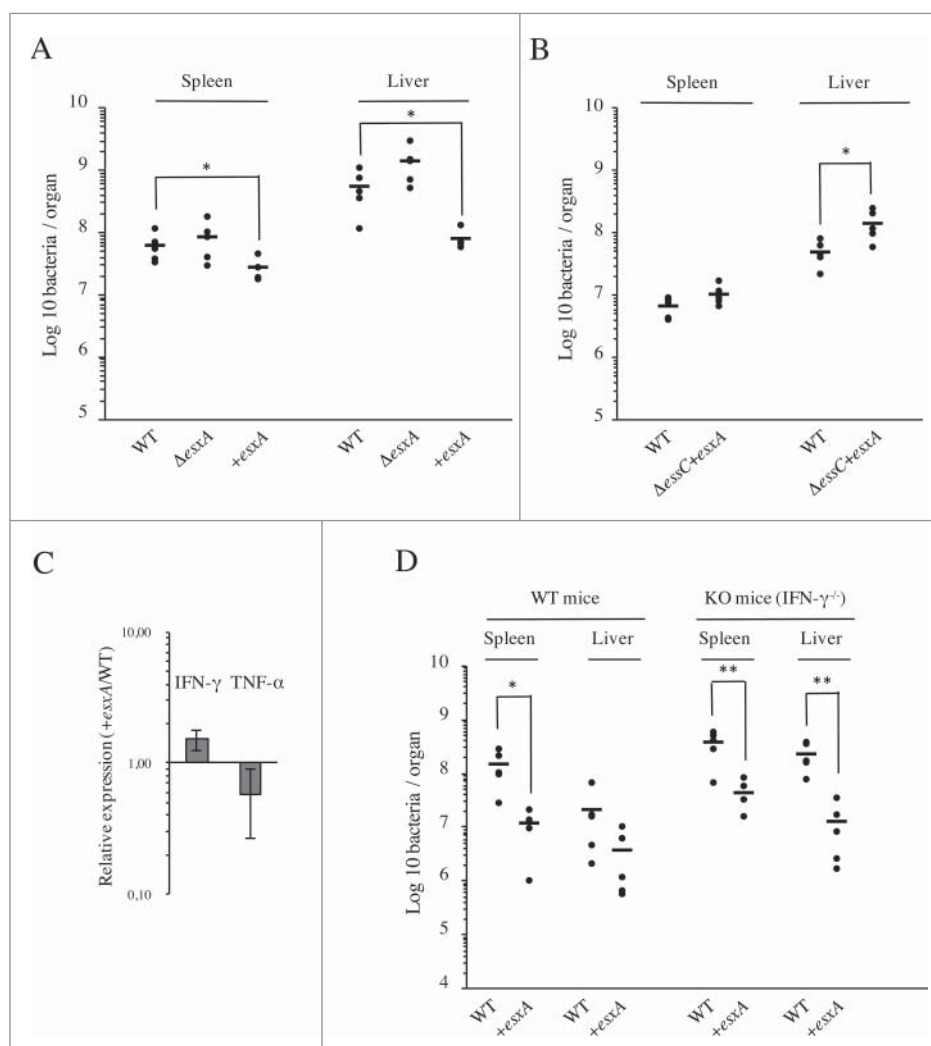
Figure 5. EsxA secretion impairs *Lm* infection *in vivo* through an IFN-γ-independent mechanism. (A) Bacterial counts for WT, Δ*esxA* and +*esxA* strains, in spleens and livers of BALB/c mice (n = 5), 72h after intravenous infection with 10⁴ bacteria. (B) Bacterial counts for WT and Δ*essC*+*esxA* strains, in spleens and livers of BALB/c mice (n = 5), 72h after intravenous infection with 10⁴ bacteria. (C) Levels of IFN-γ and TNF-α transcripts measured by qRT-PCR in livers of mice 72h after intravenous infection with either WT or +*esxA* bacteria. Expression levels in +*esxA*-infected livers were normalized to those infected with the WT strain. Values are mean ± SD (n = 3). (D) Bacterial counts for WT and +*esxA* strains in spleens and livers of WT and IFN-γ knock-out mice (n = 5), 72h after intravenous infection with 10⁴ bacteria. *, $p \leq 0.05$; **, $p \leq 0.01$.

and *essC* and *esxC* were also shown as upregulated during cell infection.[34] Altogether, these results indicate that the ESX-1 locus of *L. monocytogenes* EGDe is poorly or not expressed in most of the conditions, with some genes of the locus expressed in few conditions that could suggest a role of the ESX-1 apparatus in these specific environments.

Using a deletion mutant and complemented strain for *essC*, we demonstrated that ESX-1 is functional in *Lm*, at least for the secretion of EsxA. Even if EssC was shown to be an essential ESX-1 element also for the secretion of EsxB in *M. tuberculosis* and *S. aureus*,[5,7] the EssC-dependent secretion of EsxB remains to be confirmed in *Lm*. EsxA and EsxB are the only WXG100 proteins predicted to be encoded by the *Lm* genome. In addition to EsxA and EsxB, the EsxC protein is also a substrate for ESX-1 in *S. aureus* and confers pathogenic function to this bacterium.[11] However, an *esxC* ortholog is absent from *Lm*. Instead, an unrelated gene of unknown function (*lmo0062*) occupies the *esxC* position in the *Lm* genome (Fig. 1). Interestingly, Lmo0062 was recently predicted to be secreted,[38] therefore investigating its capacity to be secreted in an ESX-1-dependent manner could widen the spectrum of ESX-1 substrates to proteins that do not belong to the WXG100 family.

All the above observations induced a strong presumption for the involvement of ESX-1 in *Lm* pathogenicity. However, we demonstrated that this secretion system and its substrates are not required for *Lm* cell invasion, intracellular multiplication and *in vivo* infection. In agreement with our results, EsxA

was also previously shown dispensable for *Lm* mouse infection.[23] Unexpectedly, we observed a slight increase in the infection level of mutants for ESX-1 components as compared to WT bacteria. This was also previously observed for a Δ*esxA* mutant,[23] suggesting a damaging role for ESX-1 in *Listeria* infectious capacity. In agreement with this hypothesis, overexpression of *esxA* resulted in a decrease infection of *Lm* in the mouse model, confirming the adverse effect of a functional ESX-1 secretion system. Despite our attempts to elucidate the reasons of this detrimental role, we failed to find differences regarding host immune responses upon infection by WT or *esxA* overexpressing *Lm*. ESAT-6 (the mycobacterial EsxA homolog) was shown to play a pro-apoptotic role in *M. tuberculosis*.[39] A comparable role of *Lm* EsxA could result in increased bacterial recognition and clearance by the host immune system that would explain the phenotype of *esxA* overexpressing bacteria. The absence of EssC in the bacterial membrane could also disturb cell envelope homeostasis, possibly resulting in the mislocalization of some surface proteins that could induce an increased virulence. However, no difference was observed regarding the capacity of mutants for ESX-1 components to invade or multiply inside host cells as compared to WT *Lm*.

L. monocytogenes is sensitive to a broad range of antibiotics. However, resistance to several antibiotics has been reported,[40] as well as multidrug-resistant strains.[41] The ESX-1 system could appear as a potential target for innovative anti-*Listeria* drugs that, by inducing ESX-1-dependent secretion, would be capable to impair the infectious capacity of bacteria but not their viability, significantly reducing the risk of resistance development.

Listeria has maintained this locus in its genome, suggesting that it may probably be helpful in certain conditions, such as resisting to stress encountered in specific environments. However, we were unable to find any difference between the WT and *essC* mutant regarding growth in stress conditions such as low pH (pH 5.5) and high salt concentration (4.5% NaCl) (Fig. S1C). Another reason for the conservation of this locus in the *Listeria* genome could be related to a strain issue. Indeed, we tested here the role of the ESX-1 system in only one specific *Lm* strain (*L. monocytogenes* EGDe), that is one of the most commonly used laboratory strains.[42,43] Even if EsxA was also shown to be dispensable for *in vitro* and *in vivo* growth of a different widely used *Lm* strain,[23,43] this locus could play important roles in the infection capacity of other *Listeria* strains from different serotypes.

In summary, we demonstrated here that the *Lm* genome encodes a functional ESX-1 secretion system required for the secretion of WXG100 proteins, such

as EsxA. In addition, despite poorly expressed and dispensable for cell invasion, we showed that a working ESX-1 system is detrimental for *Lm* infection *in vivo*. Considering its wide distribution among Gram-positive bacteria and the lack of a convergent phenotypic trait for mutants in this pathway, ESX-1 certainly fulfils different functions that remain to be elucidated.

Materials and methods

Bacterial strains and media

Lm EGDe (ATCC-BAA-679) and *E. coli* strains were routinely cultured aerobically at 37 °C in brain heart infusion (BHI, Difco) and Lysogeny Broth (LB) media, respectively, with shaking.

The synthetic minimal medium (MM) was prepared as previously described.[44] When appropriate, the following antibiotics were included in culture media as selective agents: ampicilin (Amp), 100 μg/ml; chloramphenicol (Cm), 7 μg/ml (*Lm*) or 20 μg/ml (*E. coli*); erythromycin (Ery), 5 μg/ml. For genetic complementation purposes, colistin sulfate (Col) and nalidixic acid (Nax) were used at 10 and 50 μg/ml, respectively.

Construction and complementation of mutant strains

lmo0056, lmo0061 and *lmo0063* deletions were performed in the EGD-e background through a process of double homologous recombination mediated by the suicide plasmid pMAD as described[45] using the corresponding oligonucleotides (A-D; Table S1). Genetic complementation of the deletion mutant strains was performed using the phage-derived integrative plasmid pPL2 as described[45] using the respective oligonucleotides (Table-S1). For overexpression, target genes were cloned into the pRB474 vector as described.[46] All plasmid constructs and strains were confirmed by PCR and DNA sequencing.

Western blot analysis of EsxA

Overnight bacterial cultures grown in static conditions (final OD_{660} ∼1.0) were centrifuged (10 000 g, 10 min, 4°C) and the supernatant filtered using Millipore 0.45 μm filters. A volume of 1.6 ml of 50% trichloroacetic acid was added to 6 ml of the filtered supernatant and incubated for 1 h at 4°C. The sample was centrifuged (30 000 g, 20 min, 4°C) and the pellet washed with cold acetone, repeating the centrifugation step in same conditions. The pellet was dried, suspended in 30 μl of PBS pH 7.4 and mixed with 15 μl of 4x Laemmli buffer. A volume of 15 μl was loaded in a 12% SDS-polyacrylamide gel. For

total bacterial lysates, the bacterial pellet from 10 ml of culture was suspended in 0.8 ml of PBS pH 7.4 containing 100 μg/ml DNase and protease inhibitors. Bacteria were lysed in a FastPrep-24 homogenizer (MP Biomedicals) (30 s, maximum speed) and cell debris removed by centrifugation (3 000 g, 5 min, 4°C). A volume of 40 μl of 4x Laemmli buffer was added to 100 μl of supernatant of bacterial lysates, and 10 μl loaded into the gels. Western blotting was performed as described[46] using anti-Myc tag mouse antibody (#clone 9B11, Cell Signaling #2276).

Gene expression analyses

Bacterial RNAs were isolated from 10 ml of cultures at the desired growth phase. For quantification of cytokine expression in mouse livers, organs were homogenized in RNAlater stabilization solution (Qiagen), quick-frozen in dry ice and stored at -80°C. Total RNAs were extracted by the phenol-chloroform method as previously described,[47] and treated with DNase I (Turbo DNA-free, Ambion) as recommended by the manufacturer. Purified RNAs (1 μg) were reverse-transcribed with random hexamers, using iScript cDNA Synthesis kit (Bio-Rad Laboratories). For qualitative analysis, PCR was performed in 20-μl reactions containing 2 μl of cDNA, 10 μl of MangoMix 2 $\times$ reaction mix (Bioline) and 0.5 μM of forward and reverse primers (Table S1), using the following protocol: 1 cycle at 95 °C (5 min), 25 cycles at 95 °C (30 s), 55 °C (30 s) and 72 °C (20 s), and 1 cycle at 72 °C (5 min). Amplification products were resolved in 1% (w/v) agarose gel and analyzed in a GelDoc XR+ System (Bio-Rad Laboratories). Quantitative real-time PCR (qRT-PCR) was performed in 20-μl reactions containing 2 μl of cDNA, 10 μl of SYBR Green Supermix (Bio-Rad Laboratories) and 0.25 μM of forward and reverse primers (Table S1), using the following cycling protocol: 1 cycle at 95 °C (3 min) and 40 cycles at 95 °C (30 s), 55 °C (30 s) and 72 °C (30 s). Each target gene was analyzed in triplicate and blank (water) and DNA contamination controls (unconverted DNase I-treated RNA) were included for each primer pair. Amplification data were analyzed by the comparative threshold ($\Delta\Delta$Ct) method, after normalization of the test and control sample expression values to a housekeeping reference gene (16S rRNA).

Adhesion and invasion assays

Adhesion and invasion assays were performed as described.[45] Briefly, Caco-2 (ATCC, HTB-37) and Vero (ATCC, CCL-81) cells were seeded (in triplicate) per 24-well plates ($\sim$2 $\times$ 10^5/well) in EMEM 20% foetal bovine serum and DMEM 10% foetal bovine serum, respectively (LONZA), and propagated for

48 h. *Listeria* were grown in BHI to $OD_{600nm} = 0.8$, washed and inoculated at 50 bacteria-per-cell for 1h. For adhesion, cells were washed 3 times, lysed in 0.2% Triton X-100 and viable bacteria were enumerated after plating serial dilutions of the lysates in BHI agar media. For invasion assays, cells were infected for 1h and treated with 20 μg/ml gentamicin for 1h30 before lysis in 0.2% Triton X-100.

Intracellular multiplication

Mouse macrophage-like J774A.1 cells (ATCC TIB-67) were propagated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and infection assays were performed as described.[37] Briefly, cells ($\sim$2$\times$$10^5$/well) were infected for 45 min with exponential-phase bacteria at ˜10 bacteria/cell and treated afterwards with 20 μg/ml gentamicin for 75 min. At several time-points post-infection, cells were washed with PBS and lysed in cold 0.2% Triton X-100 for quantification of viable intracellular bacteria in BHI agar. One experiment was performed with triplicates for each strain and time-point.

Animal infections

Infections were performed in 6-to-8 week-old specific-pathogen-free females as described.[48] Briefly, wild-type BALB/c (Charles River Laboratories) or wild-type IFN-γ knockout C57BL/6J mice were infected intravenously with 10^4 CFUs in PBS, or starved 12h before gavage inoculation with 10^9 CFUs in PBS containing 150 mg/ml $CaCO_3$. The infection was carried out for 72 h, at which point the animals were euthanized by general anesthesia. The spleen and liver were aseptically collected, homogenized in sterile PBS, and serial dilutions of the organ homogenates plated in BHI agar. Mice were maintained at the IBMC animal facilities, in high efficiency particulate air (HEPA) filter-bearing cages under 12-h light cycles, and were given sterile chow and autoclaved water *ad libitum*.

Ethics statement

All the animal procedures were in agreement with the guidelines of the European Commission for the handling of laboratory animals (directive 2010/63/EU), with the Portuguese legislation for the use of animals for scientific purposes (Decreto-Lei 113/2013), and were approved by the IBMC Animal Ethics Committee, as well as by the Direcção Geral de Veterinária, the Portuguese authority for animal protection, under license PTDC/SAU-MIC/111581/2009.

Statistical analyses

Statistical analyses were performed with Prism 6 (Graph-Pad Software). Unpaired two-tailed Student's t-test was used to compare the means of 2 groups; one-way ANOVA was used with Tukey's post-hoc test for pair-wise comparison of means from more than 2 groups, or with Dunnett's post-hoc test for comparison of means relative to the mean of a control group. Mean differences were considered statistically non-significant (ns) when p value was above 0.05. For statistically significant differences: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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