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# UNRAVELLING NEW INNATE IMMUNE RESPONSES TO *LISTERIA MONOCYTOGENES* INFECTION

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# UNRAVELLING NEW INNATE IMMUNE RESPONSES TO *LISTERIA MONOCYTOGENES* INFECTION

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O autor esclarece que na elaboração desta tese foram incluídos dados da publicação abaixo indicada, e declara ter participado activamente na concepção e execução das experiências que estiveram na origem da mesma, assim como na sua interpretação, discussão e redacção.

The author clarifies that this thesis includes data from the publication listed below, and declares that she participated actively in the conception and execution of the experiments that produced such data, as well as in their interpretation, discussion and writing.

## PUBLICAÇÃO/ PUBLICATION

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# ACKNOWLEDGEMENTS

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# ABSTRACT

*Listeria monocytogenes* (*Lm*) is a major intracellular foodborne pathogen that causes listeriosis, a severe human systemic infection that is rare, but often fatal among immunocompromised hosts. *Lm,* through an arsenal of virulence factors, has the ability to transverse several physiological barriers and multiplies within phagocytic and non-phagocytic cells, to evade the host immune system and effectively disseminate throughout host tissues. In immunocompetent hosts, the innate immune response is able to contain *Lm* expansion and to activate adaptive immunity. Aiming to better understand the *Lm*-host interaction, we explored new host innate immune mechanisms used by the host to clear *Lm* infection, as well as new strategies used by *Lm* to evade the host immune system and promote bacterial multiplication. For this purpose, we assessed the impact of both host Scavenger Receptors (SRs) and new virulence factors in *Lm* pathogenicity.

SRs, an emergent family of conserved pattern recognition receptors, are involved in pathogen infections and play key functions in antimicrobial host immune response. In this work, we found for the first time a role for STABILIN-1 (STAB-1) in pathogen infection. In particular, we show that STAB-1 promotes *Lm* phagocytosis and ensures macrophage membrane integrity. Using a mouse model of infection, we demonstrate that STAB-1 is induced in response to *Lm* infection, regulates inflammatory cytokine production and controls the recruitment of myeloid cells to restrict *Lm* proliferation. We thus propose a new protective role for STAB-1 against bacterial infection.

CadC is the transcriptional regulator of CadA, an efflux pump conferring cadmium resistance. We show that during *in vivo* infection, *Lm* uses CadC to directly repress the expression of the LspB lipoprotein signal peptidase to avoid the exposure of the LpeA lipoprotein to the host immune system, thus diminishing inflammatory cytokine expression and promoting intramacrophage survival and virulence. In addition, we show that CadC controls bile salt hydrolase (BSH) activity and *Lm* resistance to bile by repressing *bsh* expression. We demonstrate that the CadC-independent expression of *bsh* induces the expression of the cholic acid efflux pump MdrT, restricting *Lm* virulence. CadC regulates additional genes, including virulence genes and $\sigma^B$-activated genes during colonization of the host intestinal lumen. Altogether, these data point out CadC as a new general repressor repurposed to fine-tune virulence gene expression over the *Lm* infectious process.

# RESUMO

A *Listeria monocytogenes* (*Lm*) é um agente intracelular patogénico transmitido através de alimentos contaminados e responsável por uma infeção sistémica humana grave, embora rara, geralmente fatal em hospedeiros imunocomprometidos, a listeriose. A *Lm,* através da expressão de inúmeros factores de virulência, tem a capacidade de atravessar diferentes barreiras fisiológicas e multiplicar-se em células fagocíticas e não fagocíticas, de forma a evitar a resposta imune gerada pelo hospedeiro, e assim disseminar-se ao longo dos tecidos. Em indivíduos imunocompetentes, a resposta imune inata é capaz de limitar a expansão de *Lm* e ativar a imunidade adaptativa. Com o objetivo de compreender melhor a interação entre a *Lm* e o hospedeiro, explorámos novos mecanismos imunológicos utilizados pelo hospedeiro para eliminar a infecção por *Lm*, bem como novas estratégias usadas pela *Lm* para contornar o sistema imunológico do hospedeiro e promover a multiplicação bacteriana. Para este propósito, avaliámos o impacto dos "Scavenger receptors (SRs)" e de novos fatores de virulência na patogenicidade de *Lm*.

Os SRs constituem uma família emergente de "pattern recognition receptors" que desempenham funções anti-microbianas cruciais para a resposta imune do hospedeiro. Neste trabalho, revelámos pela primeira vez o papel de STABILIN-1 (STAB-1) em infecções patogénicas. Em particular, mostrámos que STAB-1 promove a fagocitose da *Lm* e garante a integridade da membrana dos macrófagos. Usando murganhos como modelo de infecção, demonstrámos que a expressão de STAB-1 é induzida em resposta à infecção por *Lm*, regula a produção de citoquinas inflamatórias e controla o recrutamento de células mieloides de forma a restringir a proliferação da *Lm*. Deste modo, propomos que STAB-1 constitui um SR com um papel fundamental no controlo da infeção bacteriana.

CadC é um regulador transcricional de CadA, uma bomba de efluxo que confere resistência ao cádmio. Mostrámos que durante a infecção *in vivo*, a *Lm* usa CadC para reprimir diretamente a expressão da lipoproteína de peptidase sinal LspB, de forma a evitar a exposição da lipoproteína LpeA ao sistema imune do hospedeiro, diminuindo assim a expressão de citoquinas inflamatórias e promovendo a sobrevivência intra-macrofágica e a virulência. Além disso, mostrámos que o CadC controla a atividade da hidrolase de sais biliares (BSH) e a resistência da *Lm* à bílis, reprimindo a expressão de *bsh*. Demonstrámos que a expressão de *bsh*, independente de CadC, induz a expressão da bomba de efluxo de ácido cólico, MdrT, restringindo a virulência da *Lm*. CadC regula outros genes, incluindo genes de virulência e genes ativados por σ$^B$ durante a colonização do lúmen intestinal do hospedeiro. Deste modo, CadC atua como um novo repressor reutilizado para afinar a expressão de determinados genes de virulência ao longo do processo infeccioso de *Lm*.

# LIST OF ABBREVIATIONS

**acLDL** – acetylated low-density lipoprotein

**ActA** - actin assembly-inducing protein

**Arp2/3** – actin-related proteins 2 and 3

**ATP** - adenosine triphosphate

**BHI** – brain and heart infusion

**BSA** - bovine serum albumin

**CD** - cluster of differentiation

**CDC** - cholesterol-dependent cytolysin

**cDNA** - complementary DNA

**CLEVER-1** - common lymphatic endothelial and vascular endothelial receptor-1

**CLRs** - C-type lectin receptors

**CpG** - cytosine, phosphodiester link, guanine

**C-terminal** – carboxy-terminal

**CNS** - central nervous system

**CtaP** - cysteine transport associated protein

**DAMPs** - damage-associated molecular patterns

**DCs** - dendritic cells

**DltA** - D-alanine-activating enzyme

**dsRNA** - double-stranded RNA

**DNA** - deoxyribonucleic acid

**Ecad** – E-cadherin or epithelial cadherin

**EGF** - epidermal growth factor

**FbpA** - fibronectin-binding protein

**FEEL** - fasciclin, EGF-like, laminin type EGF-like and link domains

**GAGs** - glycosaminoglycans

**G+C** – guanine and cytosine

**GW** - glycine/tryptophan

**HDL** - high-density lipoprotein

**Hpt** - hexose phosphate transporter

**HGF** - hepatocyte growth factor

**HIV** - human immunodeficiency virus

**IL** – interleukin

**InI** – internalin

**IFN** - interferon

**IR** - inter-repeat

**IRFs** - interferon regulatory factors

**KO** - knock-out

**LAMP** - lysosome-associated membrane protein

**LAP** - *Listeria* adhesion protein

**LDL** - low-density lipoprotein

**LLO** - listeriolysin O

**LOX-1** - lectin-like oxidized LDL receptor 1

*Lm - Listeria monocytogenes*

**LPS** - lipopolysaccharide

**LPXTG** - leucine-proline-unknown-threonine-glycine

**LRR** - leucine-rich repeat domain

**LTA** - lipoteichoic acid

**MAMPs** - microorganism-associated molecular patterns

**MAPK** - mitogen-activated protein kinase

**MARCO** - macrophage receptor with collagenous structure

**MHC** - major histocompatibility complex

**MYD88** - myeloid differentiation primary-response protein 88

**NETs** - neutrophils extracellular traps

**NK** - natural killer cells

**NLRs** - NOD-like receptors

**N-terminal** - amino-terminal

**NF-kB** - nuclear factor kappa B

**oxLDL** - oxidized low-density lipoprotein

**PBS** - phosphate-buffered saline

**PCR** - polymerase chain reaction

**PI3-kinase** - phosphoinositide 3-kinase

**PrfA** - positive regulatory factor A

**qPCR** - quantitative real-time PCR

**RecA** - recombination protein recA

**RLRs** - RIG-I-like receptors

**ROS** - reactive oxygen species

**RNA** - ribonucleic acid

**rRNA** - ribosomal RNA

**RNS** - reactive nitrogen species

**RTKs** - receptor tyrosine kinases

**PAMPs** - pathogen-associated molecular patterns

**PGN** - peptidoglycan

**PL** - placental lactogen

**PRRs** - pattern-recognition receptors

**PS** - phosphatidylserine

**PI-PLC** - phosphatidylinositol-specific phospholipase C

**PplA** - peptide pheromone-encoding lipoprotein A

**SCARA** - scavenger receptor class A

**SigB ($\sigma^B$)** - sigma B

**SLAPs** - spacious *Listeria*-containing phagosomes

**SPARC** - secreted protein acidic and rich in cysteine

**SRs** - scavenger receptors

**SRCL** - scavenger receptor C-type lectin

**SRCR** - scavenger receptor cysteine-rich

**SREC** - scavenger receptor expressed by endothelial cells

**SR-PSOX** - scavenger receptor for phosphatidylserine and oxidized LDL

**ssRNA** - single-stranded RNA

**Tim4** - T-cell immunoglobulin- and mucin-domain-containing molecule

**TLRs** - Toll-like receptors

**TRIF** - TIR domain-containing adaptor protein inducing interferon α/β

**tRNA** - transfer ribonucleic acid

**TNF-α** - tumour necrosis factor alpha

**WT** - wild type

**WTAs** - wall teichoic acids

# CHAPTER I: INTRODUCTION

## A. *LISTERIA MONOCYTOGENES*

### A.1. Historic perspective

It has been over 90 years since *Listeria monocytogenes* (*Lm*) was for the first time identified by E.G.D. Murray and his colleagues, in 1926 (Figure 1A). Murray isolated the bacterium from the liver of sick rabbit upon an epidemic outbreak affecting rabbits and guinea pigs in their laboratory in Cambridge. At the time, they named it *Bacterium monocytogenes*, due to the large numbers of monocytes present in the blood of infected animals (Murray *et al.* 1926). One year later, Pirie and co-workers isolated the same specie in the liver of gerbils in South Africa and renamed it *Listerella hepatolytica*, honouring the father of antiseptic surgery, Lord Joseph Lister (Figure 1A) (Pirie 1927). However, in 1939, the International Committee on Systematic Bacteriology rejected the generic name *Listerella* and Pirie proposed the current name, *Listeria monocytogenes* (Pirie 1940).
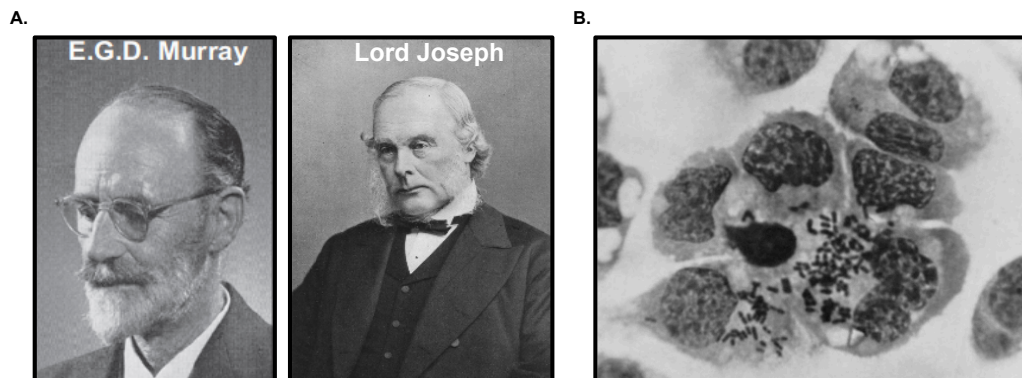


**Fig. 1.** History of *Listeria*. **A**. E.G.D. Murray and Lord Lister portraits (Cossart 2007). **B**. *Lm* resistance to intracellular killing in macrophages (Mackaness 1962).

At that time, human cases were highly sporadic and listeriosis was largely considered as a zoonosis, even upon Nyfeldt had isolated the bacterium from patients with an infectious mononucleosis-like disease (Nyfelt 1929). Later on, Macknass and his colleagues found for the first time that *Lm* was able to resist to intracellular killing in macrophages in a mouse model of infection (Figure 1B). In addition, they have shown that a primary infection by *Listeria* induced a protective cellular immune response against a secondary infection (Mackaness 1962). These pioneering studies made *Listeria* as one of the most intracellular organisms used to study the mechanisms underlying the activation of immune response (Zenewicz *et al.* 2007). It was only in 1981 that listeriosis was related with the consumption of *Listeria* contaminated foodstuffs, after a severe outbreak of the disease with a high mortality rate among the maritime province in Canada (Schlech *et al.* 1983). Afterwards, other food-related outbreaks arose and the disease was recognized as a serious public health concern (Swaminathan *et al.* 2007).

## A.2. Biodiversity, taxonomy and phylogeny

*Listeria* belongs to the Listeriaceae family, which pertains to the order Bacillales, class Bacilli and the phylum Firmicutes of the Bacteria domain. Recent progress on the phylogenetic diversity and taxonomy of the genus *Listeria* has been done. Seeliger and Rocourt have firstly identified five *Listeria* species: *L. innocua* (Seeliger 1981*), L. welshimeri*, *L. seeligeri* (Rocourt *et al.* 1983), *L. ivanovii* (Seeliger *et al.* 1984) and *L. grayi* (Larsen *et al.* 1966). Therefore, two other species were isolated – *L. marthii* and *L. rocourtiae (Graves et al. 2010;* Leclercq *et al. 2010)*; nowadays the *Listeria* genus has expanded and already comprises seventeen distinct species including *L. fleischmannii, L. weihenstephanensis* (Bertsch *et al.* 2013; Lang Halter *et al.* 2013), *L. floridensis*, *L. aquatica*, *L. cornellensis*, *L. riparia*, *L. grandensis, L. booriae* and *L. newyorkensis* (Figure 2A) (Den Bakker *et al.* 2014; Weller *et al.* 2015). Nevertheless, among these species only two of them are considered to be pathogenic: *Lm*, often associated with severe illness both in humans and animals, and *L. ivanovii,* which causes disease in livestock. The remaining species are widespread in nature, being physiologically similar and co-inhabiting in the same environments (Orsi *et al.* 2016).
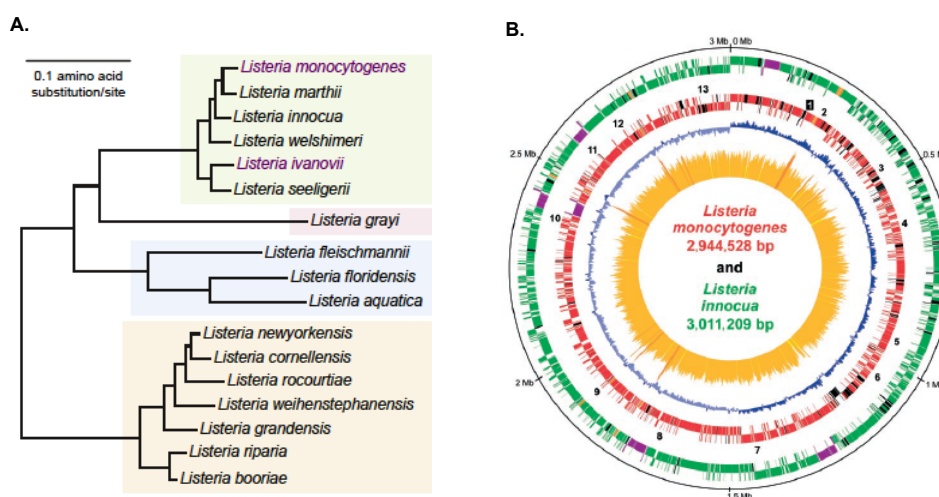


**Fig. 2.** Phylogeny and post-genomics of *Listeria*. **A.** Phylogenetic tree of the genus *Listeria* (Lebreton *et al.* 2016). **B.** Circular genome maps of *Lm* EGD-e and *L. innocua* CLIP 11262: From the outside: Circles 1 and 2, *L. innocua* and *Lm* genes on the plus and minus strands, respectively. Color code: green, *L. innocua* genes; red, *Lm* genes; black, genes specific for *Lm* or *L. innocua*; orange, rRNA operons; purple, prophages. Numbers on the second circle indicate the position of known virulence genes. Circle 3, G/C bias (G+C/G-C) of *Lm*. Circle 4, G+C content of *Lm*. The scale in megabases is indicated on the outside of the genome circles, with the origin of replication at position 0 (Glaser *et al.* 2001).

In 2001, a relevant step forward in *Listeria* research was the publication of the first complete genome sequences of *Lm* EGD-e and the phylogenetically close but non-pathogenic *L. innocua* CLIP 11262 (Figure 2B) (Glaser *et al.* 2001). Few years later, the whole-genome sequence of other *Listeria* species became available (Buchrieser *et al.* 2011; Hain *et al.* 2006; Steinweg *et al.* 2010). Comparative genomics pointed out differences that were

important to understand the phylogenetic relationship of *Listeria* spp. Schimid and his co-workers, through the comparison of multiple virulence-associated *loci* in different species, argued that *L. grayi* was the oldest branch of the genus and lose its pathogenic capacity, while *Lm*, *L. innocua*, *L. ivanovii*, *L. seeligeri* and *L. welshimeri* radiated more recently into two distinct lineages from a common ancestor (Figure 2A). Interestingly, *Listeria* genomes share a highly strong conservation in genome organization (Schmid *et al.* 2005).

## A.3. General features

*Lm* is a Gram-positive bacterium with an extremely high capacity to adapt and survive to a wide range of environmental conditions. It is a small rod-shaped (0.5 x 1-2 µm), non-spore-forming and non-encapsulated bacterium that is usually motile in environments with temperatures above 25 ºC (Seeliger *et al.* 1986). A particularity of this aerobic or facultative anaerobic bacillus is its adaptation capacity to large shifts of temperature (<0 to 45 ºC, with optimal growth at 30-37 ºC), pH (4.3 to 9, being optimal at 7) and osmotic pressure (up to 10% NaCl) (Junttila *et al.* 1988; Shahamat *et al.* 1980). This ubiquitous pathogen sustains a broad array of survival skills to manage life within diverse conditions. Indeed, *Lm* is found throughout the environment in soil, water, vegetation, sewage, animal feces, food and importantly, in several animal species and humans (Orndorff *et al.* 2006). *Listeria* genome is highly stable and conserved, therefore different species contain similar size of circular chromosomes, ranging from 2.8 to 3.0 Mb. The G+C genome content is in average 38% and encodes approximately 2900 open reading frames (Table 1). However, there are intrinsic genomic differences between the *Listeria* genomes, which are closely related to the pathogenicity of the bacterium. In fact, the most essential virulence genes present is *Lm* are all absent from the homologous regions of the non-pathogenic species (Buchrieser 2007; Schmid *et al.* 2005).

**Table 1.** General features of published *Listeria* genome sequences (Buchrieser 2007).

| | *L. monocytogenes* EGD-e (1/2a) | *L. monocytogenes* F2365 (4b) | *L. innocua* CLIP11262 |
|---|---|---|---|
| Size of the chromosome (bp) | 2 944 528 | 2 905 310 | 3 011 209 |
| G+C content (%) | 38 | 38 | 37.4 |
| G+C content protein-coding genes (%) | 38.4 | 38.5 | 38 |
| Total no. of CDS | 2853 | 2847 | 2973 |
| Prophages | 1 | 2 | 5 |
| Plasmids | - | - | 1 (79 CDS) |
| Strain-specific genes | 61 | 51 | 78 |
| Transposons | 1 (Tn916 like) | - | - |
| rRNA genes | 6 | 6 | 6 |
| tRNA genes | 67 | 67 | 66 |

* Abbreviations: bp (base pairs); G+C (guanine and cytosine); CDS (coding sequence)

## B. LISTERIOSIS

### B.1. Successive steps of the infection

Following the consumption of contaminated food products, *Lm* has an advantage of overcoming harsh gastric conditions to reach the intestinal lumen (Figure 3). The transgression of the intestinal barrier by *Lm* is host species-specific and mainly mediated by Internalin A. This bacterial protein interacts preferentially with the E-cadherin (Ecad) exposed around goblet cells, but also with the Ecad expressed by villus epithelial folds and the one surrounding extruded enterocytes at the tip and lateral sides of villi. Once *Lm* is internalized, it is rapidly transcytosed across the intestinal epithelium, being released in the lamina propria (Nikitas *et al.* 2011). Then, *Lm* enters into the bloodstream or lymph and ends up both in liver and spleen, the major target organs for bacterium colonization (Figure 3) (Cossart 2011). About 90% of the bacteria are trapped in the liver due to the high capacity of Kupffer cells to capture them, leading to a decrease on the bacterial load during the first six hours of infection (Conlan *et al.* 1991; Cousens *et al.* 2000; Mackaness 1962). *Lm* infection induces an early necroptotic death of Kupffer cells, which generates an inflammatory response that mobilize monocytes for liver repair (Bleriot *et al.* 2015). Still surviving bacteria replicate within hepatocytes and do spread to nearby cells and tissues, being released into the bloodstream and ultimately causing bacteremia (Vazquez-Boland *et al.* 2001). Importantly, circulating bacteria may also disseminate to secondary target organs and gain access to the central nervous system by transgressing the blood-brain barrier or to the uterus by crossing the placental barrier (Figure 3) (David *et al.* 2017; Lecuit 2005; Radoshevich *et al.* 2017; Vazquez-Boland *et al.* 2001).



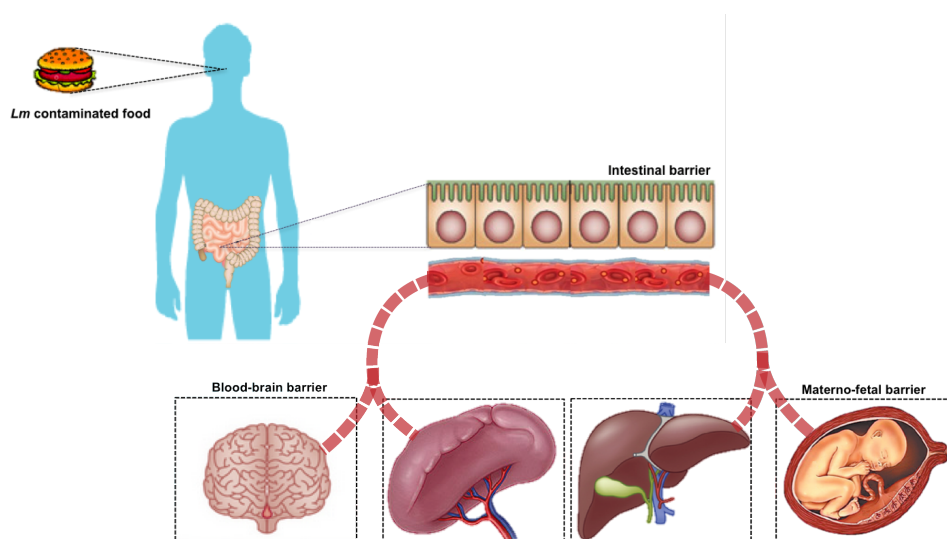**Fig. 3.** Successive steps of human listeriosis and the major organs affected by the disease. Following the ingestion of contaminated food, *Lm* translocates across the intestine, it enters into the bloodstream to travel to the liver and spleen. *Lm* could also gain access to the central nervous system by transgressing the blood-brain barrier or to the uterus by crossing the placental barrier.

**B.2. Clinical features, risk groups and treatment**

The capacity of the pathogen to cause disease depends on the bacterial load, on its own pathogenic potential and importantly, on the immunological status of the host. There are two different forms of illness caused by *Lm:* a non-invasive and an invasive form. In immunocompetent hosts, the non-invasive listeriosis can be asymptomatic or manifests itself as a typical febrile gastroenteritis, being usually a self-limiting infection. However, there are specific health conditions and groups of individuals that manifest higher susceptibility to develop the invasive form of listeriosis, including elderly, pregnant women, neonates and patients carrying diabetes *mellitus*, alcoholism, autoimmune diseases, HIV and those ones that receive immunosuppressive drugs (Allerberger *et al.* 2010; Swaminathan *et al.* 2007; Vazquez-Boland *et al.* 2001). Indeed, the primordial host defence against listeriosis is the cell-mediated immunity and therefore T-cell deficient hosts are highly prone to be infected (Zenewicz *et al.* 2007). Among these high-risk groups, listeriosis manifests as a bacteraemia that may further evolve to a septicaemia or to local organ infections, in particular in the central nervous system (CNS) or in the fetoplacental system (Allerberger *et al.* 2010). In non-pregnant adults, the most frequent form of infection, ranging from 55% to 70% of the overall cases, affects the CNS due to the tropism of *Lm* for the brain tissues. In this case, meningitis and also meningoencephalitis are the most common manifestations of the disease, often accompanied by severe changes in consciousness, movement disorders and less frequently, paralysis of the cranial nerves (Nieman *et al.* 1980; Vazquez-Boland *et al.* 2001). Listeriosis in the course of pregnancy is a serious threat to the foetus (Madjunkov *et al.* 2017). One-third of the materno-fetal cases result in abortions or stillbirth, mainly during the third trimester of pregnancy, when T-cell immunity is impaired (Al-Tawfiq 2008). *Lm* is able to go through the placenta from the maternal blood to the foetus, leading to the development of the materno-fetal infection. This early-onset neonatal listeriosis can cause abortion, birth of stillborn foetus or generalized infection in newborns (sepsis) and meningitis. Less frequent is the late-onset manifestation of the disease, which may develop in week-old neonates, probably upon having in contact with contaminated mother fluids during childbirth (Allerberger *et al.* 2010; Vazquez-Boland *et al.* 2001).

Although the intrinsic resistance to antibiotics is a serious therapeutic problem nowadays, the most clinically effective therapy to treat listeriosis involves the administration of antibiotics (Krawczyk-Balska *et al.* 2016). The combination of the β-lactams penicillin and ampicillin with the aminoglycoside gentamicin is so far, the best alternative. Nevertheless, these β-lactams are bacteriostatically efficient against *Lm,* reinforcing the importance of host defence mechanisms. Other antimicrobial drugs including erythromycin, vancomycin, trimethoprim/ sulfamethoxazole or fluoroquinolones can be used as an alternative for patients allergic to β-

lactams. Pregnant women should not be treated with gentamicin due to the teratogenic effects on the foetus (Allerberger *et al.* 2010).

## B.3. Epidemiology

It has been over almost four decades since listeriosis was associated with the consumption of *Listeria* contaminated food (Schlech *et al.* 1983). In the past few years, the number of *Lm* foodborne outbreaks has been increasing, in particular in developed countries, due to the globalization of food industry, as well as the recurrence to ready-to-eat products. Nevertheless, several cases of zoonosis have also been reported, affecting many species of animals, including domestic pets, livestock, rodents, fish and amphibians (Allerberger *et al.* 2010; Gandhi *et al.* 2007; Goulet *et al.* 2008). *Lm* has a great economical impact on food industry due to its ability to survive to the most common food-preserving methods (refrigeration and acidic or salty treatments). In addition, the bacterium persists in raw and processed food environments, such as meat, seafood, fruits, vegetables, dairy products (e.g. cheese, ham) and unpasteurized milk (Swaminathan *et al.* 2007). *Lm* is also able to produce a biofilm as a survival strategy, therefore it is important to control biofilm formation to diminish the prevalence of the bacterium in foodstuff (Zhu *et al.* 2017).

Listeriosis is a rare disease, with an incidence of 1-10 cases per million people reported every year (Denny *et al.* 2008; Goulet *et al.* 2008; Lomonaco *et al.* 2015). The rate of listeriosis has steadily increased during 2010–2014 in European Union countries. In 2010 approximately 23.150 cases of listeriosis were estimated worldwide (de Noordhout *et al.* 2014). In 2014, 2.194 confirmed cases of listeriosis were reported by 28 European Union countries, with an overall rate of 0.6 per 100.000 people. Germany and France had the highest numbers of reported cases (44.3% of all cases) (ECDC 2016). Listeriosis is an overall public health concern once it is associated with high hospitalization and mortality rates, being one of the most deadly food-borne infections, with an average rate of deaths around 20-30% (Table 2).

**Table 2.** Reported and hospitalized human cases of zoonosis in the European Union 2014 (EFSA 2015).

| Disease | Number of confirmed human cases | Reported hospitalised cases | Reported deaths |
|---|---|---|---|
| Campylobacteriosis | 236.851 | 18.303 | 25 |
| Salmonellosis | 88.715 | 9.830 | 65 |
| Yersiniosis | 6.625 | 442 | 5 |
| VTEC infections | 5.955 | 930 | 7 |
| Listeriosis | 2.161 | 812 | 210 |
| Brucellosis | 347 | 142 | 0 |
| Trichinellosis | 319 | 150 | 2 |

* Abbreviations: VTEC (Verocytotoxin - producing Escherichia coli)

*Listeria* classification is based on the serotyping of somatic (O) and flagellar (H) antigens. According this method, *Lm* comprises 13 known serotypes, but serotypes 1/2a, 1/2b, and 4b account for more than 95% of human reported cases of listeriosis (Goulet *et al.* 2008; Seeliger *et al.* 1979; Seeliger *et al.* 1989). In Portugal, listeriosis has been notifiable since April 2014, but there is no active surveillance program for the disease (Magalhaes *et al.* 2015).

## B.4. Cell biology of infection

The potential of *Lm* to cause and establish infection within different tissues is related with its ability to invade and replicate in both phagocytic (macrophages, neutrophils and dendritic cells) and non-phagocytic host cells (epithelial and endothelial cells, enterocytes, fibroblasts and hepatocytes) (Cossart *et al.* 2008; Pamer 2004). When *Lm* encounters a eukaryotic host cell, it intimately associates to its surface through the expression of specific adhesins, thereby inducing its own internalization (Figure 4). The invasion of a non-phagocytic cell is mediated by a zipper-like mechanism, where the bacterium is gradually involved by host cell membrane until being confined in a vacuole (Figure 4). This process implies the engagement of host membrane receptors with bacterial invasins, which triggers different intracellular signalling pathways that induce cytoskeletal and membrane rearrangements. Then, *Lm* is able to acidify and disrupt the vacuole, reach the host cytoplasm and actively replicate using the available cytoplasmic nutrients of the cell (Figure 4). Cytosolic bacteria polymerize actin, which confers to the pathogen intracellular and intercellular motility. When *Lm* randomly reaches the cell periphery, it pushes the cell membrane leading to the formation of a double membrane protrusion that culminates with the formation of a secondary vacuole in adjacent cells (Figure 4). Afterwards, *Lm* rapidly escapes from the newly formed vacuole by lysing the double membrane and it becomes free in the cytosol to travel through neighbouring cells without being exposed to the extracellular milieu (Figure 4) (Cossart *et al.* 2008; Vazquez-Boland *et al.* 2001).

## B.5. Major virulence factors

The successful achievement of each step of *Lm* intracellular life cycle is largely dependent on the proper spatiotemporal activation of a complex network of virulence factors (Figure 4). This section contains a brief description of the most relevant virulence factors that contribute for the progression of *Lm* infection cycle, as well as their respective regulatory mechanisms.
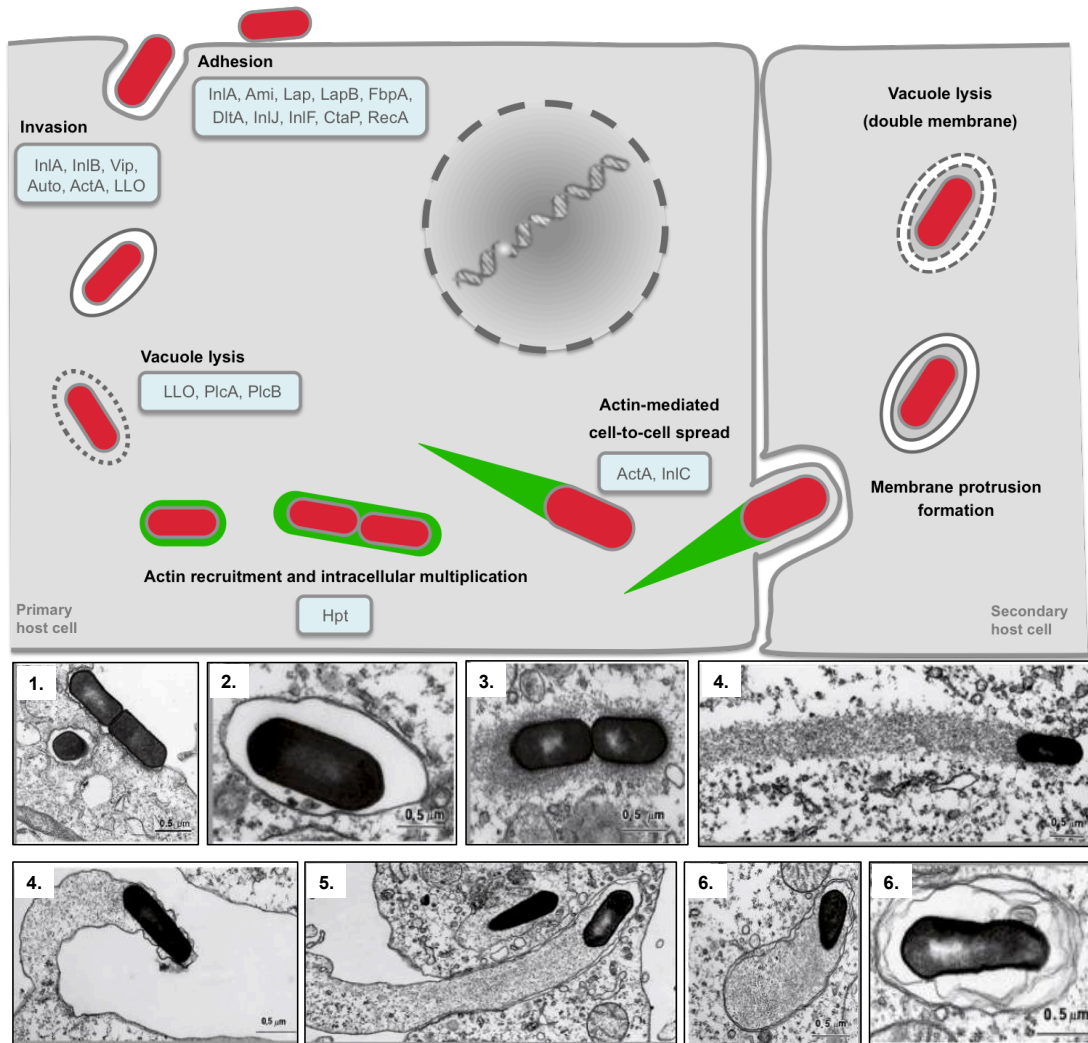
**Fig. 4.** Schematic representation of *Lm* cellular infection cycle and the major virulence factors involved in each step. *Lm* depicted in red and host actin in green. Electron microscopy images demonstrating the sequential events of *Lm* infectious cycle. Adapted from (Camejo *et al.* 2011; Cossart 2011).

### B.5.1. Adhesion

Recently, it was shown that adhesion to host cells is sufficient to mediate *Lm* internalization into epithelial cells (Ortega *et al.* 2017). The initial bacterial contact with a eukaryotic cell surface is a critical step for *Lm* cellular infection cycle and involves a number of surface adhesion factors (Figure 4). Ami is a protein with amidase activity previously implicated on bacterial adhesion and virulence (Milohanic *et al.* 2000; Milohanic *et al.* 2001). It has a N-terminal catalytic domain and a C-terminal cell wall-association domain containing eight glycine/tryptophan (GW) repeats (Braun *et al.* 1997). In addition, *Lm* Ami promotes, through glycosaminoglycans, an efficient adherence to mouse hepatocytes and enhances host innate immune responses by increasing the production of TNF-α and IL-6 (Asano *et al.* 2011; Asano *et al.* 2012). *Listeria* adhesion protein (LAP) is an alcohol acetaldehyde dehydrogenase that interacts with host cell receptor Hsp60 to promote bacterial adhesion to

intestinal epithelial cells and accelerates transepithelial translocation (Burkholder *et al.* 2010; Pandiripally *et al.* 1999). LapB was identified as a sortase-anchored LPXTG surface adhesin, found to be *up-regulated* in infected *Lm* mouse spleens and to be required for both adhesion and invasion of *Lm* into eukaryotic cells (Reis *et al.* 2010). FbpA is also an adhesin, which is required for liver and spleen colonization of mice intravenously infected. This protein seems to work not only as a fibronectin-binding protein but also as a chaperone that ensures the proper secretion of InlB and LLO (Dramsi *et al.* 2004; Osanai *et al.* 2013). So far, several other proteins were shown to significantly contribute to adhesion, including DltA, InlJ, InlF, CtaP and RecA (Abachin *et al.* 2002; Bierne *et al.* 2002; Dons *et al.* 2004; Kirchner *et al.* 2008; van der Veen *et al.* 2011; Xayarath *et al.* 2009).

### B.5.2. Internalization

The bacterium internalization by phagocytic cells is mostly driven by the cell itself. However, invasion of non-professional phagocytes is highly dependent on several *Lm* factors (Figure 4). The *inlAB* gene locus encodes two proteins, Internalins A and B, known to be involved in cell invasion and tissue tropism (Dramsi *et al.* 1995; Gaillard *et al.* 1991). Internalin family is characterized by the presence of a N-terminal domain containing a signal sequence followed by a leucine-rich repeat domain (LRR), which is variable in length and contributes for protein-protein interactions. Downstream this region, internalins have a conserved inter-repeat domain (IR) and a variable carboxy-terminal region (Cabanes *et al.* 2002; Dussurget *et al.* 2004; Seveau *et al.* 2007).

InlA is one of the proteins employed by *Lm* to trigger internalization by non-phagocytic cells. InlA-encoding gene is not found in the non-pathogenic *L. innocua* genome. It is covalently linked to the peptidoglycan meshwork of the bacterial cell wall by a LPXTG motif in its C-terminal (Dhar *et al.* 2000). The expression of *inlA* is regulated by PrfA (Lingnau *et al.* 1995) and Sigma B (SigB) (Kazmierczak *et al.* 2003). Ecad is an intercellular adhesion glycoprotein that was found to be the host cellular receptor for InlA (Mengaud *et al.* 1996). The engagement of Ecad by InlA triggers complex signalling pathways involving actin cytoskeleton rearrangements and clathrin-mediated endocytosis, which further culminates with bacteria internalization (Figure 5). The extracellular domain of Ecad is sufficient to bind InlA, whilst the intracellular one binds to catenins and promote internalization (Lecuit *et al.* 2000; Pizarro-Cerda *et al.* 2012).

InlB is encoded on the same operon of *inlA* (Gaillard *et al.* 1991). Its N-terminal LRR region is sufficient to promote *Lm* internalization in several cell types. Previous work demonstrated that InlB was noncovalently associated to the cell wall lipoteichoic acids via three GW (glycine-tryptophan) modules (Braun *et al.* 1997; Cossart *et al.* 2003; Seveau *et al.* 2007).
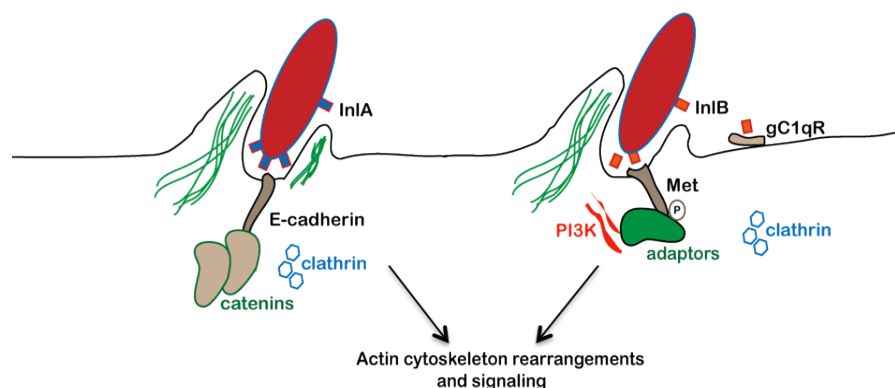
**Fig. 5.** InlA and InlB signalling pathways of *Lm* internalization into host cells. The engagement of Ecad and c-Met by InlA and InlB, respectively, triggers a series of events that induce actin cytoskeleton rearrangements and bacteria internalization. Adapted from (Pizarro-Cerda *et al.* 2006).

Recently, a glycosyltransferase involved in the LTA glycosylation process was discovered, revealing that GW-repeat-containing InlB protein can be retained in *Lm* cell wall in the absence of LTA (Percy *et al.* 2016). Remarkably, this protein is also absent from non-pathogenic *L. innocua* genome and its expression is under the regulation of both PrfA and SigB. Unlike InlA, InlB promotes bacterial invasion by interacting with different partners at the host cell surface. c-Met, which binds hepatocyte growth factor (HGF) was identified as a major InlB receptor (Shen *et al.* 2000). c-Met belongs to the family of receptor tyrosine kinases (RTKs), a group of transmembrane signalling molecules expressed on a wide diversity of cells (Seveau *et al.* 2007). In addition, InlB also binds to the glycosaminoglycans (GAGs) and to the ubiquitous complement receptor gC1qR, that are not sufficient to allow entrance but are important to cooperate with HGF receptor. The LRR domain of InlB interacts with the extracellular domain of c-Met to promote the receptor autophosphorylation in two tyrosine residues, which allows the recruitment of some adaptor molecules that subsequently lead to the activation of PI3-kinase (Dussurget 2008). This signalling cascade induces actin cytoskeleton rearrangements and clathrin-mediated endocytosis, thus promoting *Lm* internalization (Figure 5) (Cossart 2001).

The diversity of host cell receptors and their broad expression allows bacterial tropism for a panoply of different cells and tissues. InlA mainly contributes to cross the intestinal and placental barriers by triggering epithelial cells invasion (Gaillard *et al.* 1991; Lecuit *et al.* 2004), while InlB mediates *Lm* entry into a large variety of cells, including hepatocytes, fibroblasts and endothelial cells. Therefore, InlB needs to be functional not only at the placental barrier to allow maternofetal infection, but also at the blood-brain barrier (Dramsi *et al.* 1995; Dramsi *et al.* 1997; Greiffenberg *et al.* 1997; Parida *et al.* 1998; Radoshevich *et al.* 2017). Several other proteins are involved in *Lm* internalization. Auto, an autolysin absent in *L. innocua* genome, has four C-terminal GW modules responsible for the association of

the protein to the bacterial cell wall (Cabanes *et al.* 2004). Vip is an LPXTG surface protein absent from non-pathogenic *Listeria* species, which is necessary for bacteria internalization, by interacting with the endoplasmatic reticulum resident chaperone Gp96 (Cabanes *et al.* 2005; Martins *et al.* 2012).

### B.5.3. Vacuolar escape

Soon after uptake, *Lm* become engulfed within a phogocytic vacuole, which is quickly acidified (Figure 4). The disruption of the membrane is crucial for *Lm* proliferation and is highly dominated by Listeriolysin O (LLO), whose expression is positively regulated by PrfA. LLO is a secreted pore-forming toxin belonging to the cholesterol-dependent cytolysin family (CDC), which oligomerizes in the vacuole membrane as ring-like pore complexes. Although LLO was first reported to be responsible for pore formation, leading to phagosomal rupture and *Lm* escape from primary and secondary vacuoles (Shatursky *et al.* 1999; Tweten *et al.* 2001), it is now appreciated that LLO has additional functions (Osborne *et al.* 2017). Mutants of LLO-encoding gene, *hly*, do not replicate into cultured cells once *Lm* is trapped in the vacuole; they are also attenuated in virulence in a mouse model of infection (Gaillard *et al.* 1986; Gaillard *et al.* 1987; Portnoy *et al.* 1988). Calcium and potassium eflux across the plasma membrane were found to be essential for LLO-dependent internalization (Dramsi *et al.* 2003; Vadia *et al.* 2014). Apart from its pore-forming ability, LLO also contributes to activate and amplify different signalling pathways in the host cell, including the nuclear translocation of NF-kB and the secretion of proinflammatory IL-6 among other cytokines (Dewamitta *et al.* 2010; Kayal *et al.* 1999; Osborne *et al.* 2017; Tsuchiya *et al.* 2005). During infection, LLO was found to mediate apoptosis of different cell types, such as lymphocytes (Carrero *et al.* 2012). LLO also controls ROS production in *Lm*-containing phagosomes, induces autophagy, fragments the mitochondria, promotes the degradation of several host proteins and activates inflamassome (Eitel *et al.* 2010; Lam *et al.* 2011; Meyer-Morse *et al.* 2010; Samba-Louaka *et al.* 2014; Stavru *et al.* 2011). Importantly, host cells have mechanisms to protect themselves against the action of pore-forming toxins. Recently, it was convincingly shown that Gp96 is required to protect host from LLO-dependent killing (Mesquita *et al.* 2017).

Intravacuolar activity of LLO is reinforced by the secretion of two phospholipases, PI-PLC (PlcA) and PC-PLC (PlcB), which have overlapping functions although the first one highly contributes to induce primary vacuole lysis, and the second one mostly facilitates the disruption of the secondary vacuole (Smith *et al.* 1995). Two other proteins required for vacuolar escape were more recently identified. *Lm* secretes a small peptide pheromone, PplA (peptide pheromone-encoding lipoprotein A), which enhances *Lm* escape from the host

cell vacuoles and may facilitate PrfA activation (Xayarath *et al.* 2015). Additionally, it was shown that the DNA uptake competence (Com) system is essential throughout infection to promote bacterial escape from professional macrophage phagossomes, independently of DNA uptake (Rabinovich *et al.* 2012).

### B.5.4. Cytosolic life, Intracellular motility and cell-to-cell spread

Bacterial growth and replication within the cytosol is mainly dependent of an hexose phosphate transporter, Hpt, whose expression is regulated by PrfA. It imports hexose sugars available in the cytoplasm as a carbon energy source for intracellular growth (Chico-Calero *et al.* 2002; Ray *et al.* 2009).

Actin-based intracellular motility is a remarkable step of *Lm* cellular infection (Figure 4). ActA is a *Lm* PrfA-dependent virulence factor crucial to mediate bacterial propulsion along the cytosol and guide *Lm* to neighboring cells, by modulating the speed and directionality of the bacterial movement (Figure 6) (Pillich *et al.* 2017). *Lm actA* mutants are non-motile in the host cytoplasm and avirulent in a mouse model of infection (Domann *et al.* 1992; Kocks *et al.* 1992). ActA is a surface-anchored bacterial protein that interacts with the host actin nucleator Arp2/3 complex, which induces actin polimerization and thus the formation of actin filaments (Truong *et al.* 2014; Welch *et al.* 1998; Yoshikawa *et al.* 2009). Apart from its pivotal role in *Lm* motility, ActA was also implicated in *Lm* attachment and internalization into different cells, through the interaction with glycosaminoglycans (Alvarez-Dominguez *et al.* 1997). This protein plays also a role in preventing autophagy in the cytosol of macrophages, an ubiquitous process characterized by the degradation of cytosolic components in eukaryotic cells. To promote escape from autophagy, ActA may act in two major ways: confering actin-based movement to the bacteria or by actin-masking the bacteria, that no longer will be recognized by autophagy machineray (Figure 6) (Dussurget 2008; Yoshikawa *et al.* 2009). Interestingly, efferocytosis, which is the process of removing dead cells by phagocytosis, is exploited by *Lm* to favour cell-to-cell spread. LLO activity in the protrusions may induce plasma membrane damage and the consequent exofacial exposure of phosphatidylserine (PS). Then, TIM4 receptor on neighbouring macrophages mediates the uptake of these PS-positive protrusions (Czuczman *et al.* 2014). In addition, InlC is a secreted protein that alters cell rigidity and contributes to protusions formation by interacting and inhibiting Tuba, a protein essential to maintain intact the structure of the apical junctions (Rajabian *et al.* 2009). Moreover, a Rho GTPase-formin network has also a key role in protrusion formation and *Lm* cell-to-cell spread (Fattouh *et al.* 2015).
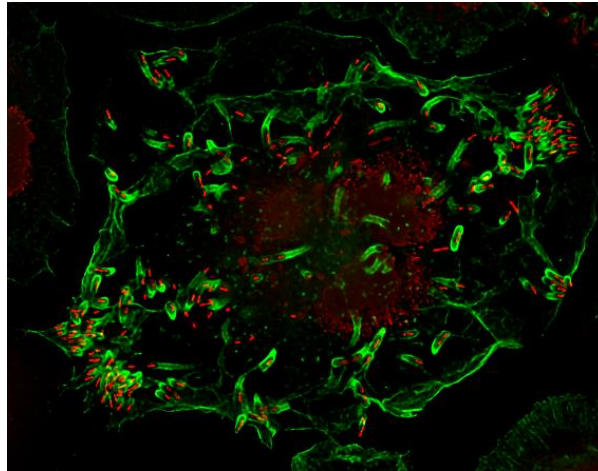
**Fig. 6.** *Lm* (red) polymerizing host cell actin (green) into clouds and comet tails (Ana Costa, 2017).

### B.5.5. Regulation of virulence factors

The expression of *Lm* virulence determinants requires a tight and coordinated regulation. PrfA (positive regulatory factor A) is the major transcriptional regulator controlling the expression of several genes whose products play a critical role in different steps of *Lm* pathogenesis (InlA, InlB, ActA, LLO, Hpt, PlcA, PlcB, Vip, among others). The self regulation of *prfA* expression and protein activity involves complex transcriptional, post-transcriptional and post-translational mechanisms (Port *et al.* 2007). The transcription of PrfA-dependent genes is activated by PrfA binding to a palindromic promoter region of a canonical sequence (tTAACanntGTtAa) named PrfA box, which is composed by seven conserved nucleotides with a tolerance of two mismatches (de las Heras *et al.* 2011; Scortti *et al.* 2007). *prfA* expression is under the control of both RNA thermosensor mechanism, that allows translation at 37°C, and by a trans-acting riboswitch (Johansson *et al.* 2002; Loh *et al.* 2009). Afterwards, bacterial and host-derived glutathione were found to be required to activate PrfA (Reniere *et al.* 2015). Importantly, most of the PrfA-dependent virulence genes were found to be highly expressed throughout macrophages infection and in mouse organs upon *Lm* infection (Camejo *et al.* 2009; Chatterjee *et al.* 2006; Rolhion *et al.* 2017).

The transcription factor SigmaB ($\sigma^B$) regulates several genes that are predicted to be important in stress tolerance, carbohydrate metabolism, transport and cell envelope processes (Hain *et al.* 2008). It is important to highlight that some of the PrfA-regulated genes display potential $\sigma^B$ promoter sequences (Milohanic *et al.* 2003). Different studies have shown that $\sigma^B$ and PrfA co-regulate genes that are important for *Lm* to switch from an extracellular to an intracellular environment (Chaturongakul *et al.* 2008; Ollinger *et al.* 2008). VirR is among the two-component systems present in *Lm*. It is a response regulator that appeared to be highly regulated during infection and whose deletion severely diminished virulence in mice (Mandin *et al.* 2005; Thedieck *et al.* 2006).

**B.5.6. Metal ion homeostasis and pathogenesis**

During infection, bacteria must compete with their hosts to acquire essential metals. Importantly, distinct locations within the host, such as infected tissues, circulatory fluids, mucosal surfaces and diverse intracellular environments have to deal with variations in the levels of available metals (Osman *et al.* 2010). Heavy metals are toxic at high concentrations and therefore its intracellular availability needs to be tightly regulated to maintain metal homeostasis (Saier *et al.* 1998). Bacteria display complex and often redundant resistance systems to fine tune metal ions concentration, that usually encode metal-specific efflux pumps, membrane-bound and cytoplasmic transporters, and metal-responsive transcriptional regulators (Silver *et al.* 1996). *Lm* is frequently challenged by variations in metal availability and thus it is forced to develop strategies to overcome metal-dependent host responses. In *Lm*, iron withholding is controlled by the iron-dependent repressor Fur and through Fur-repressed genes, such as Fri and HupC (Ledala *et al.* 2010; Rea *et al.* 2004). Furthermore, CtpA is a P-type adenosine triphosphatase involved in copper homeostasis and Zur (zinc uptake regulator) is predicted to coordinate zinc uptake from the external environment. Importantly, these proteins were shown to be required for *Lm* virulence (Dalet *et al.* 1999; Dussurget *et al.* 2005; Francis *et al.* 1997; Francis *et al.* 1997). Cadmium is dispersed into the water, air, soils and foodstuffs, once it naturally results from erosion, forest fires and volcanic eruptions. Cadmium resistance systems are usually composed by a transcriptional repressor (CadC) belonging to the ArsR-SmtB family, and a P1-type ATPase (CadA) that extrudes heavy metals from the cell (Endo *et al.* 1995; Nucifora *et al.* 1989). CadA was first identified in *Lm*74 strain isolated from a food product in France. In this case, *cadA* is encoded in an operon, together with *cadC*, in a transposable element designated Tn5422, harboured in plasmid pLm74. This CadAC system was found to be induced by cadmium and to confer resistance to this metal (Lebrun *et al.* 1994; Lebrun *et al.* 1994). More recently, it was shown that *Lm cadC* is highly expressed during infection and is required for bacterial virulence (Camejo *et al.* 2009).

## C. IMMUNOLOGICAL ASPECTS OF THE INFECTION

The concept of "immunity" relies on the global capacity of the host to resist to microbial colonization. This section describes the fundamental principles of immunology, the greatest dichotomy between innate and adaptive immunity and also the immune responses orchestrated by the host to avoid microbial predation.

### C.1. Basic principles of immunology

Efficient protection of the host against infectious agents and the damage they cause implies the complex interaction between organs, tissues, effector cells and molecules that together compose the immune system. For this purpose, our immune system should be able to fulfil four main requirements: (i) immunological recognition, which comprises the detection of the infection, not only by innate immune cells but also by the lymphocytes of an adaptive response; (ii) immune effector function, mainly characterized by the restriction and elimination of the infection; (iii) immune regulation, which is crucial to control and distinguish self from non-self antigens and (iv) immunological memory that is a hallmark of adaptive response, which allows immune system to respond more rapidly and effectively to a pathogen that has been previously encountered. Immune defence mechanisms are broadly subdivided into innate and adaptive systems (Figure 7). They have both evolved to provide host defences, repair mechanisms and ultimately maintain homeostasis (Janeway *et al.* 2002).
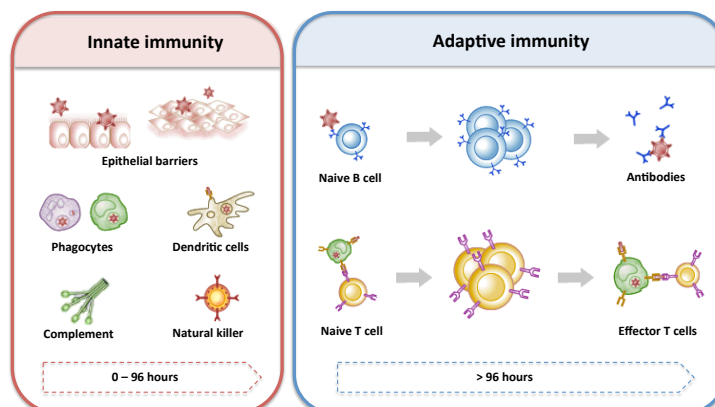


**Fig. 7.** Major cellular components of both innate and adaptive immune responses. Innate response is the first line of defence against infection and is composed by physical barriers, soluble factors and different cellular components. Adaptive immune response is generated by B and T cells and has increased antigen specificity and memory. Adapted from (Dranoff 2004).

### C.2. Innate immunity

Innate immune response relies on the initial and rapid response against any invading pathogen (Figure 7). It acts in a relatively non-specific manner and it is crucial for the induction of the adaptive immune response. Defects in host innate immune components may

lead to increased susceptibility to infection, even in the presence of a fully effective adaptive immune response (Janeway *et al.* 2002).

**C.2.1. The front line of host defence**

Disease manifests when the infectious agent succeeds in overwhelming host defences to establish infection. The initial contact of the microorganism with the host occurs through external or internal epithelial surfaces (Figure 7). For this reason, epithelial barriers such as the skin, gut, lungs, eyes, nose and oral cavities are the linings of our body creating tight physical barriers. Importantly, epithelia have the ability to secrete mucus that is composed by several glycoproteins, mucins, with a high potential to prevent microorganism adherence to the epithelium. In the respiratory tract, microbes are easily expelled in the outward flow of the mucus through the action of cilia on the mucosal epithelium. Interestingly, healthy epithelial surfaces are co-inhabited by a large population of commensal microbiota, which not only competes with pathogens for nutrients and attachment sites, but also manages the production of antimicrobial substances. Wherefore, these surfaces also exert the function of a chemical barrier since they display microbicidal mechanisms, including acidic pH, bile salts, digestive enzymes, fatty acids and antimicrobial peptides (lysozyme, phospholipase A, defensins, cathelicidins, histatins) (Brown *et al.* 2017; Janeway *et al.* 2002). Apart from these anatomical and physiological barriers, complement is a powerful mechanism of the innate immune system, composed by a number of secreted proteins widely present in the blood and other body fluids as inactive precursors (Figure 7). Nevertheless, inflammatory stimulus triggers the coordinated and sequential action of a cascade of plasma proteins, whose final outcome is the pathogen killing, either directly or by facilitating its phagocytosis (Nesargikar *et al.* 2012; Schifferli *et al.* 1986). This efficient immune surveillance system can be activated by three pathways: the classical pathway, which is an antibody-triggered pathway; the alternative pathway usually activated by the presence of the pathogen *per se*; and most recently described the lectin pathway, which is activated by the recognition and binding of lectin-type proteins to the carbohydrates present on the pathogen surface (Ricklin *et al.* 2010). Invading microbes interact with phagocytes either through direct binding to their surface receptors (nonopsonic) or through the action of opsonins (complement proteins or antibodies) that coat the bacterial surface (Moser *et al.* 2010).

**C.2.2. Pattern Recognition Receptors**

Our immune system is equipped with a diversified cell population, including dendritic (DCs) cells, cytotoxic natural killer (NK) cells, macrophages and neutrophils, which are multitasking cells that have a central role in immunity, inflammation and tissue repair (Figure 7). These

professional phagocytes are key players in antimicrobial process as they internalize, through distinct mechanisms, a wide variety of targets (Kaufmann 2008; Metchnikoff 1984). Endocytosis generally implies the internalization of small molecules, either through coated or uncoated vesicles in an actin-independent process. On the other side, the uptake of fluids or particles into large vacuolar structures are achieved by actin-driven processes known as macropinocytosis and phagocytosis, respectively (McMahon *et al.* 2011; Swanson 2008). Phagocytosis is a dynamic host-driven process that occurs through the engagement of host cell receptors by their cognate microbial ligands (Metchnikoff 1984). Remarkably, besides the engulfment of microorganisms, phagocytes have an amazing capacity to ingest dead cells and environmental debris. In addition, they are crucial not only for the processing and presentation of the antigen to T lymphocytes, but also for the secretion of pro-inflammatory cytokines that help to build up an inflammatory and an adaptive immune responses (Jaillon *et al.* 2013; Kruger *et al.* 2015; Varol *et al.* 2015). DCs are specialized cells in initiating adaptive immune response, being essential to do the link between both arms of the immune system (Steinman *et al.* 1973; Steinman *et al.* 1974).

These host immune cells broadly express a family of sensors known as pattern-recognition receptors (PRRs) (Figures 8 and 9) (Akira *et al.* 2006; Basset *et al.* 2003). PRRs are evolutionary conserved receptors able to sense conserved signature molecules named pathogen-associated molecular patterns (PAMPs). PAMPs are characterized as being invariant microbial components, crucial for pathogen survival and somehow distinguishable from "self" components. The engagement of these microbial ligands by PRRs activates different signalling pathways to invoke measured innate immune responses and subsequently shape the adaptive immunity (Fraser *et al.* 1998; Gordon 2002; Janeway 1989; Medzhitov *et al.* 2000a; Medzhitov *et al.* 2000b). Generically, PRRs have two mechanisms of action, either by a cell-intrinsic recognition assessed by intracellular cytosolic sensors in infected cells, or through cell-extrinsic recognition, which is useful to sense the pathogen when the cell expressing the receptor is not yet infected. There are an extensive repertoire of PRRs, which are grouped according their structure and cellular localization (Figures 8 and 9). Multiple PRRs may sense identical microbial PAMPs and in turn, different ligands can be recognized by the same PRR. Mechanistically, there is a strong crosstalk among PRRs either through co-activation or co-inhibition of the innate immune pathways. Co-activation implies a positive interaction between the signalling components of different PRRs that can be either mandatory if one pathway is completely dependent on the activity of another, or facultative if each pathway is activated independently of the other one. In opposition, co-inhibition results from the blocking of one signalling pathway to the detriment of another (Nish *et al.* 2011). This section describes the most consensual and characterized families of PRRs (Figures 8 and 9, Table 3) (Kumar *et al.* 2011).
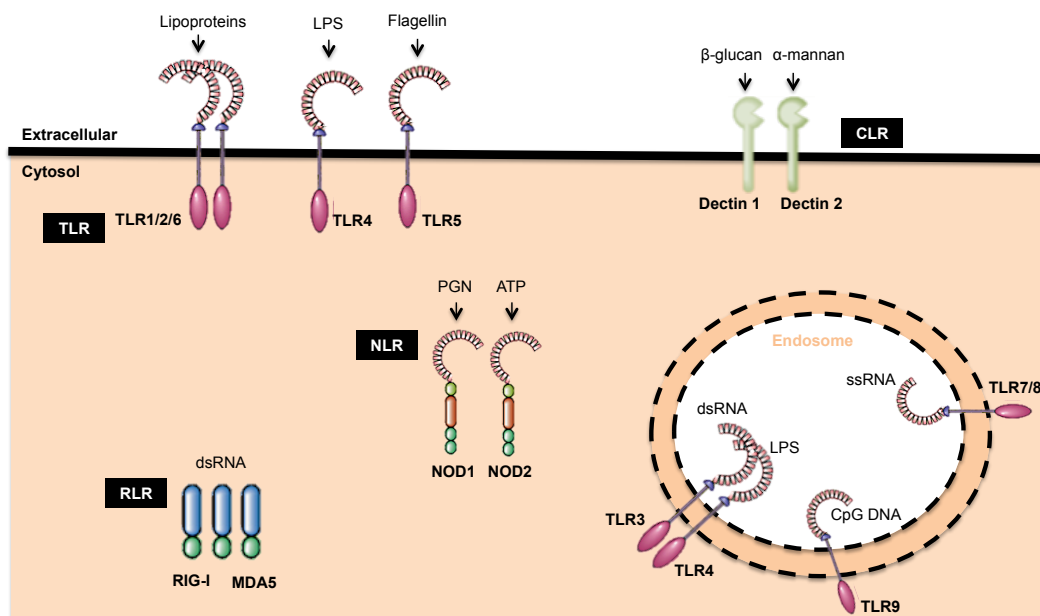
**Fig. 8.** Schematic representation of the pattern-recognition receptors and some of their ligands. Adapted from (Kvarnhammar *et al.* 2012). LPS, lipopolysaccharide; PGN, peptidoglycan; ATP, adenosine triphosphate; dsRNA, double-stranded ribonucleic acid; ssRNA, single-stranded ribonucleic acid; CpG, cytosine ("C"), phosphodiester link ("p"), guanine ("G"); DNA, deoxyribonucleic acid.

### C.2.2.1. Toll-like receptors (TLRs)

TLRs are the most extensively studied class of PRRs (Figures 8 and 9). They are expressed by several cell types including epithelial cells, mast cells, NK cells, dendritic cells, B and T cells, macrophages, monocytes, neutrophils, basophils and endothelial cells (Hopkins *et al.* 2005). TLRs are type I transmembrane glycoproteins localized either on the plasma membrane or on the endosomal membrane. They are structurally characterized by the presence of an extracellular domain containing leucine-rich repeats (LRRs) and a cytoplasmic tail composed by a conserved region called the Toll/IL-1 receptor (TIR) domain (Figure 9) (Leber *et al.* 2008). Ten human and twelve murine TLRs have been described so far and they have evolved to recognize a diversity of PAMPs from bacteria, viruses, fungi and parasites (Table 3) (Trinchieri *et al.* 2007). TLRs exposed at the plasma membrane usually recognize signature molecules expressed at the microbial surfaces, whether endosomal receptors mainly respond to microbial nucleic acids (Figure 8 and Table 3) (Akira *et al.* 2006). TLRs often sense endogenous ligands known as damage-associated molecular patterns (DAMPs), including heat shock proteins, heparin, DNA, uric acid and purine metabolites (Trinchieri *et al.* 2007). Stimulation of TLRs by their corresponding PAMPs, such as LPS, lipoproteins, flagellin, or DAMPs triggers two main pathways: MYD88 (myeloid differentiation primary-response protein 88) or TRIF (TIR domain-containing adaptor protein inducing interferon α/β), stimulating the activation of signalling transduction pathways, such as NF-κB, mitogen-activated protein kinase (MAPK) and interferon regulatory factors (IRFs)

(McGuire *et al.* 2015). Signalling through TLRs accounts for a number of cellular responses, including the production of interferons (IFNs) and the secretion of pro-inflammatory cytokines (Akira *et al.* 2006). IFNs are polypeptides secreted by infected cells with a key role on balancing innate immunity and activate adaptive response, in particular through the clearance of viral and bacterial pathogens (Hennessy *et al.* 2010; Wheelock *et al.* 1965).

### C.2.2.2. NOD-like receptors (NLRs)

NLRs are a large family of cytoplasmic sensors of several microbial and non-microbial stimuli (Figure 8 and Table 3). NOD proteins are expressed in cells that are constantly exposed to bacteria, including epithelial cells, macrophages, neutrophils and dendritic cells. NLRs are characterized by a tripartite-domain organization with a central conserved nucleotide-binding and oligomerization domain, a C-terminal LRR responsible for ligand sensing and a specific effector domain at the N-terminal: caspase recruitment domain (CARD), pyrin domains (PYDs), baculoviral inhibitor of apoptosis repeat (BIR) or the transactivation domain (AD) (Figure 9) (Kumar *et al.* 2013). NOD1 and NOD2 are the most studied receptors of this family and they are capable to detect bacterial molecules that result either from the synthesis or degradation of peptidoglycan (Figure 8 and Table 3) (Kanneganti *et al.* 2007). The stimulation of NLRs may activate NF-kB and consequently alters gene expression, or trigger the formation of a large cytosolic protein complex termed inflammasome. This complex regulates the activation of caspase-1, which in turn allows the cleavage of pro-inflammatory IL-1 family of cytokines into their bioactive forms, IL-1β and IL-18. Ultimately, these events conduct to pyroptosis, which is a form of inflammatory caspase-1-dependent cell death (Guo *et al.* 2015; Lamkanfi *et al.* 2012).
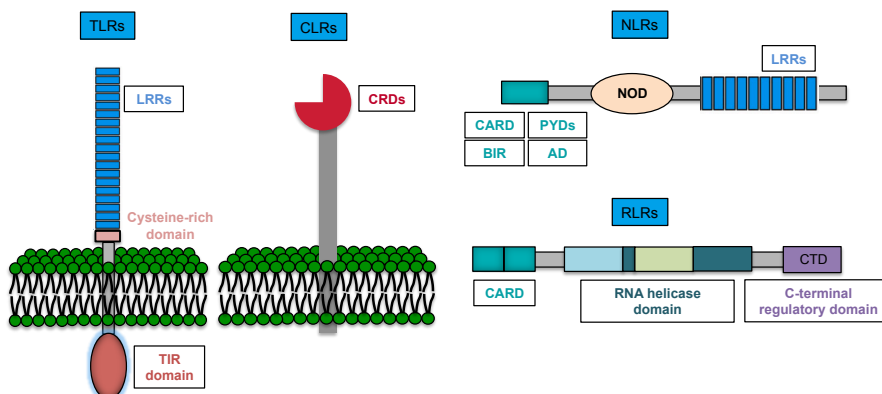


**Fig. 9.** Host innate pattern recognition receptors (PRRs) and their main domains. Toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs) and RIG-like receptors (RLRs) are expressed at the cell surface, in the endosomes, or in the cytosol of several immune cells. LRRs (leucine-rich repeats); TIR (Toll/IL-1 receptor); CRDs (carbohydrate recognition domain); CARD (caspase activation and recruitment domain); PYDs (pyrin domain); BIR (baculoviral inhibitor of apoptosis repeat); AD (transactivator domain).

### C.2.2.3. RIG-I-like receptors (RLRs)

This family of RNA sensors is expressed within the cytoplasm of nearly every mammalian cell, and it is indispensable to initiate an effective innate immune response to RNA viral infection (Figure 8 and Table 3) (Yoneyama *et al.* 2004; Yoneyama *et al.* 2005). RIG receptors are usually expressed on macrophages, dendritic cells and fibroblasts. These RNA helicases are equipped with CARDs (caspase activation and recruitment domain) at the N-terminal, a central helicase domain associated with the ATPase activity and a C-terminal domain involved on the recognition and binding specificity of the ligands (Figure 9) (Takahasi *et al.* 2008; Yoneyama *et al.* 2005). RIG-I and MDA5 sense short and long dsRNAs, respectively and trigger a serious of events, including the recruitment of adaptor proteins, kinases and transcription factors, which further lead to the production of type I interferon, pro-inflammatory cytokines, and antiviral effector gene transcription (Table 3) (Kato *et al.* 2005; Zevini *et al.* 2017).

### C.2.2.4. C-type lectin receptors (CLRs)

CLRs are membrane-bound carbohydrate receptors expressed by the majority of cells that phagocyte glycoproteins and microbes to first clear them and then present the antigens to T-lymphocytes. CLRs can be divided into type I or II depending if they are composed by multiple or a single carbohydrate recognition domain (CRDs), respectively (Figure 9) (Brown 2006; McGreal *et al.* 2005). Dectin-1 is mainly expressed by monocytes, macrophages and DCs and recognizes β-glucan, which is the main component of fungal cell wall. It triggers diverse cellular responses such as phagocytosis, cytokine production and inflammasome activation (Figure 8 and Table 3) (Plato *et al.* 2013). On the other hand, Dectin-2 was previously shown to bind α-mannan, a polysaccharide also present in fungal cell wall (Saijo *et al.* 2010).

### C.2.3. How host cells sense pathogens

In the past few years, an increasing number of PRR ligands have been discovered, but conceptually it is important to highlight that these ligands are not exclusive of pathogenic bacteria, being also present in both non-pathogenic and commensal bacteria. Having this in mind, a relevant question arises: how the host has the ability to distinguish and respond to pathogenic microorganisms, while remains tolerant to beneficial commensal bacteria? For this reason, the term PAMP for microbial ligands is now more appropriately designated as microorganism-associated molecular patterns (MAMPs) (Figure 8 and Table 3) (Medzhitov 2010).

**Table 3.** Cellular localization and microbial ligands of different PRRs. Adapted from (Drummond *et al.* 2013; Mogensen 2009).

| Receptor | Cellular localization | Microbial ligand | Origin |
|---|---|---|---|
| **TLRs** | | | |
| **TLR1/TLR2** | Cell surface | Triacyl lipopeptides | Bacteria |
| **TLR2/TLR6** | Cell surface | Diacyl lipopeptides | Mycoplasma |
| | | Lipoteichoic acid | Gram-positive bacteria |
| **TLR2** | Cell surface | Lipoproteins | Various pathogens |
| | | Peptidoglycan | Gram-positive and -negative bacteria |
| | | Lipoarabinomannan | Mycobacteria |
| | | Porins | Neisseria |
| | | Envelope glycoproteins | Viruses (e.g., measles virus, HSV, cytomegalovirus) |
| | | GPI-mucin | Protozoa |
| | | Phospholipomannan | Candida |
| | | Zymosan | Fungi |
| | | β-Glycan | Fungi |
| **TLR3** | Cell surface/endosomes | dsRNA | Viruses |
| **TLR4** | Cell surface | LPS | Gram-negative bacteria |
| | | Envelope glycoproteins | Viruses (e.g., RSV) |
| | | Glycoinositolphospholipids | Protozoa |
| | | Mannan | Candida |
| | | HSP70 | Host |
| **TLR5** | Cell surface | Flagellin | Flagellated bacteria |
| **TLR7/8** | Endosome | ssRNA | RNA viruses |
| **TLR9** | Endosome | CpG DNA | Viruses, bacteria, protozoa |
| **RLRs** | | | |
| **RIG-I** | Cytoplasm | dsRNA (short), 5 -triphosphate RNA | Viruses (e.g., influenza A virus, HCV, RSV) |
| **MDA5** | Cytoplasm | dsRNA (long) | Viruses (picorna- and noroviruses) |
| **NLRs** | | | |
| **NOD1** | Cytoplasm | Diaminopimelic acid | Gram-negative bacteria |
| **NOD2** | Cytoplasm | Muramyl dipeptide (MDP) | Gram-positive and -negative bacteria |
| **NALP1** | Cytoplasm | Muramyl dipeptide (MDP) | Gram-positive and -negative bacteria |
| **NALP3** | Cytoplasm | ATP, uric acid crystals, RNA, DNA, MDP | Viruses, bacteria, and host |
| **CLRs** | | | |
| **Mincle** | Cell surface | α- mannose | Fungi (e.g., *Candida albicans*) |
| | | Trehalose-6, 6´-dimycolate (TDM) | Mycobacteria |
| **Dectin-1** | Cell surface | β-Glycan | Fungi (e.g., *Candida albicans, Aspergillus fumigatus, Pneumocystis carinii*) |
| **Dectin-2** | Cell surface | α- mannan | Fungi (e.g., *Candida albicans*) |

* Abbreviations: HSV, Herpes simplex virus; RSV, Human respiratory syncytial virus; HCV, Hepatitis C virus

Different but complementary hypothesis for pathogen recognition have been proposed so far. Matzinger firstly launched the danger theory, which suggests that our immune system does not recognize pathogens *per se*, but somehow senses the damage they cause (Matzinger 1994). He later proposed that there are damage-associated molecular patterns (DAMPs), which are molecules released by the cells to the extracellular milieu, such as DNA, ATP, uric acid and DNA binding proteins that alert the host immune system of a threat (Matzinger 1998; Matzinger 2002). An additional model that came out to discriminate between pathogens and non-pathogens was associated with the location of recognition. This hypothesis was proposed upon the observation that invasive pathogens, such as *Listeria*, induced stronger immune response whenever they were within the host cytoplasm, which is usually devoiced of bacterial components (Sauer *et al.* 2010; Vance *et al.* 2009). A third mechanism was described based on the monitoring of bacterial viability by host cells. An

increase in bacterial viability is a signal that infection is not being effectively controlled and therefore immune response needs to be amplified (Sander *et al.* 2011). Moreover, a particular system that avoids the risk of killing beneficial commensals that reside in our body was further described: effector-triggered immunity, which is a mechanism of protective immune response that relies on the detection of the damage caused by microbial virulence factors. These effectors are translocated across the plasma membrane, gain access to the cytoplasm, interact with host proteins and ultimately activate immune response (Brodsky *et al.* 2009; Flor 1942).

## C.3. Adaptive immunity

Innate immunity, by itself, may not be sufficient to effectively protect the host from an invading pathogen. Therefore, innate defence mechanisms generate a threshold dose of a specific antigen that triggers adaptive immune response (Figure 7). When lymphocytes in circulation encounter a particular antigen in peripheral lymphoid tissues, they are induced to proliferate and differentiate into effector T and B lymphocytes, which have an enormous potential to eliminate the infectious agent (Figure 7). B cells recognize antigens and differentiate into effector plasma cells responsible for antibody production, and memory cells crucial to the immunological memory against re-infection (Kurtz 2004). On the other hand, T cells differentiate into effector T cells that have key roles to eliminate infection by killing infected cells, activating other immune cells and regulating lymphocytes. This assignment is achieved through cytotoxic (CD8), helper (CD4) and regulatory T cells, respectively. Whether B cells directly recognize antigens secreted by a pathogen or expressed on its surface, the T-cell receptor does not bind directly to the antigen molecules. Firstly, the antigens have to be processed, partly degraded and then exposed on a self-surface glycoprotein called MHC (major histocompatibility complex) to be further recognized by T-cell receptor (Hoebe *et al.* 2004; Janeway *et al.* 2002).

## C.4. Innate immune responses against *Lm* infection

*Lm* has been largely used as a model organism to explore both innate and adaptive immune responses. The early eradication of *Lm* invokes the participation of different immune cells and the activation of a number of PRRs. These mechanisms are crucial to prime the adaptive immune response. The complement system has an important protective role in listeriosis, through the opsonisation and intracellular killing of *Lm* and also by promoting leukocyte survival (Calame *et al.* 2016; Drevets *et al.* 1991; van Kessel *et al.* 1981).

Macrophages, monocytes and neutrophils play a central role in the initial immune response against *Lm*. Neutrophils, which are among the first cells to migrate toward the inflammation

site, are able to phagocyte *Lm* and produce reactive oxygen and nitrogen species (ROS and RNS) that exert antimicrobial effects against intracellular bacteria (Figure 10) (Conlan *et al.* 1994). Moreover, neutrophils are capable to release extracellular traps (NETs), which are granule-derived proteins and chromatin that involve and kill extracellular bacteria (Brinkmann *et al.* 2004). Neutrophils are particularly important in the liver and during high-dose *Lm* inoculum (Carr *et al.* 2011) and mice deficient in these cells are more susceptible to *Lm* infection (Rogers *et al.* 1993; Williams *et al.* 2012; Witter *et al.* 2016). Macrophages are the primordial replication cells for *Lm.* Kupffer cells are liver resident macrophages responsible for the initial killing of the majority of the bacteria, through direct phagocytosis or by secreting TNF-α and IL-12 that conduct NK cells to release IFN-γ, which in turn leads to the activation of other macrophages (Havell 1987; Tripp *et al.* 1993). Importantly, activated macrophages also produce RNS and ROS upon bacterial uptake aiming to prevent bacterial escape into the cytoplasm (MacMicking *et al.* 1995). Cytosolic live *Lm* targets DCs that, once activated, migrate from tissues to lymph nodes, where they largely contribute to present captured ligands to naive T cells (Feng *et al.* 2005; Zenewicz *et al.* 2007). Currently, it is well established that certain cytokines have an important role in controlling *Lm* infection. Type II IFN-γ is highly secreted by NK cells and T lymphocytes and it is essential to restrict primary response, although it is less involved in protective immunity against re-infection. Mice that are deficient in IFN-γ are more susceptible to *Lm* infection (Harty *et al.* 1995; Huang *et al.* 1993). In addition, *Lm* triggers the expression of type I IFN genes (IFN-α, IFN-β), which are beneficial for the bacteria, once directly promote their growth and down-regulate immune responses responsible for bacterial load control (Auerbuch *et al.* 2004; Snyder *et al.* 2017). More recently, it was shown that type I IFNs induce T cell apoptosis during early *Lm* infection, leading to IL-10 secretion by phagocytic cells, which thus dampen the innate immune response (Carrero *et al.* 2006; Witter *et al.* 2016).

Importantly, several PRRs were shown to be involved in the recognition of *Lm*. NOD2 is a cytoplasmic sensor that was previously demonstrated to be relevant in the context of intragastric *Lm* infection, but not when mice were challenged with intravenous or intraperitoneal injection (Figure 10). Strikingly, NOD2 knock-out (KO) mice display increased bacterial numbers both in the liver and spleen and are unable to generate an adaptive response to *Lm* in the intestine (Dolowschiak *et al.* 2010). NLRP6-deficient mice are highly resistant to *Lm* infection and infected KO mice had increased numbers of monocytes and neutrophils in circulation, suggesting that NLRP6 is a negative regulator of inflammatory signalling (Anand *et al.* 2012). Members of the intracellular NLR family highly contribute to *Lm* immune responses through the activation of NF-κB, type I interferon and inflammasome. In particular, *Lm* induces inflammasome upon triggering of NLRP1, NLRP3, NLRC4 and AIM2 (absent in melanoma 2) receptors, leading to the activation of pro-inflammatory

caspase-1, processing and secretion of IL-1β and IL-18, and ultimately cell death by pyroptosis (Figure 10) (Kim *et al.* 2010). Cytosolic flagellin is responsible for the activation of NLRC4 and the bacterial DNA from ruptured phagosomes is the primary agonist triggering caspase-1 activation through AIM2 inflammasome (Wu *et al.* 2010). Moreover, RIG-I expression is *up-regulated in vivo* in hepatic Kupffer cells and in splenic reticular cells of mice infected with *Lm* (Imaizumi *et al.* 2006). RIG-I is important for *Lm* detection in intestinal epithelial cells due to the recognition of small RNAs secreted during active bacterial infection (Figure 10) (Abdullah *et al.* 2012).
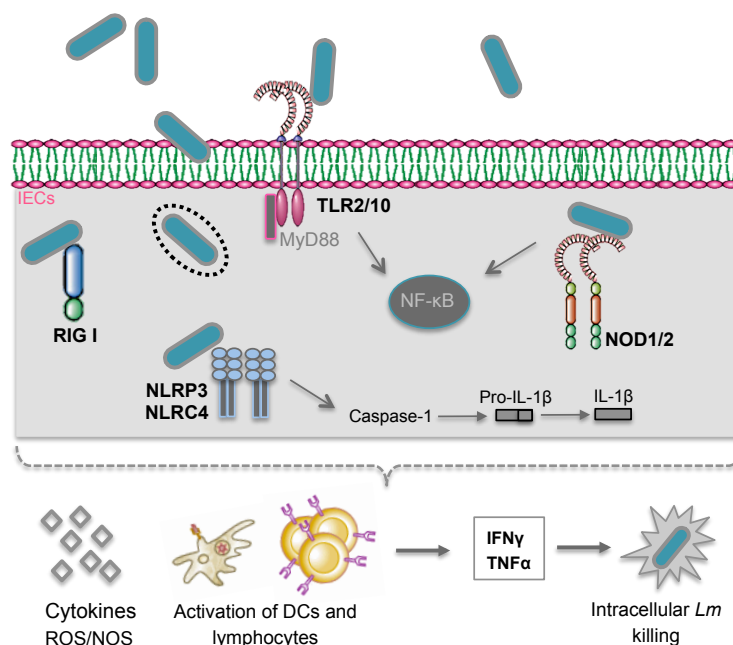


**Fig. 10.** Different innate immune mechanisms to recognize and clear *Lm*. Within intestinal epithelial cells (IECs), several PRRs are triggered upon the recognition of bacterial ligands. Adapted from (Regan *et al.* 2014).

TLRs also participate in *Lm* recognition and although the bacterium does not have the prototypical TLR ligand LPS, it does express other PAMPs including peptidoglycan, flagellin and bacterial DNA. TLR2 mediates *Lm* recognition through bacterial lipoproteins and the lipidation of pre-lipoproteins is a process quite necessary to activate NF-κB via TLR2 (Figure 10). Secreted *Lm* lipoproteins were shown to induce inflammatory cytokines production (TNF-α and IL-6) in a TLR2-dependent manner during infection (Machata *et al.* 2008). Additionally, CD14 is the TLR2 co-receptor essential for *Lm* recognition, and therefore mice deficient on TLR2 or with reduced expression of CD14 present heightened mortality (Janot *et al.* 2008; Torres *et al.* 2004). In fact, TLR2-deficient macrophages secrete less IFN-γ, TNF-α and IL-1β upon *Lm* infection *in vitro* (Ozoren *et al.* 2006). Moreover, TLR10 usually recognizes profilin-like proteins and it is a key mediator of the inflammatory response against *Lm,* both in intestinal epithelial cells and in macrophages (Figure 10) (Regan *et al.* 2013). Mice deficient in the key adaptor molecule, MyD88, which is important for signalling of several TLRs, are highly susceptible to *Lm* infection (Edelson *et al.* 2002).

## C.5. Immuno-evasive strategies employed by *Lm*

*Lm* has evolved different strategies to overcome immune response and avoid detection by host innate receptors (Figure 11). The intracellular niche that it establishes is, *per se,* an efficient mechanism employed by *Lm* to escape from immune detection. Furthermore, *Lm* is able to modify bacterial ligands to impede their recognition by PRRs (Boneca *et al.* 2007; Rae *et al.* 2011). Peptidoglycan (PGN), which is an essential component of the bacterial cell wall readily exposed to the host, constitutes an important target for the innate immune system. Remarkably, *Lm* is able to modify its PGN through the N-deacetylation process, by the action of N-acetylglucosamine deacetylase (Pgd) (Figure 11). *pgdA* mutation demonstrated the crucial role of this PGN modification in bacterial virulence, once the mutant strain is more sensitive to the bacteriolytic action of lysozyme and less efficient to grow upon oral and intravenous mice infections. Once inside macrophage vacuoles, *pgdA* mutant had defects on intracellular growth and induced a massive IFN-β response in a TLR2 and NOD1-dependent manner, being immediately destroyed (Boneca *et al.* 2007; Regan *et al.* 2014). *Lm* also evades innate defences through PGN O-acetylation. PGN O-acetyltransferase, OatA confers resistance to different antimicrobial compounds and contributes not only to intracellular survival in macrophages *in vitro* but also to the suppression of IL-6 secretion *in vivo* (Aubry *et al.* 2011). In addition, to avoid recognition by TLR2 and NOD1, *Lm* can directly *up-regulate* TLR-inhibitory proteins such as DC-SIGN, a C-type lectin receptor expressed by DCs and known to interact with a large array of pathogens (Figure 11) (Geijtenbeek *et al.* 2000). P60 is an extracellular protein encoded by *Lm iap* gene (invasion associated protein) that possesses a murein hydrolase activity important for septum formation and thus essential for cell division (Wuenscher *et al.* 1993). The secretion of P60 by *Lm* largely enhances NK cell activation, increases the production of IFN-γ and other pro-inflammatory cytokines that consequently promote bacterial expansion (Humann *et al.* 2007; Sashinami *et al.* 2010). Moreover, *Lm* manipulates host gene expression through histone modification, thereby regulating gene transcription and DNA replication or repair (Hamon *et al.* 2008; Schmeck *et al.* 2005). Particularly, eukaryotic DNA is packed into the organized structure of chromatin composed by histones and chromatin-remodelling proteins. *Lm* induces the dephosphorylation of histone H3 and deacetylation of histone H4 by the secretion of LLO before invasion, leading to the down-regulation of key inflammatory signalling pathways, such as TLR2 and NOD1-induced responses (Figure 11) (Hamon *et al.* 2007; Hamon *et al.* 2008). *Lm* evades autophagy mechanisms, mainly through ActA by recruiting host proteins in a complex that somehow protects bacteria (Birmingham *et al.* 2008a; Yoshikawa *et al.* 2009). Curiously, it was observed that *Lm* replicates within vacuoles in liver granuloma macrophages, termed spacious *Listeria*-containing phagosomes (SLAPs),

a process that is mediated by LLO. LLO impedes phagosome maturation, allowing bacterial replication and survival within these compartments, thereafter evading host immune responses and establishing a persistent infection (Birmingham *et al.* 2008b). Apoptosis is a program of cell death with a pivotal role along the lymphocyte development. *Lm* has evolved strategies to induce immune cells apoptosis, limiting inflammation and creating a permissive environment for bacterial growth (Carrero *et al.* 2006).
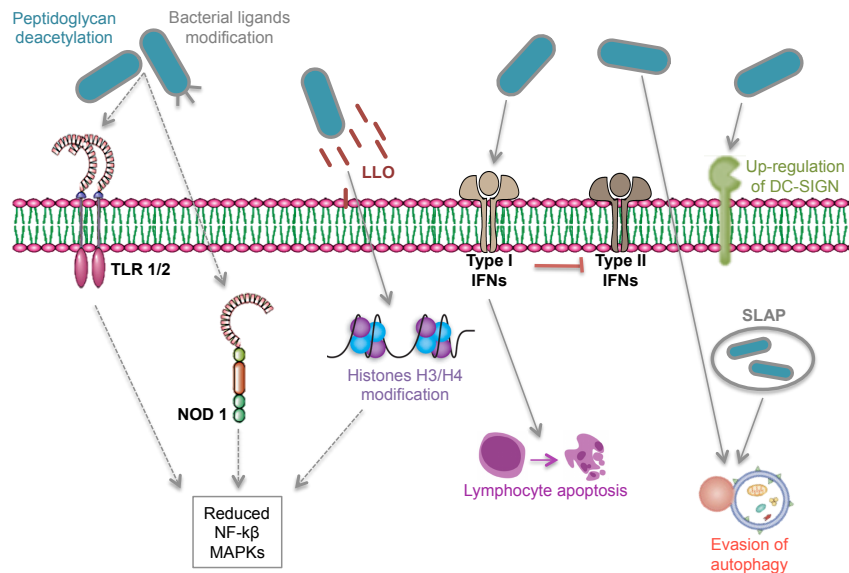


**Fig. 11.** Strategies employed by *Lm* for the evasion of host innate immune response. Adapted from (Regan *et al.* 2014).

# D. SCAVENGER RECEPTORS

This section aims to address the role of an emergent family of PRRs, named scavenger receptors, their structural and biochemical properties, as well as their most common mechanisms of action.

## D.1. General properties

Scavenger Receptors (SRs) compose a diverse family of evolutionary conserved proteins that are functionally and structurally distinct. They are soluble or cell surface proteins able to bind to a wide range of ligands and stimulate the removal of both non-self and modified-self targets. Although SRs were originally implicated on the recognition and binding of modified lipoproteins, it is now appreciated that they identify and remove unwanted entities, mainly polyionic ligands, such as apoptotic cells, mineral-laden debris, damaged proteins, cholesterol ester, phospholipids, proteoglycans, ferritin and carbohydrates, or non-self ligands including microorganisms and foreign particles (Areschoug *et al.* 2008; Areschoug *et al.* 2009; Mukhopadhyay *et al.* 2004; Pluddemann *et al.* 2006). The removal of these ligands is usually undertaken by different mechanisms, including endocytosis or more complexes processes such as phagocytosis or macropinocytosis (Prabhudas *et al.* 2014). SRs are usually expressed in cells patrolling potential portals of pathogen entry, including macrophages, neutrophils, dendritic cells, microglia and B cells. Nonetheless, there are also evidences that they are expressed in several endothelial and epithelial cells (Mukhopadhyay *et al.* 2004; Murphy *et al.* 2005; Platt *et al.* 2001).

## D.2. Classification, genetics and expression

Currently, SRs are categorized into 10 classes (A-J) grouped according to their sequence similarity or common structural features (Figure 12) (Prabhudas *et al.* 2014).

### D.2.1. Class A

The members of this class are type II membrane proteins composed by a N-terminus with a short cytoplasmic domain, a single transmembrane region and a long extracellular domain responsible for ligand recognition. A particular signature of this class is the presence of a collagen-like domain with collagen-binding activity; it comprises SCARA1, SCARA3, SCARA5, SRCL and MARCO (Figure 12) (Gowen *et al.* 2001; Kodama *et al.* 1990; Zani *et al.* 2015).

SR-A (SCARA1) gene is expressed mainly on tissue macrophages and some macrophages subsets, including kupffer cells, cortical and medullary thymic macrophages. In addition, SR-A is expressed by DCs, and by vascular smooth muscle and endothelial tissues (Becker *et*

*al.* 2006; Hughes *et al.* 1995). SR-A was the first SR to be cloned and displays three alternative splice variants of the gene, namely SR-AI, SR-AII and SR-AIII. SR-AII and SR-AIII isoforms express shorter or truncated C-terminus, respectively but SR-AIII is non-functional and remains trapped in the endoplasmic reticulum (Kodama *et al.* 1990). This SR was previously shown to recognize modified LDL, both Gram-positive and Gram-negative bacteria, hepatitis C virus, heat shock proteins, proteoglycans, β-amyloid and different microbial components, including LPS, double strand RNA, DNA and unmethylated bacterial CpG (Pluddemann *et al.* 2007). Besides the participation of this receptor in atherosclerosis and in the maintenance of homeostasis, it is actively involved in cancer proliferation (Canton *et al.* 2013; Gordon 2002; Suzuki *et al.* 1997). Higher levels of SR-A are correlated with more aggressive cancer phenotype (Ohtaki *et al.* 2010; Yeung *et al.* 2015; Yoshikawa *et al.* 2012). Genetic variations of SR-A-encoding gene have been associated with multiple tumour susceptibility phenotypes (Low *et al.* 2011). In fact, tumour progression and metastasis are inhibited in SR-A knock-out mice *in vivo* in models of both ovarian and pancreatic cancer (Neyen *et al.* 2013).
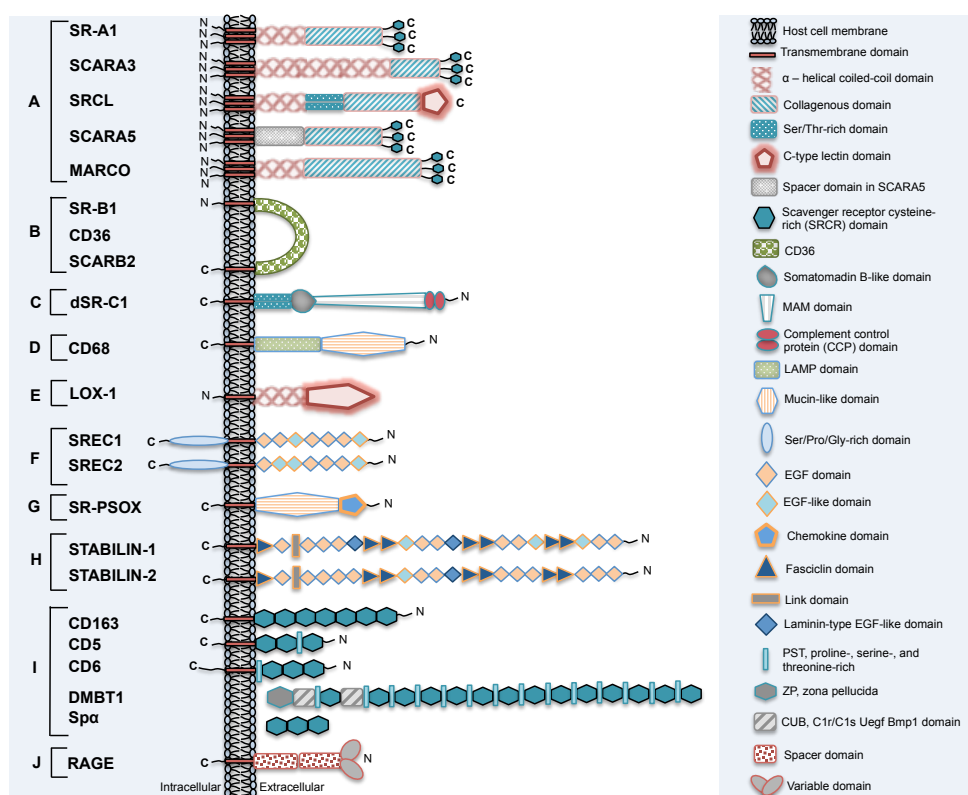


**Fig. 12.** Schematic overview of SRs families. SRs are either transmembrane or soluble proteins distributed into 10 classes, from A to J, according to their sequence or structural similarities. Protein and carbohydrate domains that compose SR members are indicated.

SR-A3 (SCARA3) transcription is stimulated by oxidative stress, thus protecting cells from the harmful effects of ROS (Canton *et al.* 2013). It was previously proposed that this SR

might work as a potent tumour suppressor in prostate cancer (Yu *et al.* 2006). Its expression is distributed in the lung, placenta, heart, intestine and epithelial cells (Whelan *et al.* 2012).

SR-A4 (SRCL) is an endocytic receptor for lipoproteins that contains a C-type lectin domain. It is widely expressed in placenta, umbilical cord, lung, skeletal muscle and heart and its expression is induced by oxidative and hypoxic stress (Selman *et al.* 2008).

SR-A5 (SCARA5), which appears do not endocytose modified LDL particles, is able to scavenge serum ferritin for iron transport. It is exclusively expressed in epithelial cells within testis, airway, thymus and the adrenal gland (Li *et al.* 2009).

SR-A6 (MARCO) is highly expressed by macrophages from liver and spleen, lymph nodes and peritoneum, being considered a dominant receptor for unopsonized particles (Elomaa *et al.* 1995). Moreover, this receptor was previously implicated in the regulation of DCs function and in antitumor immunity (Granucci *et al.* 2003).

**D.2.2. Class B**

The members that belong to class B SRs are currently characterized by the presence of two transmembrane domains flanking an extracellular loop, with both the amino and carboxyl terminal located within the cytosolic region (Figure 12). These short N- and C-terminus are involved on the regulation of trafficking and signal transduction, whereas the extracellular region of these receptors is highly N-linked glycosylated being essential to confer protection against the proteases generally found into the inflammatory sites.

SR-BI (SCARB1) gene and its alternative spliced form (SR-BII) bind high-density lipoprotein (HDL), viral and bacterial particles. It is ubiquitously expressed in multiple tissues, although it is highly exposed by organs that participate in cholesterol metabolism, including liver, adrenal and gonad (Landschulz *et al.* 1996). Mutations on this SR are commonly associated with higher risk of atherosclerosis, infertility and an impaired innate immune response (Guo *et al.* 2011; Scarselli *et al.* 2002; Yates *et al.* 2011).

CD36 (SR-B2) is the prototype class B SR and was firstly identified as a receptor for thrombospondin with the ability to modulate angiogenesis and cell-to-cell interactions (Asch *et al.* 1987). This SR has an important role to recognize and uptake oxidized phospholipids, modified LDL, apoptotic cells and amyloid proteins. Notably, it is also crucial to control atherosclerosis, Alzheimer´s disease progression, platelet activation, angiogenesis and inflammation (Liani *et al.* 2012; Silverstein *et al.* 2010; Stewart *et al.* 2010). CD36 may also play a key role as a co-receptor of TLR4 and TLR6, thus augmenting the pro-inflammatory signalling in response to oxLDL (Stewart *et al.* 2010).

SR-B3 (SCARB2/LIMP2) is mainly expressed in brain, liver, heart and macrophages and binds HDL particles (Eckhardt *et al.* 2004; Ishikawa *et al.* 2009; Tabuchi *et al.* 1997).

### D.2.3. Class C

Class C SRs have been only identified so far, in insects such as fruit flies and mosquitoes, as being involved in innate immune response against certain pathogens. The composed members of this class are either type I membrane proteins or soluble secreted proteins with an extracellular domain at the N-terminus, containing a complement control protein (CCP) prior to a MAM (meprin, A5 antigen and receptor tyrosine phosphatase Mu) motif (Figure 12) (Pearson *et al.* 1995). The lack of dSR-C1 in fruit flies showed reduced phagocytosis of both fungus and bacteria (Ramet *et al.* 2001).

### D.2.4. Class D

CD68, the only member of class D SR, is a glycosylated type I membrane protein that belongs to the lysosome-associated membrane protein family of molecules (LAMP) (Song *et al.* 2011). CD68 is composed by a short cytoplasmic tail and a large extracellular region rich in threonine and serine and mainly useful as an attachment site of carbohydrates (Figure 12). Moreover, this SR is widely expressed in monocytes and in tissue-specific macrophages in the liver, brain, bone marrow, lungs and peritoneum. It is also expressed in Langerhans cells, microglia, osteoclasts and dendritic cells (Prabhudas *et al.* 2014). It was previously demonstrated that CD68 could promote oxLDL binding, phagocytosis and also work as a cancer biomarker (Ashley *et al.* 2011; da Silva *et al.* 1999; Makitie *et al.* 2001; Ramprasad *et al.* 1996).

### D.2.5. Class E

This class of SRs contains type II membrane proteins with C-type lectin-like domains (Figure 12). The lectin-like oxidized LDL receptor 1 (LOX-1/OLR1) is expressed on macrophages, neutrophils, dendritic cells, platelets, vascular endothelial cells, smooth muscle cells and adipocytes. LOX-1 has been implicated on the recognition of oxLDL and other ligands, such as phosphatidylserine, bacteria and heat shock proteins, thus contributing for immune surveillance (Wu *et al.* 2011; Yoshimoto *et al.* 2011). The recognition of oxLDL by LOX-1 promotes endothelial dysfunction, foam cell progression and vascular smooth muscle apoptosis (Kataoka *et al.* 2001; Kume *et al.* 2001).

### D.2.6. Class F

Class F mainly comprises two members, SREC-I (SCARF1) and SREC-II (SCARF2). They are type I membrane proteins containing an extracellular domain with multiple EGF-like repeats, a single transmembrane region and a large cytoplasmic domain (Figure 12) (Adachi

*et al.* 1997; Ishii *et al.* 2002). Both receptors are predominantly expressed on DCs, macrophages, endothelial cells in the heart, ovary, placenta, lung, kidney, spleen and small intestine. Contrarily to SREC-I that binds and internalizes modified LDL, SREC-II is not able to internalize these molecules. SREC-I was shown to be an effective receptor for fungal pathogens and heat shock proteins (Means *et al.* 2009).

### D.2.7. Class G

SR-PSOX (CXCL16) is currently the only member of class G that exists in both membrane and soluble forms (Matloubian *et al.* 2000; Shimaoka *et al.* 2000). It is a type I transmembrane glycoprotein that contains a CXC chemokine motif followed by a mucin-like stalk region, a transmembrane and soluble domains. This SR is generically expressed on vascular smooth muscle cells, endothelial cells, DCs, B cells, monocytes and macrophages and it has a pro-atherogenic function (Gutwein *et al.* 2009; Wagsater *et al.* 2004). It is expressed by several tumour cells and plays a role as an adhesion molecule to activate primary and secondary T cell responses (Matsumura *et al.* 2008).

### D.2.8. Class H

Class H of SRs is particularly relevant in context of the work that we developed. It comprises two members, SR-H1 and SR-H2, which are large type I membrane glycoproteins or soluble secreted proteins. These receptors are currently named as FEEL-1/Stabilin-1 (STAB-1)/CLEVER-1 and FEEL-2/Stabilin-2 (STAB-2), which consist of **F**asciclin, **E**pidermal Growth Factor (EGF)-like, laminin type **E**GF-like and **L**ink domains (FEEL), or **C**ommon **L**ymphatic **E**ndothelial and **V**ascular **E**ndothelial **R**eceptor-1 (CLEVER-1) (Figures 12 and 13) (Irjala *et al.* 2003; Politz *et al.* 2002). STAB-1 is predominantly expressed in macrophages, mononuclear cells, hematopoietic stem cells and endothelial cells, whilst STAB-2 is mainly found in sinusoidal endothelial cells (Figure 13). Furthermore, it appears that certain organs highly express STAB-1 including spleen, liver, placenta, prostate, colon, lymph nodes and bone marrow (Adachi *et al.* 1997; Adachi *et al.* 2002). These multifunctional SRs can recognize a wide variety of ligands, such as oxLDL, acLDL, heparin, matricellular protein like SPARC (secreted protein acidic and rich in cysteine), and promote endocytic and phagocytic clearance of unwanted-self components (Qian *et al.* 2009; Workman *et al.* 2011). Generically, STAB-1 and STAB-2 were implicated in lymphocyte adhesion, transmigration, angiogenesis, intracellular trafficking and apoptotic cell clearance (Figure 13) (Prabhudas *et al.* 2014). The prototype member of this class, STAB-1, was discovered in 1991 and named, at that time MS-1 antigen. Goerdt and his colleagues found that mouse monoclonal antibody MS-1, raised against human spleen, specifically detected an endothelial cell antigen amply

expressed throughout the sinusoidal endothelia of spleen, lymph node, liver and adrenal cortex, but contrarily it was absent from non-sinusoidal continuous endothelia in these organs.
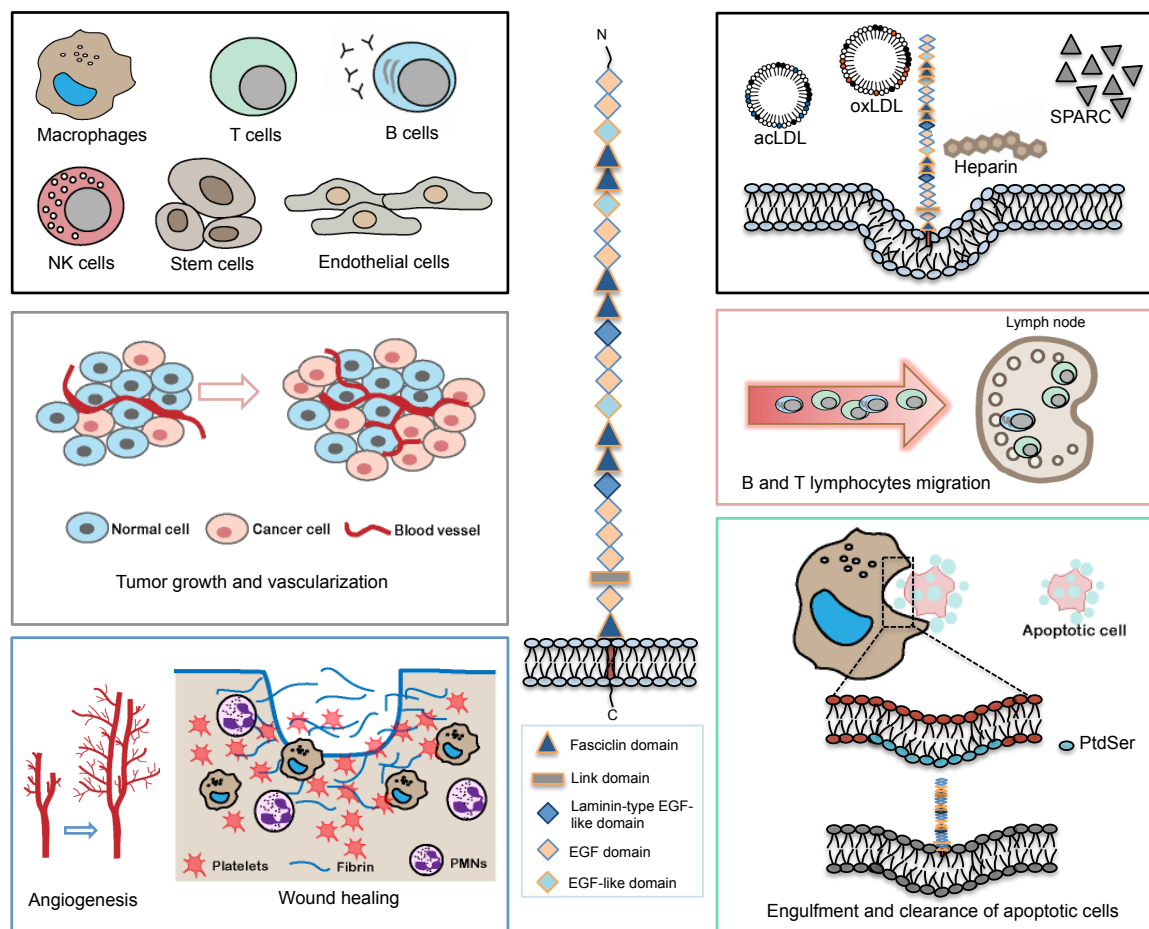


**Fig.13.** Stabilin-1 domain organization is represented in the middle. Stabilin-1 is expressed by different immune cells (macrophages, T and B cells, NK, stem cells and endothelial cells) and has a panoply of ligands (acLDL, oxLDL, SPARC and heparin). Stabilin-1 plays a role in tumour growth and vascularization, angiogenesis, wound healing, migration of T and B lymphocytes and it also contributes for apoptotic cells clearance.

Moreover, they observed by immunohistochemistry and immunoelectron microscopy, that the MS-1 antigen was usually deposited at intercellular contact regions between adjacent sinusoidal endothelial cells. Interestingly, MS-1 antigen was purified by mass spectrometry and a protein with a predicted molecular weight of 280 kDa arose as STAB-1 (Goerdt *et al.* 1991). Later on, the homologous protein STAB-2 was also cloned and described as a hyaluronan receptor of hepatic sinusoidal endothelial cells (McCourt *et al.* 1999; Politz *et al.* 2002). STAB-1 and STAB-2 share an identity at the protein level of 55%. They kept a high evolutionary conservation once the homology with their respective murine proteins is about 86% for STAB-1 and 79% for STAB-2. STAB-1 has a multitude of immunoregulatory functions in cells and it is located in several vesicular compartments. Indeed, when STAB-1 recognizes extracellular endocytic ligands, they are internalized and delivered to early

endosomes. At this point, a portion of ligand-free receptor can recycle back to the cell surface via recycling endosomes or being targeted to late endosome to be subsequently degraded in lysosomes. Then, part of the ligand that is not degraded can be delivered by Stabilin-1 to trans-Golgi network (TGN), a compartment where the receptor dissociates from the ligand allowing its secretion to the extracellular space through storage vesicles. Additionally, Stabilin-1 was implicated in intracellular sorting process, shuttling itself between endosomes and TGN (Kzhyshkowska *et al.* 2004). STAB-1 mediates the endocytosis of acLDL, SPARC, and growth hormone family member placental lactogen (PL) (Adachi *et al.* 2002; Kzhyshkowska *et al.* 2006; Kzhyshkowska *et al.* 2008). The sortin nexin 17 (SNXs), a protein involved in intracellular membrane trafficking was shown to interact with STAB-1, thus altering its intracellular recycling and degradation in endothelial cells (Adachi *et al.* 2010). It was previously demonstrated that STAB-1 is expressed on blood vessels being involved in angiogenesis phenomena, which includes wound healing, tumour vascularization and chronic inflammation of the skin, such as psoriasis (Figure 13) (Adachi *et al.* 2002; Salmi *et al.* 2004).

Heat shock proteins play important roles in cell signalling and immunity. Remarkably, STAB-1 avidly binds Hsp70-peptide complexes and promotes their efficient internalization (Theriault *et al.* 2006). *In vivo* models demonstrated that STAB-1 mediates the migration of T and B lymphocytes to the draining lymph nodes (Figure 13). In fact, using an antibody that blocks STAB-1, peritonitis is efficiently inhibited in mice due to the decreased migration of granulocytes, monocytes and lymphocytes into the inflamed peritoneum (Karikoski *et al.* 2009). Also relevant is the fact that *STAB-1* expression defines a subset of macrophages, which mediates tissue homeostasis and prevents fibrosis in chronic liver injury. Thereby the deficiency of this SR exacerbates fibrosis and delays fibrosis resolution throughout the recovery phase (Rantakari *et al.* 2016). Several authors have been associating STAB-1 with cancer. Indeed, it was shown that STAB-1 knock-out mice developed smaller primary and metastatic tumours than the wild-type mice, suggesting that STAB-1 is an immunosuppressive molecule (Figure 13) (Karikoski *et al.* 2014). In addition, STAB-1 is expressed in human breast cancer and potentiates tumour growth in mammary adenocarcinoma mouse model (Riabov *et al.* 2016). It has the ability to mediate the engulfment and clearance of apoptotic bodies, a process that is highly dependent on PS, known as an "eat-me" signal molecule. PS interacts directly with STAB-1 and is sufficient for STAB-1-mediated phagocytosis, thus contributing for the maintenance of tissue homeostasis and prevention of autoimmunity (Park *et al.* 2009). Moreover, STAB-1 expressed by hepatic sinusoidal endothelial cells is critical to clear damaged or aged red blood cells in mouse liver (Lee *et al.* 2011).

**D.2.9. Class I**

CD163 (M130) is the prototype of class I SR and a type I transmembrane protein composed by an extracellular region with nine scavenger receptor cysteine-rich (SRCR) domains, a transmembrane domain followed by a short intracellular cytoplasmic tail. CD163 is exclusively expressed by monocytes and macrophages and binds to haptoglobin/hemoglobin (Hgb) complexes to eliminate them by endocytosis during intravascular haemolysis (Kristiansen *et al.* 2001). It exists not only as a membrane-bound molecule but also as a soluble form in plasma and other tissue fluids (Etzerodt *et al.* 2010). M160 is a long tail variant of CD163 also expressed by myeloid cells and intimately associated with monocyte differentiation into macrophages (Gronlund *et al.* 2000).

The CD5 and CD6 belong to class I and are scavenger receptor cysteine-rich (SRCR) that are constitutively expressed on T cells and B cells, which are extremely important to regulate lymphocyte selection and immune tolerance (Aruffo *et al.* 1997; Padilla *et al.* 2000; Tarakhovsky *et al.* 1995). DMBT1 (gp340) also belongs to the SRCR and is located on B- and T-lymphocytes but also on macrophages (Holmskov *et al.* 1997).

**D.2.10. Class J**

The only member of this class is the receptor for advanced glycation end-products (RAGE/AGER), which is a multi-ligand transmembrane receptor that belongs to the immunoglobulin gene superfamily and is expressed by endothelial cells, hepatocytes, smooth muscle cells and monocytes (Ramasamy *et al.* 2009). RAGE has a short cytoplasmic domain and an extracellular region essential to recognize diverse ligands, including advanced glycation end-products, glycosaminoglycan, collagen and complement C3 (Yu *et al.* 2015). This SR is mainly associated with the recognition of some endogenous molecules usually released in conditions of physiological stress, infection or chronic inflammation (Ibrahim *et al.* 2013).

**D.3. Scavenger receptors and disease**

Regarding the enormous repertoire of ligands that SRs recognize, it is not surprising that they are intimately associated with the maintenance of host homeostasis and pathogenesis of several diseases.

**D.3.1. Atherosclerosis**

Coronary artery disease and cerebrovascular disease are the most common forms of cardiovascular disease and the pathological process behind this life threatening is

atherosclerosis, which underlies the leading cause of death in industrialized societies (Lloyd-Jones *et al.* 2010). Atherosclerosis is a chronic inflammatory disease characterized by an inflammatory response of endothelial cells, resultant from the circulating LDL particles that accumulate over time in the arterial intima. These LDL particles undergo several chemical modifications, including oxidation and acetylation (Sawamura *et al.* 1997; Steinbrecher *et al.* 1984). This phenomena leads to increased expression of adhesion molecules and chemokines, which are responsible for the recruitment and infiltration of circulating monocytes and T-cells that promote local inflammation. The accumulation of both lipids and immune cells in the artery wall, together with the formation of a fibrous cap mainly composed by collagen, constitute the atherosclerotic plaque. Subsequently, a more complex necrotic core in the lesion is formed with the accumulation of apoptotic cells, necrotic cells and cell debris (Hansson *et al.* 2011). In the intima, macrophages *up-regulate* their SRs that further uptake modified LDL particles, leading to intracellular cholesterol deposition. The eventual conversion of subendothelial macrophages into cholesterol-laden foam cells, which is characteristic of the atherosclerotic lesion, results from the inability of macrophages to properly process modified lipoproteins (Murphy *et al.* 2005).

### D.3.2. Type 2 diabetes

Diabetes mellitus is a common metabolic disorder worldwide, characterized by chronic hyperglycaemia caused by the accumulation of fatty acids and lipid metabolites that alter insulin signalling (Kennedy *et al.* 2011). CD36 was found to be a fatty acid translocase in certain insulin-sensitive tissues such as adipocytes (Abumrad *et al.* 1993). Strong evidences show that this transporter protein taken up nearly 70% of fatty acids in the heart. Actually, the continuous accumulation of lipids is the primary cause of insulin resistance (Coort *et al.* 2002). *In vivo* studies on spontaneous hypertensive rats containing a *CD36* gene mutation revealed a defective transport of long-chain fatty acids by adipocytes leading to type 2 diabetes (Aitman *et al.* 1999).

### D.3.3. Alzheimer´s disease

Alzheimer´s disease is one of the most prevalent forms of dementia nowadays, which consists of senile plaques that contain β-amyloid fibrils, microglia and astrocytes (Heneka *et al.* 2007; Wilkinson *et al.* 2012). Interestingly, it was previously reported that microglia bind to β-amyloid fibrils through SR-AI and CD36 (Chung *et al.* 2001; El Khoury *et al.* 2003). Indeed, CD36 is capable to form a complex with both TLR4 and TLR6, in the presence of β-amyloid that leads to the secretion of IL-1β and production of ROS. These molecules and

other inflammatory products are responsible for neuronal cell death that is a hallmark of this disease (Stewart *et al.* 2010).

### D.3.4. Cancer

As previously mentioned, SRs are involved in cancer progression possibly because they control pro-inflammatory responses that are deregulated in cancer (Bak *et al.* 2007). Distinct physiological and pathological conditions, such as cellular adhesion, antigen presentation and apoptotic cell clearance are key functions of SRs to regulate cancer development (Murshid *et al.* 2012; Park *et al.* 2007; Santiago-Garcia *et al.* 2003; Shimaoka *et al.* 2004). Several authors have revealed that SRs expression on macrophages is important for tumour progression and metastasis both *in vitro* and *in vivo.* Remarkably, some SRs have been exploited as specific diagnostic or prognostic markers in several cancer pathologies (Yu *et al.* 2015).

### D.4. SRs: Promiscuous Players During Microbial Pathogenesis

This section explores important findings regarding the role of SRs in pathogen infection. This version will be submitted to the Virulence Journal and is presented bellow.

# Scavenger Receptors: Promiscuous Players During Microbial Pathogenesis

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Innate immunity is the most broadly effective host defence, being essential to clear the majority of microbial infections. Scavenger Receptors (SRs) comprise a family of sensors expressed in a multitude of host cells, whose dual role during microbial pathogenesis gained importance over recent years. SRs regulate the recruitment of immune cells and control both host inflammatory response and bacterial load. Contrarily, bacteria have evolved different strategies to overcome immune response, avoid recognition by SRs and exploit them to favour infection. Here, we discuss the most relevant findings regarding the interplay between SRs and pathogens, revealing how these multifunctional proteins recognize a panoply of ligands and act as bacterial phagocytic receptors.

## Scavenger Receptors: an emergent family of Pattern Recognition Receptors

Host cells are effective guardians of the immune response through the expression of complex surveillance systems, including the Pattern Recognition Receptors (PRRs) (Pluddemann *et al.* 2011). SRs compose a diverse and evolutionary conserved family of PRRs that are functionally and structurally distinct. They are soluble or cell surface associated proteins originally implicated on the recognition and binding of modified lipoproteins. However, it is now appreciated that SRs eliminate a number of altered self and non-self ligands (extensively reviewed elsewhere) (Areschoug *et al.* 2008; Areschoug *et al.* 2009; Mukhopadhyay *et al.* 2004; Pluddemann *et al.* 2006). Diverse cell processes ranging from endocytosis to phagocytosis or macropinocytosis are usually undertaken to eliminate unwanted ligands and maintain homeostasis (Prabhudas *et al.* 2014). Importantly, SRs are widely expressed in cells patrolling potential portals of pathogen entry, such as macrophages, neutrophils, dendritic cells, microglia and B cells, but they are also expressed in endothelial and epithelial cells (Mukhopadhyay *et al.* 2004; Murphy *et al.* 2005; Platt *et al.* 2001). Currently, SRs are categorized into 10 classes (A-J) grouped according to their sequence similarity or common structural features (Figure 1) (Prabhudas *et al.* 2014). Several SRs have been reported to play opposite roles during bacterial infection: whereas at portals of pathogen invasion SRs recognize a myriad of microbial proteins activating downstream immune responses to fight and eliminate the pathogen, some pathogens hijack SRs function exploiting them to bind and

invade cells, thus promoting intracellular survival and proliferation. This review will focus on SRs, as an emergent family of PRRs, and their interplay with different pathogens to either promote an effective innate immune response or by the contrary, favour microbial pathogenesis.
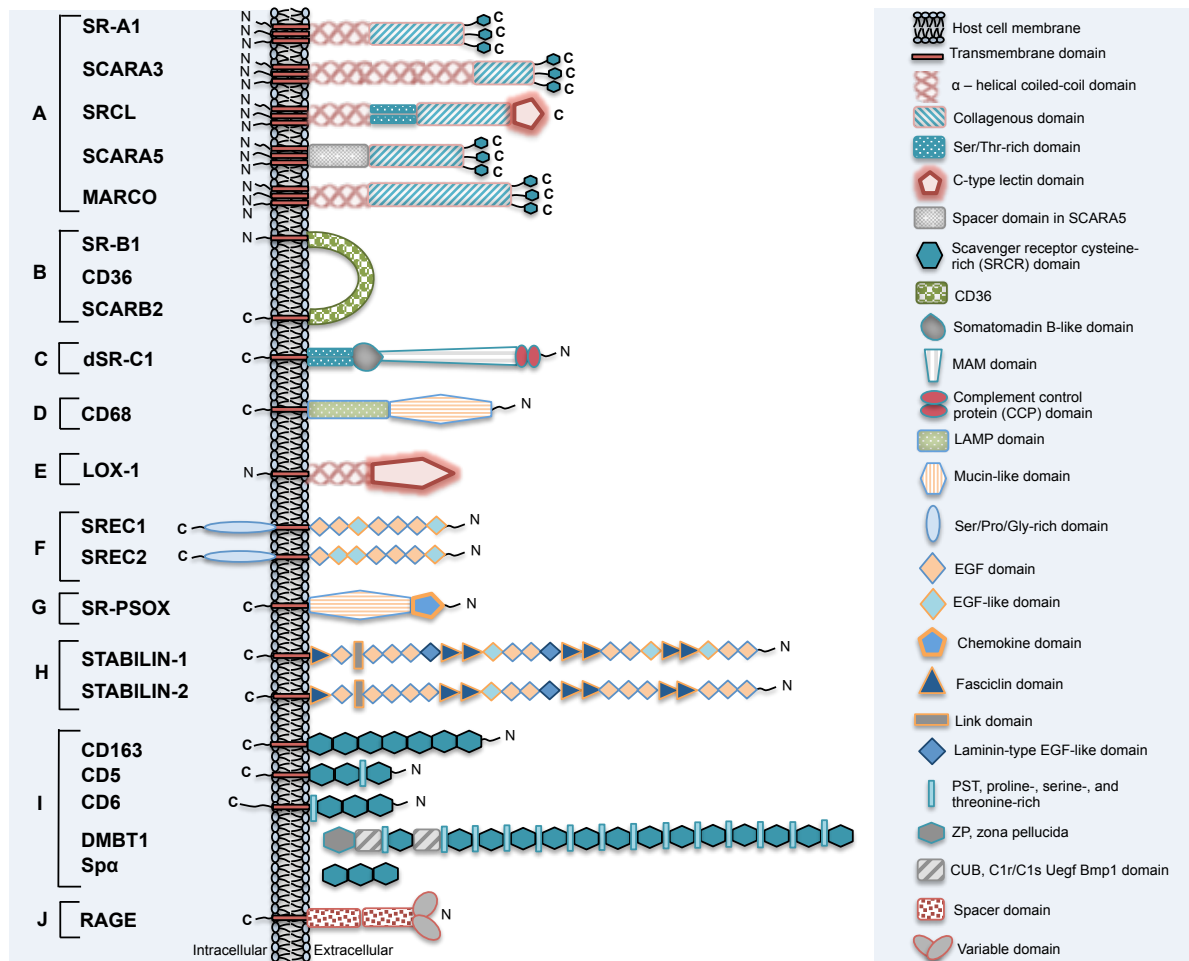


**Fig. 1**. Schematic overview of SRs families. SRs are either transmembrane or soluble proteins distributed into 10 classes, from A to J, according to their sequence or structural similarities. Protein and carbohydrate domains that compose SR members are indicated.

## Scavenger Receptors from Class A

Class A SR includes five type II membrane proteins containing a collagen-like domain with collagen-binding activity (Figure 1) (Gowen *et al.* 2001; Kodama *et al.* 1990; Zani *et al.* 2015). These proteins are primarily expressed in tissue-resident macrophages and dendritic cells (Hughes *et al.* 1995). Their role in bacterial pathogenesis was essentially reported for Scavenger Receptor A (SR-A) and Macrophage Receptor with Collagenous Structure (MARCO).

## SR-A

SR-A has long been shown to bind to *Escherichia coli* lipopolysaccharide (LPS) and lipoteichoic acids from some Gram-positive bacteria and was thus implicated in host defence

(Dunne *et al.* 1994; Hampton *et al.* 1991). Further studies revealed its involvement in the pathogenesis of different microorganisms and importantly, pointed the SR-A dual role in infection, either by favouring the host response contributing for pathogen elimination or promoting survival and pathogen dissemination.

In particular, SR-A was reported detrimental for the host during **Mycobacterium tuberculosis** (*Mtb*) infection of alveolar macrophages. Indeed, SR-A knock-out (KO) mice display increased survival to pulmonary tuberculosis (Ulrichs *et al.* 2006). Histopathology analysis of infected lungs showed *Mtb* within cholesterol clefts and multinucleated foam cells in SR-A KO mice, whilst necrotic macrophages obstructing alveolar and bronchial spaces were detected in wild type (WT) mice (Sever-Chroneos *et al.* 2011). In addition, the analysis of cell populations in infected lungs revealed increased recruitment of CD4+ lymphocytes and antigen-presenting cells (APCs) in SR-A KO mice, suggesting SR-A as a negative regulator of pulmonary adaptive immunity during chronic *Mtb* infection (Figure 2) (Sever-Chroneos *et al.* 2011). SR-A was also reported to be disadvantageous for the host during **Cryptococcus neoformans** and **Pneumocystis carinii** infections. As described for Mtb infection, SR-A KO mice displayed improved pulmonary fungal clearance, which is intimately associated with higher accumulation of CD4$^+$ T cells and CD11b$^+$ myeloid cells in the lungs (Hollifield *et al.* 2007; Qiu *et al.* 2013). So far, these reports suggest that SR-A confers an advantage for pathogens infecting host lungs and reinforce the idea that SRs can be exploited by pathogens to promote their survival within the host. The impact of SR-A on **Brucella abortus** infection was also addressed. SR-A KO mice were less prone to *B. abortus,* which is probably due to decreased bacterial internalization and intracellular replication within SR-A-deficient macrophages (Kim *et al.* 2004). Importantly, bacteria evolved different mechanisms to avoid host cells recognition. The role of SR-A in the non-opsonic phagocytosis of two major Gram-positive pathogens, **Streptococcus agalactiae** (GBS) and **Streptococcus pyogenes***,* by murine bone marrow-derived macrophages was also investigated. The polysaccharide capsule or the bacterial surface lipoprotein Blr of GBS and the surface M protein of *S. pyogenes* were shown to prevent SR-A-mediated recognition and non-opsonic phagocytosis (Figure 2) (Areschoug *et al.* 2008; Carlsson *et al.* 2005).

Reversely, SR-A also appears to be crucial to protect the host from pathogen damage. The role of SR-A was assessed in the context of infection by **Neisseria meningitidis**, the leading cause of life-threatening meningococcal meningitis and septicemia, especially in infants. *N. meningitidis* was shown to bind bone marrow-derived macrophages almost exclusively through SR-A and independently from LPS, suggesting that SR-A-expressing macrophages may be critical in the innate host immune response to meningococci (Peiser *et al.* 2002). Additionally, binding assays aiming to uncover SR-A ligands were performed, identifying three *N. meningitidis* proteins: NMB1220, NMB0278 and NMB0667 (Figure 2). Soluble forms of these

proteins were shown to block the binding of meningococci to CHO cells stably transfected with SR-A. Nevertheless, the authors claimed that only NMB1220 induced SR-A-mediated endocytosis in macrophages (Peiser *et al.* 2006). The first line of defence against invading *N. meningitidis* is the highly phagocytic dendritic cells (DCs). It was demonstrated that SR-A-mediated phagocytosis of viable *N. meningitidis* highly stimulates DCs to the release of pro-inflammatory cytokines, including TNF-α, IL-1β and IL-6 (Figure 2). Interestingly, SR-A was reported transiently dephosphorylated shortly after SR contact with the bacteria, suggesting that SR-A engagement in response to bacterial binding elicits intracellular signalling to trigger a cellular response (Villwock et al. 2008). Importantly, *N. meningitidis* infected SR-A KO mice showed reduced survival as compared to WT mice, exhibiting higher levels of bacteraemia and circulating IL-6, which is commonly associated with meningococcal septicaemia in humans (Figure 2) (Pluddemann *et al.* 2009; Prins *et al.* 1998). Additionally, the lack of SR-A was reported to impair host survival against infections by **Mycoplasma pulmonis** and **Staphylococcus aureus** (Figure 2) (Booth *et al.* 2014; Thomas *et al.* 2000).

Concerning infection by **Listeria monocytogenes** different studies pointed to the beneficial role of different SR-A isoforms in host protection. SR-AI KO mice showed higher susceptibility to *L. monocytogenes* infection, with highly increased bacterial burden and decreased host survival (Suzuki *et al.* 1997). The increased susceptibility of SR-AI KO mice to *L. monocytogenes* infection was proposed to be related with a defect in the uptake or killing of bacteria by macrophages (Figure 2) (Suzuki *et al.* 1997). Mice KO for both SR-AI and SR-AII were also shown to be more susceptible to *L. monocytogenes* infection and displayed increased hepatic granuloma formation regarding their number, dimension and persistence throughout the infection (Figure 2) (Ishiguro *et al.* 2001). Concomitantly, Kupffer cells and peritoneal macrophages from SR-AI/II KO mice showed decreased *L. monocytogenes* phagocytosis. Moreover, the listericidal phagocytic activity of WT macrophages was impaired in the presence of an anti-SR-AI/II blocking antibody (Ishiguro *et al.* 2001). Strikingly, SR-AI/II were thus proposed to play a crucial role in host defence against *L. monocytogenes* infection not only by acting as a receptor for its phagocytosis, but also by mediating listericidal mechanisms (Figure 2) (Ishiguro *et al.* 2001).

Notably, the role of SR-A in regulating the crosstalk between innate and adaptive immune response to pathogen infection remains unclear. SR-A KO mice displayed exacerbated death upon infection of the parasite **Schistosoma japonicum**. SR-A was elegantly shown to directly interact with interferon-regulatory factor 5 (IRF5) in *S. japonicum* infected macrophages and suppress IRF5 nuclear translocation, which shifts macrophage polarization from M1 towards M2, thus stimulating T-helper responses from type 2. In this context, SR-A is thus able to modulate macrophage polarization and fine-tune T-cell differentiation (Xu *et al.* 2017).

Pulmonary surfactant is composed by a variety of lipids and proteins, including surfactant protein A (SP-A), which prevents alveoli collapsing during expiration (Kuroki *et al.* 1994). It was reported that SP-A increases cell surface localization of SR-A and potentiates SR-A-mediated phagocytosis of **Streptococcus pneumoniae** by alveolar macrophages promoting bacterial clearance (Kuronuma *et al.* 2004). In agreement, SR-A KO mice displayed increased mortality rate upon *S. pneumoniae* infection, possibly due to an impaired phagocytosis, that lead to reduced clearance of live bacteria from the lungs and highly increased pneumonic inflammation (Figure 2) (Arredouani *et al.* 2006). Altogether, the data compiled above show that SR-A enhances host resistance to several pathogens, being essential to limit the severity of certain infections.
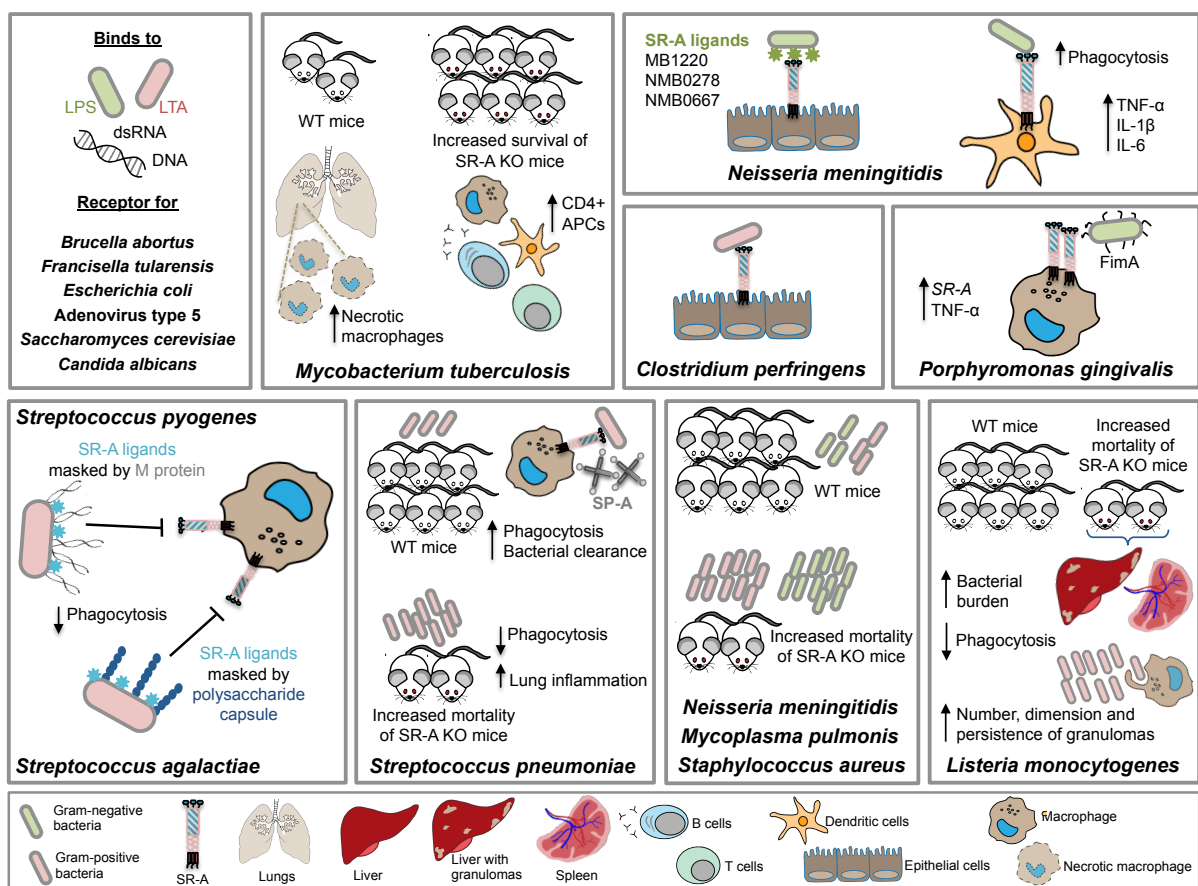


**Fig. 2. The most relevant functions of SR-A in microbial pathogenesis.** SR-A binds to different pathogen components and act as receptor for different bacteria, virus and yeasts. SR-A is a negative regulator of chronic *Mtb* infection, as SR-A KO mice show increased lymphocyte and APCs recruitment. Contrarily, SR-A positively regulates immune response against *S. pneumoniae, N. meningitidis, M. pulmonis, S. aureus* and *L. monocytogenes* mainly by potentiating bacterial phagocytosis and clearance. *In vitro* assays show that SR-A exposed at the surface of epithelial cells mediates adherence to *C. perfringens* and in the case of *N. meningitidis* and *P. gingivalis*, SR-A interacts with bacterial proteins, stimulating inflammatory response. *S. pyogenes* and *S. agalactiae* display evasion mechanisms to avoid SR-A-mediated phagocytosis through the expression of M protein and polysaccharide capsule, respectively. SR-A, Scavenger Receptor A; WT, wild-type; KO, knock-down; LPS, lipopolysaccharide; LTA, lipoteichoic acid; dsRNA, double-stranded ribonucleic acid; DNA, deoxyribonucleic acid; APCs, antigen-presenting cells; FimA, fimbrillin; SP-A, surfactant protein A. See text for details.

Other reports point to the interplay between SR-A and other pathogens, however specific outcomes in the context of pathogen-infection need to be further explored. ***Clostridium perfringens***, which has the ability to survive within murine macrophage-like cell lines (O'Brien *et al.* 2000), was shown to bind to CHO cells in a SR-A-dependent manner (Figure 2) (O'Brien *et al.* 2003). ***Porphyromonas gingivalis*** induces the expression of SR-A by macrophages (Bodet *et al.* 2007). This increase is partially dependent on the major fimbriae of *P. gingivalis* FimA and promotes TNF-α secretion, thus implicating SR-A as a regulator of inflammation (Figure 2) (Baer *et al.* 2009).

Finally, SR-A was found to act as a receptor for pathogen phagocytosis. This was shown for ***Escherichia coli*** and ***Francisella tularensis*** in dendritic cells and macrophages, respectively (Amiel *et al.* 2007; Pierini 2006). Binding and phagocytosis of yeast ***Saccharomyces cerevisiae*** and ***Candida albicans*** were also shown to be dependent on SR-A (Figure 2) (Wang *et al.* 2010). In addition, SR-A was reported to bind bacterial DNA (Zhu *et al.* 2001) and double-stranded RNA (DeWitte-Orr *et al.* 2010; Limmon *et al.* 2008). SR-A was involved in the virus recognition, namely in the uptake of **Adenovirus type 5** (Ad5) and endocytosis of **Adeno-associated virus serotype 8** (Haisma *et al.* 2009; van Dijk *et al.* 2013).

SR-A appears thus as a SR capable to recognize a wide range of microbes, and contributes to pathogen containment by modulating the recruitment and the activation of phagocytic cells and regulating inflammatory response through cytokine secretion. To counteract this function, some microbes evolved strategies to evade SR-A dependent recognition and phagocytosis.

**MARCO**

MARCO also has an ambiguous involvement in pathogen infections, either being crucial or detrimental for host response. MARCO was primarily reported to bind soluble LPS and intact Gram-positive and Gram-negative bacteria, but not yeast (Elomaa *et al.* 1995; Sankala *et al.* 2002). From that point forward several studies have been doing an effort to clarify the impact of this SR in pathogen infection. The lack of MARCO was shown to induce different outcomes upon SR interaction with bacteria, virus, fungus and even parasites.

Influenza is considered nowadays a public health concern worldwide (Bridges *et al.* 2003). MARCO induces increased morbidity and mortality of mice affected by **Influenza A** pneumonia, due to a diminished neutrophilic inflammatory response (Ghosh *et al.* 2011; Xu *et al.* 2017). MARCO is also exploited by **Herpes Simplex Virus** type 1 glycoprotein C to promote cell surface adsorption and infection in the skin (Figure 3) (MacLeod *et al.* 2013). Additionally, it was demonstrated that MARCO significantly enhances **Adenovirus** infection, due to an efficient virus recognition by macrophages, through the cytoplasmic DNA sensor cGAS, that in turn potentiates a pro-inflammatory response (Maler *et al.* 2017).

In opposition, MARCO improves host resistance to several pathogens. MARCO was largely implicated in the recognition, binding and internalization of different bacteria. Aiming to unravel the role of alveolar macrophages expressing MARCO in host innate defence against pneumococcal infection, WT and MARCO KO mice were submitted to intranasal instillation of *S. pneumoniae* suspension. MARCO KO mice displayed a diminished survival rate, due to an impaired ability to clear bacteria from the lungs, characterized by increased pulmonary inflammation (Figure 3). Both *in vitro* binding of *S. pneumoniae* and *in vivo* uptake of unopsonized particles by MARCO KO alveolar macrophages were drastically impaired (Arredouani *et al.* 2004). Later, MARCO was revealed as a crucial component against *S. pneumoniae* response in the murine nasopharynx colonization, with MARCO KO mice showing a defect on bacterial clearance. In addition, MARCO deficiency abrogates cytokine production and cellular recruitment to the nasopharynx following colonization. Maximal TLR2- and NOD2-dependent NF-κB activation to ultimately clear *S. pneumoniae* was shown to be dependent on MARCO (Figure 3) (Dorrington *et al.* 2013).

MARCO-deficient macrophages do not mount an efficient inflammatory response to **Mtb** infection. MARCO, which is a tethering receptor for the cell wall glycolipid TDM (trehalose 6,6′-dimycolate), presents these lipids to the CD14/TLR2 complex (Bowdish *et al.* 2009). MARCO is essential for phagocytosis of **Mycobacterium marinum** by zebrafish macrophages, being crucial to control bacterial growth and inflammatory response (Benard *et al.* 2014). Moreover, peritoneal macrophages from MARCO-deficient mice showed impaired **Clostridium sordellii** phagocytosis, being KO mice more susceptible to *C. sordellii* uterine infection than WT mice. Moreover, MARCO also efficiently contributes to fungal containment during **Cryptococcus neoformans** infection, thus controlling the recruitment of monocytes and dendritic cells and regulating the levels of IFN-γ. Additionally, MARCO is involved in *C. neoformans* phagocytosis by resident pulmonary macrophages and dendritic cells (Xu *et al.* 2017). These findings confer an important role for MARCO as a phagocytic receptor of macrophages, essential to clear pathogens (Thelen *et al.* 2010).

Different microbial infections induce an increased expression of MARCO, suggesting a role for this SR in host defence. *In vivo* evidences demonstrated a marked and transient expression of MARCO, induced in liver Kupffer cells and red pulp macrophages of the spleen, following the intravenous administration of **L. monocytogenes** (Figure 3) (Ito *et al.* 1999). More recently, MARCO/SR-A double knock-out mice were challenged intraperitoneally with *L. monocytogenes* and, at three and five days post-infection, there was a trend toward higher bacterial levels in double KO mice spleen and liver, although WT and KO mice had equal survival rates (Chen *et al.* 2010). The expression of MARCO is rapidly induced on macrophages including Kupffer cells, upon **Bacillus Calmette-Guérin** (BCG) infection or after the injection of purified LPS (van der Laan *et al.* 1999).
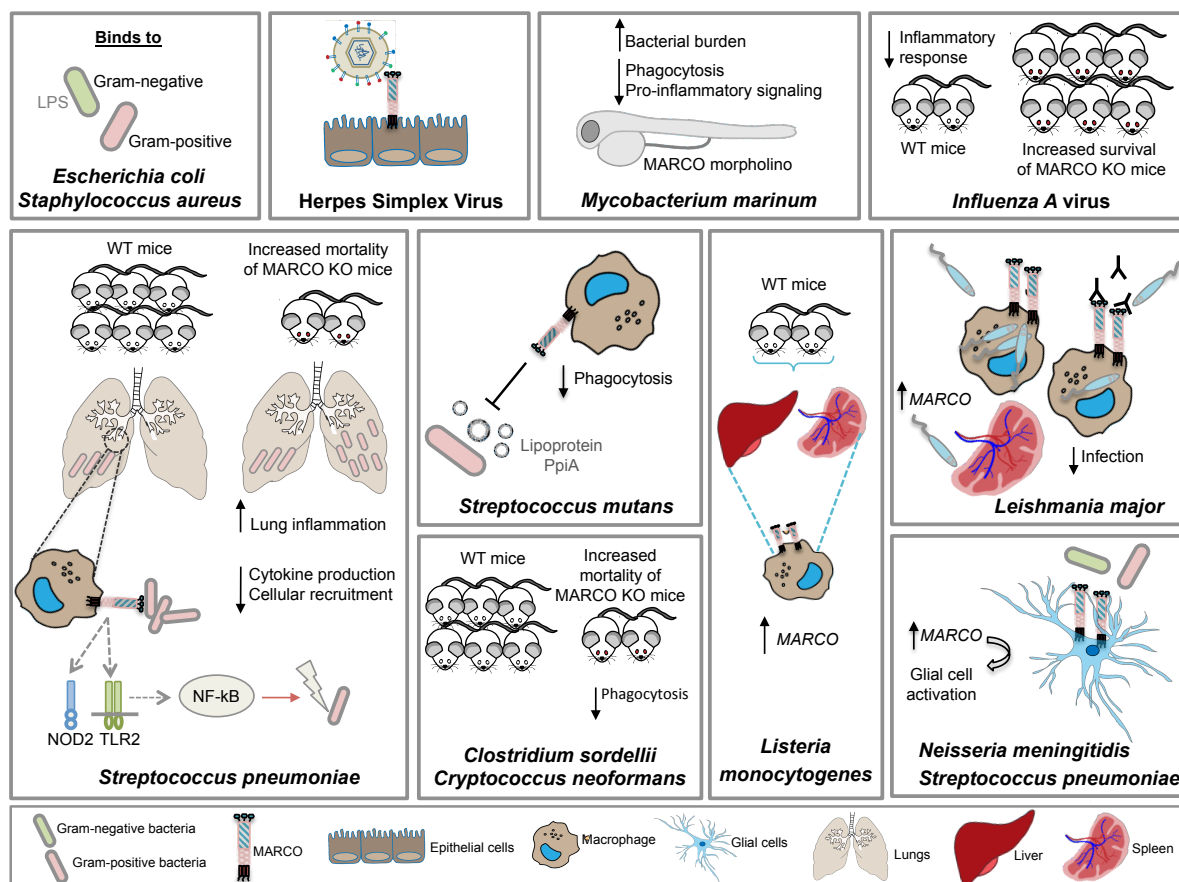
**Fig. 3. The most relevant functions of MARCO in microbial pathogenesis.** MARCO binds to *E. coli* LPS and to *S. aureus* and it promotes cell surface adsorption and skin infection by HSV. MARCO is a negative regulator of the inflammatory response against Influenza A virus. However, MARCO is essential to control host immune response to *M. marinum, S. pneumoniae, C. sordellii, L. monocytogenes* and *C. neoformans* infections, being WT mice or WT morpholino highly resistant to infection. *S. pneumoniae* clearance, which is MARCO-dependent, stimulates TLR2- and NOD2-dependent NF-κB activation. MARCO expression is *up-regulated* in macrophages, spleen and glial cells in response to *L. major, N. meningitidis, L. monocytogenes* and *S. pneumoniae* infections. The lipoprotein PpiA of *Streptococcus mutans* contributes to the anti-phagocytic activity mediated by MARCO. MARCO, macrophage receptor with collagenous structure; WT, wild-type; KO, knock-down; LPS, lipopolysaccharide; NOD2, nucleotide-binding oligomerization domain-containing protein 2; TLR2, *Toll-like receptor 2; NF-kB,* nuclear factor kappa B; PpiA, peptidyl-prolyl *cis/trans*-isomerase. See text for details.

MARCO was also implicated in the recognition of ***N. meningitidis*** independently of LPS (Mukhopadhyay *et al.* 2006). A strong increase of *MARCO* expression in meningococcal meningitis co-localized to astrocytes was detected, thus demonstrating that *N. meningitidis* induces glial cell activation *via* MARCO (Figure 3) (Braun *et al.* 2011). Furthermore, MARCO expression in mouse macrophages is increased in response to both *in vitro* and *in vivo* ***Leishmania major*** infections and the parasite infection of macrophages is partially reduced by using a specific anti-MARCO monoclonal antibody, supporting the role of MARCO in macrophage infection by *L. major* (Figure 3) (Gomes *et al.* 2009).

Contrarily, the lipoprotein PpiA of ***Streptococcus mutans*** negatively regulates the expression of MARCO at the transcriptional and translational levels, which in turn contributes for the

suppression of MARCO-mediated phagocytosis of this bacterium by macrophages (Figure 3) (Mukouhara *et al.* 2011).

So far, MARCO has been involved in the recognition of both Gram-positive and Gram-negative bacteria but also in the pathogenesis of different virus. It usually behaves as a positive regulator of pathogen infection, stimulating phagocytosis, cellular recruitment and cytokine production. Several studies have shown that MARCO is differentially regulated in response to certain pathogen infections, revealing its potential as a host innate immune receptor. This SR is also exploited by some pathogens to promote infection.

**Other class A SRs**

From the less known SRs from class A, it was reported that SCARA5, which is exclusively expressed by populations of epithelial cells, binds to heat-killed *E. coli* and *S. aureus* (Jiang *et al.* 2006). SRCL has the ability to bind and phagocytose bacteria and yeast, suggesting a possible role for this SR in host defence (Jang *et al.* 2009; Nakamura *et al.* 2001; Ohtani *et al.* 2001).

# Scavenger Receptors from class B

The members that belong to class B SRs are currently characterized by the presence of two transmembrane domains flanking an extracellular loop, with both the amino and carboxyl terminal located within the cytosolic region (Figure 1) (Asch *et al.* 1987). CD36 is the prototype class B SR and has been largely involved in microbial pathogenesis.

**SR-B**

Importantly, SR-B, whose expression is modulated upon cell contact with **Hepatitis C virus** (HCV), was previously recognized to be essential for virus uptake, cell-to-cell transmission and cross-presentation by human dendritic cells. This may have an impact on the design of HCV vaccines and immunotherapeutic approaches (Barth *et al.* 2008; Fan *et al.* 2017; Schwarz *et al.* 2009).

SR-B is a multi-recognition receptor crucial to potentiate host response against bacterial pathogens. SR-BI KO mice showed increased mortality throughout ***Klebsiella pneumoniae*** infection, characterized by higher bacterial burden in the lung and in the blood, increased serum cytokines and neutrophils recruitment to the infected airspace, impaired phagocytic clearance and markedly organ injury (Gowdy *et al.* 2015).

Moreover, SR-B is required for ***Chlamydia pneumoniae*** infection, being important for bacteria attachment, internalization and growth into epithelial cells, and for ***Brucella abortus*** phagocytic activity by trophoblasts giant cells (Korhonen *et al.* 2012; Watanabe *et al.* 2010).

SR-B is also involved in **Mtb** recognition, although no relevant outcome in anti-mycobacterial immunity was revealed so far (Schafer *et al.* 2009).

Recently, an interesting study has claimed that SR-BI prevented tissue damage without altering pathogen burden, after intraperitoneal mice infection with increasing loads of **L. monocytogenes**. Importantly, it was shown that autophagy mechanisms were activated by SR-BI to protect organs, such as the liver and spleen, selectively from the collateral damage induced by antibacterial defences (Vishnyakova *et al.* 2006). SR-BI functions as a regulator of lipid domain formation, thus increasing cellular uptake mechanisms, such as macropinocytosis and promoting autophagic flux. Induced autophagy then suppresses tissue damage by preventing necrotic cell formation in the core of infectious foci, stimulating apoptotic cell clearance and decreasing not only the tissue infiltration but also the accumulation of neutrophils and inflammatory macrophages (Pfeiler *et al.* 2016).

The interplay between SR-B and parasites was also addressed. SR-BI significantly boosts hepatocyte permissiveness to **Plasmodium falciparum**, **P. yoelii**, and **P. berghei** entry and promotes parasite development. Interestingly, SR-B is responsible for the regulation of CD81 localization at the plasma membrane, mediating a membrane rearrangement that is more permissive to sporozoites penetration (Yalaoui *et al.* 2008). So far, these data indicate that SR-B predominantly favours host response mechanisms by containing pathogen infection.

**CD36**

CD36 has been shown to be a sensor of both Gram-positive LTA and Gram-negative LPS, working as a phagocytic receptor for a number of pathogenic bacteria, such as **E. coli**, **Klebsiella pneumoniae**, **Salmonella typhimurium**, **S. aureus** and **Enterococcus faecalis** (Figure 4) (Baranova *et al.* 2008). In particular, bacterial chaperonin 60, GroEL and LPS could potentially contribute to bacteria-induced inflammation *in vitro* and in *vivo*, acting via SR-B (Baranova *et al.* 2012). Strikingly, CD36 has a strong impact in innate immunity and host defence by recognizing both bacterial cell wall and cytosolic GroEL.

**S. aureus** is the primary cause of skin infections, characterized by a local inflammatory response that often precedes the formation of necrotic lesions. This dermonecrosis is mainly dependent on the action of a pore-forming toxin α-hemolysin (Hla). Using a mouse model of *S. aureus* dermonecrosis, CD36 was found to negatively regulate dermonecrosis following infection with Hla-producing *S. aureus.* This regulation is largely independent of bacterial load because dermonecrosis was also limited following subcutaneous intoxication with *S. aureus*–secreted and purified Hla. CD36 KO mice intoxicated with sterile *S. aureus* supernatant had enhanced dermonecrosis, with increased neutrophil accumulation and local IL-1β expression. The contribution of neutrophils to tissue injury was confirmed since dermonecrosis was almost abolished upon neutrophil depletion (Figure 4) (Castleman *et al.* 2015). Tet38 efflux pump,

which is involved in *S. aureus* internalization by lung epithelial cells, was unveiled to play a role in bacterial internalization by interacting with CD36 (Truong-Bolduc *et al.* 2017). Moreover, CD36 also provides host protection against **K. pneumoniae** and **S. pneumoniae** intrapulmonary infections, modulating host inflammatory response and enhancing macrophage phagocytosis (Olonisakin *et al.* 2016; Sharif *et al.* 2013). In opposition, CD36 deficiency confers resistance to **Mtb** infection. Thus, CD36 KO mice displayed reduced bacterial burden in both spleen and liver, decreased density of granulomas and diminished levels of circulating tumour necrosis factor (TNF) (Figure 4) (Hawkes *et al.* 2010). Furthermore, **P. gingivalis** infected macrophages *up-regulate* CD36 expression via ERK/NF-kB pathway (Liang *et al.* 2016). FACS analysis demonstrated that the uptake of **L. monocytogenes** was 20-fold higher in HeLa cells overexpressing CD36 when compared with mock-HeLa cells (Vishnyakova *et al.* 2006).

Regarding the relevance of this SR for parasite infection, CD36 was demonstrated to be the most common target of the PfEMP1 proteins of **P. falciparum**, tethering parasite-infected erythrocytes to endothelial receptors, thus preventing their splenic clearance and allowing increased parasitaemia (Figure 4) (Hsieh *et al.* 2016). Importantly, CD36 appears to have a dual role in infection, being useful or not for pathogen elimination.

## Scavenger Receptors from classes D and I

CD68, which is the only member of class D SR, is a glycosylated type I membrane protein that belongs to the lysosome-associated membrane protein family (LAMP) (Figure 1) (Song *et al.* 2011). Class I SRs are also type I transmembrane proteins characterized by an extracellular region with scavenger receptor cysteine-rich (SRCR) domains (Figure 1) (Kristiansen *et al.* 2001). Less is known about the role of these classes in microbial pathogenesis.

Malaria still remains a devastating disease worldwide and new target drugs need to be identified (White 2017). **Plasmodium** sporozoites enter the blood circulation and infect the liver. The synthetic peptide P39 was shown to interact with CD68 at the surface of Kupffer cells, inhibiting sporozoite entry and liver infection. Understanding the underlying molecular mechanisms may lead to the development of new therapeutic strategies to control malaria (Cha *et al.* 2015).

CD163 was found to be important in the inflammation process, but it could also function as a receptor for Gram-positive and Gram-negative bacteria. In particular, it was demonstrated that soluble CD163 recognizes a specific fragment of fibronectin bound to **S. aureus** surface molecules, leading to increased phagocytosis and antimicrobial defences. (Fabriek *et al.* 2009; Kneidl *et al.* 2012; Law *et al.* 1993).

Although CD5 and CD6 are both implicated on the modulation and signalling of T and B cell receptors, CD5 is well adapted to interact only with fungal associated ligands, while CD6 has

evolved to recognize bacterial ones (Soldevila *et al.* 2011). On the other hand DMBT1 was previously found to inhibit **HIV-1** infection (Chu *et al.* 2013) and to be associated with the activation of the complement (Leito *et al.* 2011). Human Spα has the same domain organization as the extracellular region of CD5 and CD6 and may regulate monocyte activation, function and survival (Figure 1) (Gebe *et al.* 1997). Human recombinant Spα, that is the homologue of the mouse protein AIM, was found to interact *in vitro* with Gram-negative and Gram-positive bacteria, including **L. monocytogenes** (Bessa Pereira *et al.* 2016; Gebe *et al.* 2000; Sarrias *et al.* 2005).

## Scavenger Receptors from classes E and F

Class E of SRs contains type II membrane proteins with C-type lectin-like domains, whether class F SRs are type I membrane proteins, containing an extracellular domain with Epidermal Growth Factor (EGF)-like repeats (Figure 1) (Politz *et al.* 2002; Sawamura *et al.* 1997).

### LOX-1

LOX-1 was previously implicated on adhesion of **S. aureus** and **E. coli** (Shimaoka *et al.* 2001). Expression of this SR was shown to be *up-regulated* in response to **Aspergillus fumigatus**, **C. pneumoniae** and **Herpes simplex virus I** (HSV-1) infection, which may reflects its role in host defence mechanisms against pathogen infections (Campbell *et al.* 2013; Chirathaworn *et al.* 2004; Gao *et al.* 2016; Li *et al.* 2015; Yoshida *et al.* 2006). In addition, surface-associated GroEL in *E. coli* was recognized by LOX-1 on macrophages, enhanced their phagocytic capacity and made mice more susceptible to bacterial-induced peritonitis (Figure 4) (Zhu *et al.* 2013). OmpA from **K. pneumoniae** was reported to bind LOX1 and SREC, activating macrophages and dendritic cells (DCs) in a TLR2-dependent way. Cellular recognition of *K. pneumoniae* OmpA activates a pro-inflammatory mechanisms beneficial for host innate immune response (Jeannin *et al.* 2005).

### SREC

SREC was previously reported to bind heat-killed **S. aureus** and **E. coli** (Jeannin *et al.* 2005). In addition, SREC was described to recognize and internalize molecular chaperones and heat shock proteins (Gong *et al.* 2009). Curiously, **N. meningitidis** expressing the serotype A of the major outer membrane porin PorB (PorB$_{IA}$) interacts with SREC via Gp96 mainly allowing adherence to host cells. However, the invasion process requires the dissociation of Gp96 from SREC, since SREC is masked by surface-exposed Gp96, impairing PorB$_{IA}$ binding (Figure 4).
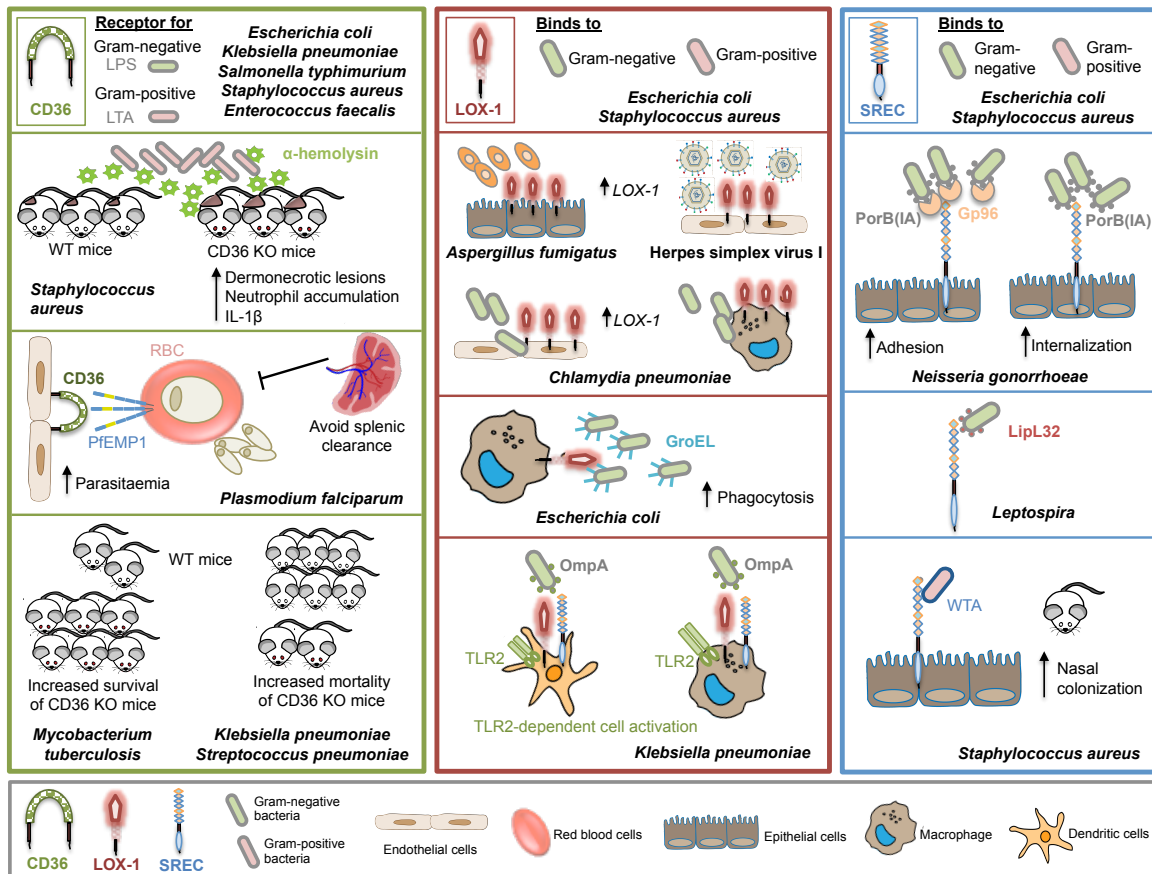
**Fig. 4. The most relevant functions of CD36, LOX-1 and SREC in microbial pathogenesis.** CD36 is a receptor for a number of Gram-positive and Gram-negative bacteria. CD36 negatively regulates dermonecrosis upon mice intoxication with *S. aureus*-producing α-hemolysin. PfEMP1 proteins of *Plasmodium falciparum* target CD36, tethering parasite-infected red-blood cells to endothelial receptors to avoid splenic clearance. CD36 plays a crucial role in host defence against *K. pneumoniae* and *S. pneumoniae,* while it diminishes mice survival upon *M. tuberculosis* infection. LOX-1 and SREC bind to *E. coli* and *S. aureus. LOX-1* expression is increased in response to *A. fumigatus*, HSV and *C. pneumoniae* within different cell types. GroEL from *E. coli* is recognized by LOX-1 and stimulates bacterial phagocytosis, whether OmpA from *K. pneumoniae* interacts with both LOX-1 and SREC to activate cells in a TLR2-dependent way. PorB$_{IA}$ from *N. gonorrhoeae* interacts with SREC via Gp96 allowing adherence to host cells. For bacterial invasion Gp96 needs to dissociate from SREC. SREC also interacts with LipL32 from *Leptospira* and with WTA of *S. aureus*, stimulating nasal colonization. CD36, cluster of differentiation 36; LOX-1, oxidized low-density lipoprotein receptor 1; SREC, *scavenger receptor expressed by endothelial cells*; WT, wild-type; KO, knock-down; RBC, red-blood cell; pfEMP1, *Plasmodium falciparum erythrocyte membrane protein 1; TLR2, toll-like receptor 2; GroEL, large oligomeric chaperone; OmpA, o*uter membrane protein A*;* PorB (IA), serotype A of the major outer membrane porin; Gp96, glycoprotein 96; LipL32, leptospiral lipoprotein; WTA, wall teichoic acids. See text for details.

In that sense, the depletion of Gp96 from host cells prevented adherence but significantly triggered gonococcal invasion (Rechner *et al.* 2007). Gp96 is an endoplasmic reticulum (ER) resident chaperone previously reported to modulate the interaction between pathogens with their host cells. It was shown to interact with the Outer Membrane Protein A (OmpA) of ***E.coli*** and with the Vip surface protein of *L. monocytogenes*, thereby supporting invasion (Cabanes *et al.* 2005; Prasadarao *et al.* 2003). SREC may also have a role in Leptospirosis since it has the potential to bind to ***Leptospira*** LipL32, which is an immunogenic outer membrane protein of the pathogen (Figure 4) (Chaemchuen *et al.* 2011). Notably is the cooperation of both SR-

AI and SREC-I with TLR2 to recognize **Hepatitis C virus** non-structural protein 3 (NS3) and induce myeloid cell activation (Beauvillain *et al.* 2010). More recently, SREC-I was found to bind **S. aureus** wall teichoic acids (WTAs) and mediate adhesion to nasal epithelial cells *in vitro* (Figure 4). Additionally, the inhibition of WTA-mediated adhesion with a specific SREC-I antibody reduces nasal colonization in the animal model (Baur *et al.* 2014).

## Concluding Remarks

Throughout lifetime we are permanently in contact with a multitude of microbial species, which are usually targeted by our immune defences in an effective way to prevent infection. PRRs are key players in the initiation of the host innate immune response. SRs compose a diverse family of PRRs mainly expressed in cells patrolling pathogen invasion and with an increasing role in pathogen recognition and elimination. Some microbes developed strategies to evade SR-dependent recognition. In turn, SRs recognize a wide range of microbes and contribute to pathogen containment by modulating the recruitment and the activation of phagocytic cells and regulating inflammatory responses. This suggests that, in addition to other PRRs, the host displays a range of SRs to recognize specific ligands and activate downstream signalling pathways accordingly to their tissue-specific and cell type-specific expression.

The discovery of new SRs implicated in microbial pathogenesis increases our understanding of the host/pathogen interplay and provide crucial insights into the immune responses orchestrated by the host to avoid microbial predation. Understanding the complexity of this network may pave the way for the identification of novel targets and pathways to limit pathogenic infection by amplifying protective host cell responses.

# CHAPTER II:  PROJECT PRESENTATION

*Lm* is an opportunistic human foodborne pathogen that causes listeriosis, which is highly prevalent among immunocompromised hosts and high-risk groups (Allerberger *et al.* 2010). *Lm* is among the best-characterized bacterial pathogens concerning its molecular mechanisms and intracellular parasitism. Studies involving *Lm* infection *in vivo* have also provided fundamental insights on the immune system, establishing *Lm* as a useful model to elucidate host-pathogen interactions. Although the host possesses sophisticated and effective defences against *Lm* infection, bacteria developed molecular mechanisms to overcome host defences. By evaluating the contribution of new host cell receptors and new *Lm* virulence factors, the purpose of this project was to unravel new host innate immune responses to *Lm* infection and new *Lm* strategies to evade the host immune system.

The first goal of this work was to unveil the role of Scavenger receptors (SRs) in human listeriosis. During infection, host cells react to the presence of microorganisms through the activation of pattern recognition receptors (PRRs), which are evolutionary conserved receptors able to sense invariant microbial components, named pathogen-associated molecular patterns (PAMPs). The engagement of these microbial ligands by PRRs activates different signalling pathways to control innate immune responses and subsequently shape the adaptive immunity (Fraser *et al.* 1998; Gordon 2002; Janeway 1989; Medzhitov *et al.* 2000a; Medzhitov *et al.* 2000b). SRs compose an emergent family of PRRs able to bind to a wide range of ligands and stimulate the removal of both non-self and modified-self targets (Areschoug *et al.* 2008; Areschoug *et al.* 2009; Mukhopadhyay *et al.* 2004). They are involved in pathogen infection and play key functions in antimicrobial host immune response (Areschoug *et al.* 2009). Nevertheless, the role of SRs in infection by *Lm* is still poorly understood. We identified and characterized SRs involved in host interaction with *Lm*.

The second goal of this project was to find new *Lm* strategies to promote bacterial survival. *Lm* has the competitive advantage to effectively disseminate throughout host tissues by evading host immune response and multiplying within phagocytic and non-phagocytic cells (Cossart *et al.* 1998; Cossart 2011; Lecuit 2007). Our previous *in vivo* transcriptomic analysis of the *Lm* genome revealed genes highly induced during mouse infection, including *cadC*, which encodes the putative repressor of the cadmium efflux pump, CadA. We characterized the *Lm* cadmium efflux system and explored the role of CadC in *Lm* immune evasion and pathogenicity.

# CHAPTER III: RESULTS

The results produced by this work are divided in two main parts:

## Part I - Unravelling the Role of Scavenger Receptors in *Listeria monocytogenes* Infection

**I.1. Stabilin-1 Regulates Host Innate Immune Response and Contributes to *Listeria monocytogenes* Clearance During Infection**. We unravel the participation of STABILIN-1 in the regulation of host innate immune response and in bacterial clearance during infection. These findings were presented here according to the version submitted to Journal of Infectious Disease (29 November 2017).

**I.2. Scavenger Receptors are Required for Epithelial Cell Infection by *Listeria monocytogenes*.** This part includes unpublished data from ongoing work that highlight the relevance of SRs in the infection of epithelial cells with *Lm.*

## Part II - Role of CadC in *Listeria monocytogenes* Resistance to Cadmium and in Bacterial Pathogenicity

**II.1. *Listeria monocytogenes* CadC Regulates Cadmium Efflux and Fine-tunes Lipoprotein Localization to Escape the Host Immune Response and Promote Infection**. We characterize the *Lm* cadmium resistance system and reveal an unexpected role of CadC in *Lm* pathogenicity. We show that CadC is a metal efflux pump regulator repurposed during infection to fine-tune immunogenic lipoprotein processing and host responses. These findings were published on Journal of Infectious Disease (1 May 2017) and the published version is presented here.

**II.2. Negative Regulation of *Listeria monocytogenes* Bile Salt Hydrolase is Required to Promote Systemic Infection.** In this section we describe the involvement of CadC as a negative regulator of the *Lm* bile salt hydrolase required to promote systemic infection. These data are confined to a manuscript that is being prepared for submission.

# PART I.1.

## Unravelling the Role of Scavenger Receptors in *Listeria monocytogenes* Infection

Stabilin-1 Regulates Host Innate Immune Response and Contributes to *Listeria monocytogenes* Clearance During Infection

# Stabilin-1 Regulates Host Innate Immune Response
# and Contributes to *Listeria monocytogenes* Clearance During Infection

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## ABSTRACT

Innate immunity is crucial for the clearance of microbial pathogens including *Listeria monocytogenes* (*Lm*), a major intracellular human food-borne pathogen. The innate immune response depends on complex surveillance systems expressed by host cells. Scavenger Receptors (SRs) are important pattern recognition receptors, which play key functions in the antimicrobial host defense. Here, we show that STAB-1 promotes *Lm* phagocytosis and ensures macrophage membrane integrity. Using a mouse model of infection, we demonstrate that STAB-1 is induced in response to *Lm* infection, regulates inflammatory cytokine production and controls the recruitment of myeloid cells to restrict *Lm* proliferation. We thus propose STAB-1 as a new SR playing a protective role against bacterial infection.

**BACKGROUND**

*Listeria monocytogenes* (*Lm*) is a human food-borne pathogen that causes listeriosis, which is highly prevalent among high-risk groups including immunocompromised people, elderly, pregnant women and neonates [1]. Listeriosis is an overall public health concern associated with high hospitalization and mortality rates, being the most deadly food-borne infection in Europe [2]. Manisfestations of the disease may range from a self-limiting febrile gastroenteritis to septicemia, meningitis and encephalitis [3]. The most severe manifestations are related to the *Lm* capacity to cross the intestinal, blood-brain and maternal-fetal barriers, and to effectively disseminate throughout host tissues evading the host immune response and multiplying within phagocytic and non-phagocytic cells [1]. These characteristics are shaped by an arsenal of virulence factors [4].

Host innate immune response is critical to elicit an early defense towards *Lm* and the infection containment invokes the activation of a number of pattern recognition receptors, such as scavenger receptors (SRs) [5]. SRs compose a diverse and conserved family of proteins, able to bind to a wide range of ligands and stimulate the removal of non-self and modified-self targets [6]. They contribute to maintain homeostasis and to control pathogen infections, playing key functions in the antimicrobial host immune response [6, 7]. The impact of SRs in *Lm* infection was first revealed for SR-A, demonstrating that SR-AI/II KO mice were more susceptible to *Lm* infection and displayed increased hepatic granuloma formation [8]. Other SRs (MARCO, CD36 and SR-BI) were then shown to bind *Lm* and to be involved in *Lm* pathogenicity [9-11].

STABILIN-1 (STAB-1) is a SR expressed in macrophages, mononuclear cells, hematopoietic stem cells and endothelial cells. STAB-1 has been implicated in lymphocyte adhesion, transmigration, angiogenesis and apoptotic cell clearance [12]. Importantly, STAB-1 was previously found to bind Gram-positive and Gram-negative bacteria in vitro [13].

Here, we show that STAB-1 promotes *Lm* phagocytosis and ensures macrophage membrane integrity. We reveal that STAB-1 KO mice display deregulated inflammatory cytokine production, impaired cellular recruitment of myeloid cells and increased susceptibility to *Lm,* thus revealing the key role of STAB-1 in restraining bacterial infection.

**METHODS**

**Bacteria and cells**

*Listeria monocytogenes* EGD BUG 600 (*Lm*) and the non-pathogenic *Listeria innocua* (*Li*) CLIP 11262 were grown in Brain Heart Infusion (BHI) (BD-Difco) at 37°C. Human acute monocytic leukemia cells, THP-1 (ATCC TIB-202), were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Lonza) supplemented with 10% fetal bovine serum (FBS) (BioWest). Before bacterial infection, THP-1

cells were differentiated with 10 nM phorbol 12-myristate 13-acetate for 48h [14]. Murine macrophages J774 A.1 (ATTC TIB-67) and Raw 264.7 (ATTC TIB-71) were cultured in Dulbecco´s modified Eagle medium (DMEM) (Lonza), supplemented with 10% FBS.

**Animal infections**

STABILIN-1 full knock-out (STAB-1 KO) mice and their wild-type (WT) littermates, both with a C57BL/6N, 129SvJ mixed background, were bred from a breeding pair provided by S. Jalkanen (University of Turku, Finland). Infections were done as described [15]. Briefly, intravenous infections were performed through the tail vein with $5 \times 10^5$ colony-forming units (CFUs) in PBS. Mice were euthanized 72h post-infection, spleens and livers were aseptically collected and CFUs counted. Blood was recovered from mice heart. Mouse survival was assessed upon intravenous infection of $10^5$ CFUs. Animals were intraperitoneally injected with $10^5$ CFUs (*Lm*) or 15 mg/kg of LPS from *Escherichia coli* 055:B5 (L2880 Sigma) in PBS and euthanized 6h or 24h later. Animal procedures followed European Commission (directive 2010/63/EU) and Portuguese (Decreto-Lei 113/2013) guidelines and were approved by the IBMC Ethics Committee and Direção Geral de Veterinária (license 015301).

**Macrophage infection**

Macrophages were incubated for 30 min with: 100 µg/ml of fucoidan (Sigma-Aldrich), 50 µg/ml of poly(I) or poly(C) (Santa-Cruz-Biotechnology). Cells were infected for 30 min with exponential-phase bacteria at a multiplicity of infection (MOI) of 2 and treated with 20 µg/ml of gentamicin (Lonza) for 60 min as described [16]. Cells were washed and lysed for CFU quantification.

**Bone marrow-derived macrophages (BMDMs)**

Mouse femurs were removed and flushed with Hank's Buffered Salt Solution (HBSS-Lonza) as described [17]. Bone marrow cells were collected by centrifugation and cultured overnight in DMEM supplemented with 10 mM HEPES (Gibco), 1 mM sodium pyruvate (Lonza), 10% FBS and 10% L929 cell-conditioned medium (LCCM). Non-adherent cells were collected and seeded. Upon 4 days of differentiation, 10% of LCCM was added and on day 7 the medium was renewed. At day 10, exponential-phase bacteria were added at MOI 50 (20 min of infection plus 10 min with 20 µg/ml gentamicin) or at MOI 10 (30 min of infection plus 30 min or 9h30 with 20 µg/ml gentamicin). Macrophages were washed and lysed for CFU quantification.

**Membrane integrity assay**

PI staining (10 µg/ml) was carried out 1h and 10h post-infection in HBSS, during 30 min as described

[18]. Cells were fixed with 3% paraformaldehyde (PFA) for 15 min, permeabilized with triton 0.1%, and stained with DAPI (Sigma) and Alexa Fluor 488-conjugated phalloidin (Invitrogen) diluted in PBS containing 1% BSA. Total number of cells and PI$^+$ cells were quantified in Olympus-BX53 fluorescence microscope. At least 1000 cells were analyzed for each sample in three independent experiments.

### RNA techniques

RNAs were extracted from non-infected and *Lm*-infected cells (TripleXtractor, GRISP), as recommended by the manufacturer. Spleens and livers were collected in liquid nitrogen and 50-100 mg of tissues were homogenized in TripleXtractor. Purified RNAs were reverse-transcribed (iScript, Bio-Rad-Laboratories) and analyzed by qPCR as described [19]. Gene expression data were analyzed by comparative Ct method [20], normalized to *hprt1* expression. For qualitative analysis, PCR was performed on cDNA (KAPA2G Mix, GRISP). Amplification products were resolved in 1% (w/v) agarose gel and analyzed with GelDoc XR+ System (Bio-Rad Laboratories). Forward and reverse primers are listed in Table S1.

### Western blot

BMDMs were infected with *Lm* for indicated periods. Cells were lysed with Laemmli buffer and extracts resolved by SDS-PAGE on 8% gel as described [21]. Samples were transferred onto nitrocellulose membrane and blotted with rabbit anti-STAB-1 (1:500, Millipore), followed by HRP-conjugated goat anti-rabbit IgG (1:2000, P.A.R.I.S). Signals were detected using ECL (Thermo-Scientific) and digitally acquired in a ChemiDoc XRS+ system (Bio-Rad Laboratories). Signal intensity was quantified using Image J.

### Cytokine ELISA

Lysis buffer 2x (200 mM Tris, 300 mM NaCl, 2% triton, pH 7.4) and Complete proteinase inhibitor (Roche) were added to homogenized organs for 30 min on ice. Supernatants were collected upon centrifugation and stored (-80ºC). Mouse serum was recovered after blood centrifugation. Cytokine production was determined using murine ELISA kit (eBioscience).

### Flow cytometry

Mouse spleens were collected in ice-cold storage solution (PBS 2% FBS) and single-cell suspensions prepared using cell strainers (BD-Falcon). Cells were washed upon red blood cells lysis (150 mM $NH_4Cl$, 10 mM $KHCO_3$, pH 7.2 in $H_2O$) and cell viability was assessed by trypan blue (Life-technologies) exclusion method. Peritoneal cells were collected by washing peritoneal cavities with 5 ml of storage

solution, pelleted by centrifugation, washed and cell viability was assessed. Cells were labeled with brilliant violet (BV) 510-conjugated anti-CD11b, clone M1/70; BV 421-conjugated anti-CD11c, clone N418; allophycocyanin (APC)-conjugated anti-Ly6G, clone 1A8; APC with cyanin-7 (APC/Cy7)-conjugated anti-F4/80, clone BM8; and phycoerythrin (PE)-conjugated anti-Ly6C, clone HK1.4 (BioLegend). Data were acquired in a FACSCanto II flow cytometer (BD-Biosciences) and analyzed using FlowJo software (TreeStar Inc.). To determine cell numbers, event number for each cell population was normalized to the total cell number.

**Statistics**

Statistics were carried out with Prism (GraphPad), using unpaired two-tailed Student's *t*-test to compare means of two groups, and one-way ANOVA with Tukey's post-hoc test for pairwise comparison of means from more than two groups, or with Dunnett's post-hoc test for comparison of means relative to the mean of a control group.

**RESULTS**

**Scavenger Receptors are required for *Lm* uptake by macrophages**

SRs have been implicated in the recognition of a multitude of pathogens [6], although their role in *Lm* infection is still poorly understood. We first evaluated if SRs could play a role in *Lm* uptake by macrophages. SRs were chemically saturated with different pleiotropic compounds known to inhibit SRs [22], before macrophage infection with *Lm.* We found that pre-treatment of THP-1 cells with fucoidan severely impaired *Lm* uptake when compared to non-treated cells (Figure 1A). In addition, the number of intracellular bacteria was reduced upon SR saturation with Poly (I), but not with control Poly (C) (Figure 1A). Pre-treatment of murine macrophage-like cells with fucoidan also compromised *Lm* uptake, being the uptake defect more dramatic in Raw than in J774 cells (Figure 1B). These data suggested that *Lm* uptake by macrophages depends on some SRs.

To identify SRs potentially involved in *Lm* uptake, we assessed SR transcription profiles on total RNAs isolated from either uninfected or *Lm*-infected human (THP-1) and murine (J774 and Raw) macrophages. Our analysis revealed that although some of the selected SRs appeared broadly expressed, each cell line presented a specific SR expression profile (Figure 1C). In the conditions tested, *SR-A*, *SRCL*, *SREC*, *LOX1* and *STAB-1* appeared to be expressed in all cell lines. Only STAB-1 showed slightly decreased transcript levels upon *Lm* challenge (Figure 1C and 1D). Interestingly, STAB-1 was previously showed to bind to Gram-positive and Gram-negative bacteria in vitro, suggesting a role for this receptor in bacterial recognition [13].

These results pointed out STAB-1 as a new SR potentially involved in macrophage response to *Lm* infection.

## STAB-1 promotes *Lm* uptake and protects macrophage membrane integrity

To confirm that *Lm* infection impacts *STAB-1* expression in macrophages, mouse BMDMs were incubated with pathogenic (*Lm)* or non-pathogenic (*Listeria innocua - Li*) *Listeria* [23]. *STAB-1* transcript levels were assessed by qRT-PCR on RNAs extracted either from non-infected and infected macrophages. As previously observed in macrophage cell lines (Figure 1C and 1D), *Lm* infection efficiently down-regulated *STAB-1* expression in BMDMs, while *STAB-1* transcript levels were only slightly reduced upon *Li* infection (Figure 2A). STAB-1 expression was also analyzed at the protein level by western blot. As expected, STAB-1 protein levels were significantly diminished upon BMDM infection by *Lm* (Figure 2B), while they were not affected upon *Li* incubation, thus confirming the results obtained by qRT-PCR (Figure 2A). Together, these results indicate that STAB-1 expression by macrophages is reduced upon *Lm* infection, this decreased expression being dependent on *Lm* characteristics not present in *Li*.

SRs are expressed by most tissue macrophages and have an important role as phagocytic receptors for pathogenic microorganisms [24]. To directly address the impact of STAB-1 in macrophage response against *Lm* infection, BMDMs from both WT and STAB-1 KO mice were infected either with *Lm* or *Li*. STAB-1 KO macrophages displayed a defect in *Listeria* uptake that was more severe in the case of *Lm* (Figure 2C). We then assessed the role of STAB-1 in macrophage viability by assessing plasma membrane integrity through propidium iodide (PI) permeability assays in WT and STAB-1 KO BMDMs infected with *Lm* during 1 or 10 hours. Upon *Lm* infection, STAB-1 KO BMDMs showed a significant increased number of PI-positive cells as compared to WT macrophages, indicating that *Lm*-infected STAB-1 KO BMDMs display increased disruption of plasma membrane integrity, which may correlate with increased cell death (Figure 2D).

Taken together, these data indicate that STAB-1 promotes *Listeria* uptake into macrophages and has a protective role against *Lm*-induced membrane permeability.

## STAB-1 regulates inflammatory cytokine production upon *Lm* infection

The role of STAB-1 in *Lm* infection was then assessed in vivo, using WT and STAB-1 KO mice. We first analyzed *STAB-1* expression in uninfected mouse spleen and liver by RT-PCR. *STAB-1* transcripts were detected in organs from WT mice, while absent, as expected, from STAB-1 KO mice (Figure 3A). Additionally, *STAB-1* expression appeared higher in the liver than in the spleen (Figure 3B). WT mice were intravenously infected with *Lm*, and the expression of *STAB-1* was analyzed in both spleen and

liver isolated from uninfected and infected mice. *Lm* infection resulted in a significant up-regulation of *STAB-1* expression in the spleen (Figure 3C), strongly suggesting a potential role for STAB-1 in host response to *Lm* infection.

WT and STAB-1 KO mice were then intravenously infected with *Lm* and three days post-infection, the production of cytokines in the spleen, liver and serum of *Lm*-infected mice was evaluated by ELISA. As compared to WT animals, infected STAB-1 KO mice displayed a deregulated inflammatory cytokine expression pattern, with an overall defect on TNF-α, IL-6 and IL-10 production, and a relative increase of local IL-1β and IFN-γ (Figure 4). No significant differences regarding cytokine production were observed between non-infected WT and STAB-1 KO mice (Figure S1). In addition, the deregulated inflammatory cytokine expression pattern of infected STAB-1 KO mice was to a large extend confirmed at the transcriptional level (Figure S2).

Altogether, these data suggest that, in vivo, STAB-1 is overexpressed in response to *Lm* infection and regulates inflammatory cytokine production in mouse organs targeted by *Lm*.

**STAB-1 controls immune cell accumulation in *Lm*-infected spleens**

The recruitment of myeloid cells, namely inflammatory neutrophils, to sites of *Lm* infection is crucial to promote host survival [25]. As STAB-1 appears to regulate the innate immune response to *Lm* infection by altering cytokine production, we hypothesized that STAB-1 could impact innate immune cells throughout the infection. WT and STAB-1 KO mice were intravenously infected with *Lm.* Three days post infection, single-cell spleen suspensions were analyzed regarding myeloid cell populations by flow cytometry. As compared to WT mice, *Lm*-infected STAB-1 KO mice showed a clear defect on myeloid CD11b$^{int/hi}$ cells in the spleen, which resulted from diminished number of neutrophils (CD11b$^{hi}$Ly6G$^{hi}$) and macrophages (CD11b$^{hi}$CD11c$^{lo}$) (Figure 5B and 5C). Interestingly, within the macrophage population, the number of inflammatory macrophages (CD11b$^{hi}$Ly6C$^{hi}$) was also reduced in infected STAB-1 KO animals (Figure 5B and 5C). Importantly, similar cell populations were detected in spleens of non-infected WT and STAB-1 KO mice (Figure 5A).

Taken together, these data demonstrate that STAB-1 is essential to control the accumulation of myeloid cells in the spleen of *Lm*-infected mice.

**STAB-1 promotes early myeloid cells recruitment**

STAB-1 was previously shown to mediate the migration of T- and B- lymphocytes to the draining lymph nodes [26]. To evaluate the role of STAB-1 in the trafficking of myeloid cells to the site of *Lm* infection, WT and STAB-1 KO mice were intraperitoneally infected with *Lm* and myeloid cell populations were analyzed by flow cytometry at 6h or 24h post-infection. *Lm* infection induced increased numbers of

recruited cells to the focus of infection, mainly neutrophils (CD11b$^{hi}$Ly6G$^{hi}$) and inflammatory macrophages (CD11b$^{hi}$Ly6C$^{hi}$) (Figure 6). Additionally, we detected a slight defect on myeloid cells (CD11b$^{int/hi}$) recruitment in STAB-1 KO mice upon *Lm* infection, both at 6h and 24h post-infection (Figure 6B-C).

Lipopolysaccharides (LPS) are potent inducers of inflammation that stimulate immune cells and induce the migration of myeloid cells to the mouse abdominal cavity when injected intraperitoneally [27]. We used this experimental model to confirm the potential involvement of STAB-1 in the process of innate immune cells recruitment. Purified LPS were intraperitoneally injected in WT and STAB-1 KO mice and, 6h post-stimulation, exudate cells from the peritoneal cavity were recovered to evaluate myeloid cell populations. As compared to WT, STAB-1 KO mice showed a significant reduction in myeloid cell (CD11b$^{int/hi}$) population, due to a decreased number of recruited neutrophils (CD11b$^{hi}$Ly6G$^{hi}$), dendritic cells (CD11b$^{int}$CD11c$^{hi}$) and inflammatory macrophages (CD11b$^{hi}$Ly6C$^{hi}$) (Figure 6D).

Altogether, these results indicate that in vivo STAB-1 potentiates the recruitment of immune cells to the site of *Lm* infection.

## STAB-1 limits *Lm* infection

To further explore if the STAB-1-dependent recruitment of immune cells to the infection site could have an impact on *Lm* systemic infection in vivo, WT and STAB-1 KO mice were intravenously infected with *Lm*. Three days later, mice were sacrificed and the bacterial load in the spleen and liver was quantified. Bacterial numbers appeared significantly higher in organs of STAB-1 KO mice (Figure 7A), suggesting a role for STAB-1 in the control of *Lm* infection. To test if this defect in infection control may lead to increased mortality, mice were intravenously infected with *Lm* and mouse survival was followed overtime. Whereas WT mice survived throughout the infection, STAB-1 KO mice started to die from day 8, to reach 80% of mortality by day 18 (Figure 7B).

By regulating the production of inflammatory cytokines and promoting the recruitment of myeloid cells, STAB-1 appears thus as a new SR with a protective role against *Lm* infection.

## DISCUSSION

The role of SRs during microbial pathogenesis was explored over recent years [6]. Here, we revealed that STAB-1 is crucial to promote *Lm* phagocytosis and preserve macrophage membrane integrity. We demonstrated that STAB-1 is induced in response to *Lm* infection and contributes to infection containment by regulating inflammatory cytokine production and controlling myeloid cell recruitment (Figure S3).

The expression of SRs, such as SR-A, MARCO and LOX-1, is modulated by microbial infection, either favouring host immune response or promoting pathogen survival [28-30]. The regulation of SR expression has been associated with the action of different bacterial proteins. *Porphyromonas gingivalis* induces the expression of SR-A by macrophages through FimA, promoting TNF-α production, thus implicating SR-A as an inflammation regulator [28]. Contrarily, the PpiA lipoprotein of *Streptococcus mutans* negatively regulates MARCO expression, inducing the suppression of MARCO-mediated phagocytosis [31]. Bacteria also evolved mechanisms to avoid SR recognition. The *Streptococcus agalactiae* polysaccharide capsule or Blr lipoprotein and the *Streptococcus pyogenes* surface M protein were shown to prevent SR-A-mediated recognition and non-opsonic phagocytosis [32, 33]. We found here that STAB-1 expression is down-regulated in macrophages solely in response to *Lm* infection, suggesting that *Lm* displays effective mechanisms to hijack STAB-1 to be less recognized by macrophages.

Although STAB-1 expression is diminished in infected macrophages in vitro, in vivo infection by *Lm* induces an overall up-regulation of STAB-1 in major target organs. This could suggest that, whereas the host responds to *Lm* infection by a general increase of STAB-1 expression as part of the innate immune response, locally *Lm* develops strategies to reduce STAB-1 expression by macrophages to diminish phagocytosis. In vivo evidences also demonstrated a marked expression of MARCO in response to *Leishmania major* and *Lm* infections [9, 34]. Further studies are required to unravel putative *Lm* virulence factors responsible for STAB-1 regulation.

SRs act as phagocytic receptors of microorganisms, including *Neisseria meningitidis, Clostridium sordellii and Mycobacterium marinum* [35-37]. SR-A-deficient macrophages showed decreased listericidal capacity due to a higher proportion of perforated *Lm*-bearing phagosomes and lower frequency of perforated phagosomes fused with lysosomes [8]. STAB-1 was previously found to bind Gram-positive and Gram-negative bacteria in vitro [13]. We show that STAB-1 KO macrophages have reduced phagocytic capacity, strongly suggesting that STAB-1 acts as a phagocytic receptor for *Lm*. Macrophage cell death is an important mechanism for the down-regulation of inflammatory responses to prevent sepsis, whereas decreased *Lm*-induced apoptosis enhances bacterial clearance [38]. Our findings reveal that STAB-1 contributes to preserve macrophage membrane integrity upon *Lm* infection, suggesting that STAB-1 may enhance anti-microbial activity through the inhibition of macrophage cell death.

STAB-1 regulates inflammatory cytokine response in *Lm*-targeted mouse organs. SRs are strong players in the regulation of inflammation, such is the case of SR-A in *N. meningitidis* and *P. gingivalis* infections [28, 39] or CD36 in *Staphylococcus aureus* infection [40]. We show that STAB-1 is important for the expression of TNF-α, IL-6 and IL-10 upon *Lm* infection. In agreement, IL-6- and TNF-α -deficient

mice are more susceptible to *Lm* infection, with increased bacterial burden in the spleen and liver, and deficient neutrophil recruitment into the blood [41, 42], thus suggesting that these cytokines may induce cell recruitment. STAB-1 was previously shown to control the activation of several pro-inflammatory cytokines in human monocytes. However, in this case STAB-1 seems to act as an immunosuppressive molecule [43]. The pro-/anti-infammatory cytokine balance influences the severity of infectious diseases [44]. IL-10 is a potent inhibitor of innate immunity and IL-10 deficiency improves resistance to infection, including *Lm* [45]. *STAB-1 appears thus as an important regulator of the inflammatory response during Lm infection.*

MARCO- and CD36-deficient mice displayed diminished survival rate resultant from impaired cell recruitment [40, 46]. In vitro data showed that STAB-1 promotes the recruitment of regulatory T cells through human hepatic sinusoidal endothelial cells, and in vivo models demonstrated that STAB-1 mediates T- and B-lymphocytes migration to draining lymph nodes [26, 47]. Our findings indicate that STAB-1 is required for myeloid cells recruitment in infected organs. Neutrophils and macrophages, which are effective microbicidal cells, are among the first cells involved in the *Lm*-immune response. Mice deficient for these cells present increased mortality and bacterial burden [48]. STAB-1 KO mice have impaired myeloid cell recruitment in response to LPS, suggesting that STAB-1 could be involved in cell recruitment in response to different stimuli. Importantly, we cannot exclude that impaired cell recruitment in STAB-1 KO mice might result from the deregulated cytokine production. Although anti-STAB-1 treatment of mice was not shown to impair clearance of *Staphylococcus aureus,* it clearly diminished granulocytes migration [26].

Here, we highlight for the first time the crucial involvement of STAB-1 in pathogen infection. By promoting bacterial phagocytosis and macrophage membrane integrity, and by regulating the inflammatory response and the recruitment of myeloid cells, STAB-1 is a new SR with a protective role against *Lm* infection. Amplifying this type of host defenses may represent innovative strategies against pathogens.

**REFERENCES**

1. Cossart P. Illuminating the landscape of host-pathogen interactions with the bacterium Listeria monocytogenes. P Natl Acad Sci USA 2011;108(49):19484-91.

2. EFSA. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2014. 2015. p. 4.

3. Swaminathan B, Gerner-Smidt P. The epidemiology of human listeriosis. Microbes Infect 2007;9(10):1236-43.

4. Carvalho F, Sousa S, Cabanes D. How Listeria monocytogenes organizes its surface for virulence. Front Cell Infect Microbiol 2014;4:48.

5. Gordon S. Pattern recognition receptors: Doubling up for the innate immune response. Cell 2002;111(7):927-30.

6. Areschoug T, Gordon S. Scavenger receptors: role in innate immunity and microbial pathogenesis. Cell Microbiol 2009;11(8):1160-9.

7. PrabhuDas MR, Baldwin CL, Bollyky PL, Bowdish DME, Drickamer K, Febbraio M, et al. A Consensus Definitive Classification of Scavenger Receptors and Their Roles in Health and Disease. J Immunol 2017;198(10):3775-89.

8. Ishiguro T, Naito M, Yamamoto T, Hasegawa G, Gejyo F, Mitsuyama M, et al. Role of macrophage scavenger receptors in response to Listeria monocytogenes infection in mice. Am J Pathol 2001;158(1):179-88.

9. Ito S, Naito M, Kobayashi Y, Takatsuka H, Jiang S, Usuda H, et al. Roles of a macrophage receptor with collagenous structure (MARCO) in host defense and heterogeneity of splenic marginal zone macrophages. Arch Histol Cytol 1999;62(1):83-95.

10. Pfeiler S, Khandagale AB, Magenau A, Nichols M, Heijnen HF, Rinninger F, et al. Distinct surveillance pathway for immunopathology during acute infection via autophagy and SR-BI. Sci Rep 2016;6:34440.

11. Vishnyakova TG, Kurlander R, Bocharov AV, Baranova IN, Chen ZG, Abu-Asab MS, et al. CLA-1 and its splicing variant CLA-2 mediate bacterial adhesion and cytosolic bacterial invasion in mammalian cells. P Natl Acad Sci USA 2006;103(45):16888-93.

12. Kzhyshkowska J. Multifunctional receptor stabilin-1 in homeostasis and disease. ScientificWorldJournal 2010;10:2039-53.

13. Adachi H, Tsujimoto M. FEEL-1, a novel scavenger receptor with in vitro bacteria-binding and angiogenesis-modulating activities. The Journal of biological chemistry 2002;277(37):34264-70.

14. Park EK, Jung HS, Yang HI, Yoo MC, Kim C, Kim KS. Optimized THP-1 differentiation is required for the detection of responses to weak stimuli. Inflamm Res 2007;56(1):45-50.

15. Cabanes D, Lecuit M, Cossart P. Animal models of Listeria infection. Curr Protoc Microbiol 2008;Chapter 9:Unit9B 1.

16. Carvalho F, Atilano ML, Pombinho R, Covas G, Gallo RL, Filipe SR, et al. L-Rhamnosylation of Listeria monocytogenes Wall Teichoic Acids Promotes Resistance to Antimicrobial Peptides by Delaying Interaction with the Membrane. PLoS pathogens 2015;11(5):e1004919.

17. Pombinho R, Camejo A, Vieira A, Reis O, Carvalho F, Almeida MT, et al. Listeria monocytogenes CadC Regulates Cadmium Efflux and Fine-tunes Lipoprotein Localization to Escape the Host Immune Response and Promote Infection. The Journal of infectious diseases 2017;215(9):1468-79.

18. Leitao E, Costa AC, Brito C, Costa L, Pombinho R, Cabanes D, et al. Listeria monocytogenes induces host DNA damage and delays the host cell cycle to promote infection. Cell Cycle 2014;13(6):928-40.

19. Pinheiro J, Reis O, Vieira A, Moura IM, Zanolli Moreno L, Carvalho F, et al. Listeria monocytogenes encodes a functional ESX-1 secretion system whose expression is detrimental to in vivo infection. Virulence 2016:1-12.

20. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25(4):402-8.

21. Martins M, Custodio R, Camejo A, Almeida MT, Cabanes D, Sousa S. Listeria monocytogenes triggers the cell surface expression of Gp96 protein and interacts with its N terminus to support cellular infection. The Journal of biological chemistry 2012;287(51):43083-93.

22. Cui YQ, Luo DZ, Wang XM. [Fucoidan: advances in the study of its anti-inflammatory and anti-oxidative effects]. Yao Xue Xue Bao 2008;43(12):1186-9.

23. Glaser P, Frangeul L, Buchrieser C, Rusniok C, Amend A, Baquero F, et al. Comparative genomics of Listeria species. Science 2001;294(5543):849-52.

24. Conlan JW. Early pathogenesis of Listeria monocytogenes infection in the mouse spleen. J Med Microbiol 1996;44(4):295-302.

25. Rosen H, Gordon S, North RJ. Exacerbation of murine listeriosis by a monoclonal antibody specific for the type 3 complement receptor of myelomonocytic cells. Absence of monocytes at infective foci allows Listeria to multiply in nonphagocytic cells. J Exp Med 1989;170(1):27-37.

26. Karikoski M, Irjala H, Maksimow M, Miiluniemi M, Granfors K, Hernesniemi S, et al. Clever-1/Stabilin-1 regulates lymphocyte migration within lymphatics and leukocyte entrance to sites of inflammation. Eur J Immunol 2009;39(12):3477-87.

27. Miyazaki S, Ishikawa F, Fujikawa T, Nagata S, Yamaguchi K. Intraperitoneal injection of lipopolysaccharide induces dynamic migration of Gr-1high polymorphonuclear neutrophils in the murine abdominal cavity. Clin Diagn Lab Immunol 2004;11(3):452-7.

28. Baer MT, Huang N, Gibson FC, 3rd. Scavenger receptor A is expressed by macrophages in response to Porphyromonas gingivalis, and participates in TNF-alpha expression. Oral Microbiol Immunol 2009;24(6):456-63.

29. Braun BJ, Slowik A, Leib SL, Lucius R, Varoga D, Wruck CJ, et al. The formyl peptide receptor like-1 and scavenger receptor MARCO are involved in glial cell activation in bacterial meningitis. J Neuroinflammation 2011;8(1):11.

30. Gao X, Zhao G, Li C, Lin J, Jiang N, Wang Q, et al. LOX-1 and TLR4 affect each other and regulate the generation of ROS in A. fumigatus keratitis. Int Immunopharmacol 2016;40:392-99.

31. Mukouhara T, Arimoto T, Cho K, Yamamoto M, Igarashi T. Surface lipoprotein PpiA of Streptococcus mutans suppresses scavenger receptor MARCO-dependent phagocytosis by macrophages. Infect Immun 2011;79(12):4933-40.

32. Areschoug T, Gordon S. Pattern recognition receptors and their role in innate immunity: focus on microbial protein ligands. Contrib Microbiol 2008;15:45-60.

33. Carlsson F, Sandin C, Lindahl G. Human fibrinogen bound to Streptococcus pyogenes M protein inhibits complement deposition via the classical pathway. Mol Microbiol 2005;56(1):28-39.

34. Gomes IN, Palma LC, Campos GO, Lima JG, TF DEA, JP DEM, et al. The scavenger receptor MARCO is involved in Leishmania major infection by CBA/J macrophages. Parasite Immunol 2009;31(4):188-98.

35. Benard EL, Roobol SJ, Spaink HP, Meijer AH. Phagocytosis of mycobacteria by zebrafish macrophages is dependent on the scavenger receptor Marco, a key control factor of pro-inflammatory signalling. Dev Comp Immunol 2014;47(2):223-33.

36. Thelen T, Hao Y, Medeiros AI, Curtis JL, Serezani CH, Kobzik L, et al. The class A scavenger receptor, macrophage receptor with collagenous structure, is the major phagocytic receptor for Clostridium sordellii expressed by human decidual macrophages. J Immunol 2010;185(7):4328-35.

37. Villwock A, Schmitt C, Schielke S, Frosch M, Kurzai O. Recognition via the class A scavenger receptor modulates cytokine secretion by human dendritic cells after contact with Neisseria meningitidis. Microbes and infection 2008;10(10-11):1158-65.

38. O'Connell RM, Saha SK, Vaidya SA, Bruhn KW, Miranda GA, Zarnegar B, et al. Type I interferon production enhances susceptibility to Listeria monocytogenes infection. J Exp Med 2004;200(4):437-45.

39. Pluddemann A, Hoe JC, Makepeace K, Moxon ER, Gordon S. The macrophage scavenger receptor A is host-protective in experimental meningococcal septicaemia. PLoS pathogens 2009;5(2):e1000297.

40. Castleman MJ, Febbraio M, Hall PR. CD36 Is Essential for Regulation of the Host Innate Response to Staphylococcus aureus alpha-Toxin-Mediated Dermonecrosis. J Immunol 2015;195(5):2294-302.

41. Dalrymple SA, Lucian LA, Slattery R, McNeil T, Aud DM, Fuchino S, et al. Interleukin-6-deficient mice are highly susceptible to Listeria monocytogenes infection: correlation with inefficient neutrophilia. Infect Immun 1995;63(6):2262-8.

42. Rothe J, Lesslauer W, Lotscher H, Lang Y, Koebel P, Kontgen F, et al. Mice lacking the tumour necrosis factor receptor 1 are resistant to TNF-mediated toxicity but highly susceptible to infection by Listeria monocytogenes. Nature 1993;364(6440):798-802.

43. Palani S, Elima K, Ekholm E, Jalkanen S, Salmi M. Monocyte Stabilin-1 Suppresses the Activation of Th1 Lymphocytes. J Immunol 2016;196(1):115-23.

44. Walley KR, Lukacs NW, Standiford TJ, Strieter RM, Kunkel SL. Balance of inflammatory cytokines related to severity and mortality of murine sepsis. Infect Immun 1996;64(11):4733-8.

45. Silva RA, Appelberg R. Blocking the receptor for interleukin 10 protects mice from lethal listeriosis. Antimicrob Agents Chemother 2001;45(4):1312-4.

46. Xu J, Flaczyk A, Neal LM, Fa Z, Eastman AJ, Malachowski AN, et al. Scavenger Receptor MARCO Orchestrates Early Defenses and Contributes to Fungal Containment during Cryptococcal Infection. J Immunol 2017;198(9):3548-57.

47. Shetty S, Weston CJ, Oo YH, Westerlund N, Stamataki Z, Youster J, et al. Common lymphatic endothelial and vascular endothelial receptor-1 mediates the transmigration of regulatory T cells across human hepatic sinusoidal endothelium. J Immunol 2011;186(7):4147-55.

48. Witter AR, Okunnu BM, Berg RE. The Essential Role of Neutrophils during Infection with the Intracellular Bacterial Pathogen Listeria monocytogenes. J Immunol 2016;197(5):1557-65.

## Table S1 - Primers

| Primer | Sequence (5´ to 3´) |
| --- | --- |
| hHPRT1 Fw | GGCGTCGTGATTAGTGATG |
| hHPRT1 Rv | CACCCTTTCCAAATCCTCAG |
| hSRA Fw | TTGAATACCACATTGCTTGATT |
| hSRA Rv | CTGATTTCCTCTTGTTGTTTGA |
| hSCARA5 Fw | TTCATCTTAGCAGTGTCCAG |
| hSCARA5 Rv | ATTCAGCCGGTTCACATT |
| hSRCL Fw | AGTAGCCAACTTATCAGTGATTATG |
| hSRCL Rv | CGGTGGACCTTGTAGTATTGTA |
| hCD6 Fw | GTTCAGACAGTCACTATAGAATCT |
| hCD6 Rv | AGGGGATGAGGAGCATTAG |
| hCD163 Fw | AACTTGAGTCCCTTCACCAT |
| hCD163 Rv | TTGTCTGTTCCTCCAAGAGAA |
| hM160 Fw | CTCCTTCTCCTGGTTCTGTT |
| hM160 Rv | TCCTTCTGGTTGAAACTCTGA |
| hSREC Fw | ACTCCTTCTCATCCGATCC |
| hSREC Rv | GGACCATCCCTTCTTGGG |
| hLOX1 Fw | AAAGCTAAAGGTCTTCAGTTTC |
| hLOX1 Rv | CATAATGGTCACTACTAATCCC |
| hSTAB1 Fw | TAACCAATTCACGAAATACTCCTA |
| hSTAB1 Rv | CCATTAGCTGCTATGTTGTTG |
| mHPRT1 Fw | TGATTAGCGATGATGAACCA |
| mHPRT1 Rv | GTCTTTCAGTCCTGTCCATAA |
| mSRA Fw | CTGAATATGACACTGCTTGATG |
| mSRA Rv | ATTTACTGATGTCCTCCTGTTG |
| mSRCL Fw | TCAGTGGTTATGGAAGAGATG |
| mSRCL Rv | CAGGAGGACCTTGTAGAATG |
| mCD5 Fw | GTCTGCTTATCCAGCTCTG |
| mCD5 Rv | AGGTCATAGTCACTGTCAG |
| mCD163 Fw | GTGTGATTTGCTTAGAGGGA |
| mCD163 Rv | CACTTCCAATCTTCCTGAACA |
| mSREC Fw | TTCTCTTCTGATCCTGACTC |
| mSREC Rv | CATAGGGACCATCTCTTCTC |
| mLOX1 Fw | CCTGCTGCTATGACTCTG |
| mLOX1 Rv | ATACCTGGCGTAATTGTGT |
| mSTAB1 Fw | AGGGGACTCCAAGAAAAC |
| mSTAB1 Rv | CCACAGTTCTCCAGGATC |
| IFN gamma Fw | CGTCATTGAATCACACCTG |
| IFN gamma Rv | GTTGTTGACCTCAAACTTGG |
| TNF alpha Fw | CCAAAGGGATGAGAAGTTC |
| TNF alpha Rv | GAGAAGATGATCTGAGTGTG |
| IL-6 Fw | GACCTGTCTATACCACTTCAC |
| IL-6 Rv | GCCATTGCACAACTCTTTTC |
| IL-1B Fw | TTTGAAGAAGAGCCCATCC |
| IL-1B Rv | GTAGTGCAGTTGTCTAATGG |
| IL-10 Fw | AGCCAGGTGAAGACTTTCT |
| IL-10 Rv | GCAACCCAAGTAACCCTTAAAG |
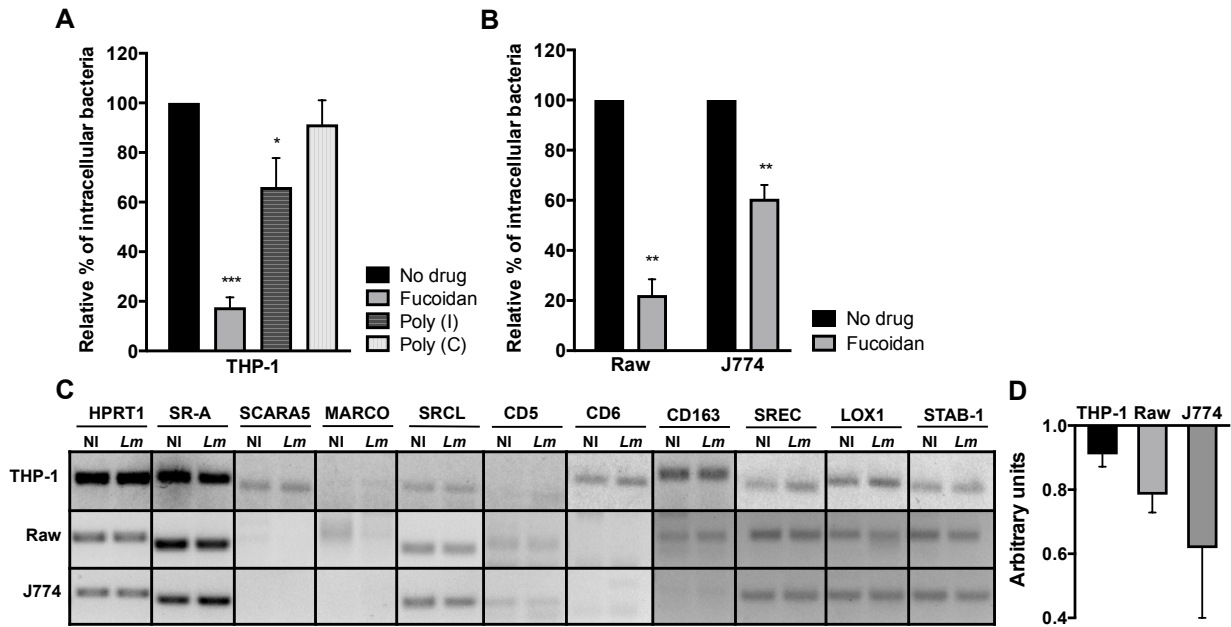
**Figure 1. SRs are required for *Lm* uptake by macrophages.** (A-B) Chemical saturation of macrophage SRs impairs bacterial uptake. (A) Human THP-1, (B) murine Raw and J774 macrophage-like cells were left untreated or pre-treated with fucoidan, poly(I) and its control poly(C), infected by *Lm* for 30 min, incubated with gentamicin, washed and lysed to quantify intracellular bacteria. Values are expressed relative to no drug values arbitrarily fixed to 100%. Values are mean ± SD of three independent assays. *$p<0.05$; **$p<0.01$; ***$p<0.001$. (C) Expression of SR genes in non-infected (NI) and *Lm*-infected (*Lm*) conditions. Gene expression was assessed by RT-PCR analysis of THP-1, Raw and J774 total RNAs, using HPRT1 as the reference gene. Representative of two assays. (D) Quantification of *STAB-1* expression in infected THP-1, Raw and J774 macrophages, normalized to expression in NI cells.
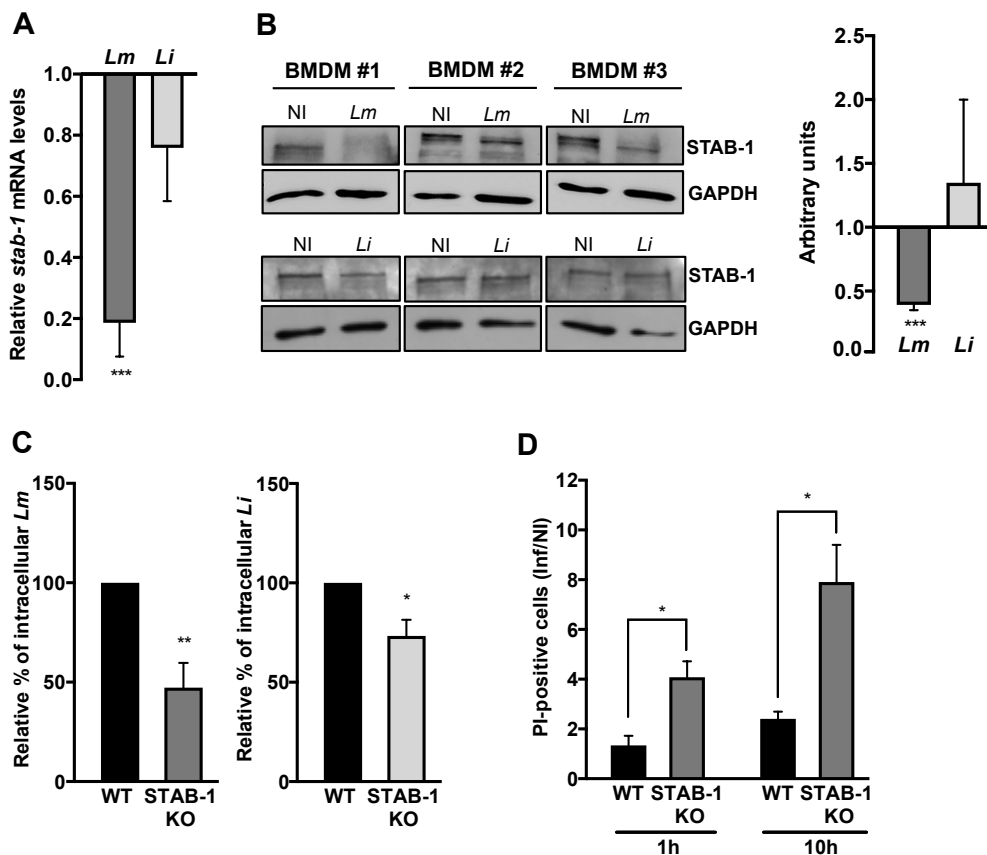
**Figure 2. STAB-1 promotes *Lm* uptake and preserves macrophage membrane integrity.** (A-C) Assessment of STAB-1 expression by (A) quantitative RT-PCR and (B) Western Blot. (A) Quantification of *STAB-1* mRNA levels on RNAs extracted from BMDMs infected either with *Lm* or incubated with the non-pathogenic *Li,* for 30 min. *STAB-1* expression levels in infected conditions were normalized to those in non-infected BMDMs, arbitrarily fixed to 1. (B) Independent immunoblots to detect STAB-1 protein in BMDMs left uninfected (NI) or infected with *Lm* and *Li* for 30 min. Immunoblots quantification showing STAB-1 signal intensity in infected macrophages, normalized to GAPDH. (C) Quantification of intracellular bacteria in WT and STAB-1 KO BMDMs infected either with *Lm* or *Li* for 30 min. Values are expressed relative to WT arbitrarily fixed to 100%. (D) Membrane integrity assay. WT and STAB-1 KO BMDMs were infected with *Lm* during 1 h and 10 h. Macrophages with disrupted plasma membrane were discriminated by immunofluorescence through PI incorporation, and normalized for the total population of cells in each condition. Values are mean ± SD of three to four independent experiments. Statistical significance is indicated as compared to NI or to WT BMDMs. *$p<0.05$; **$p<0.01$; ***$p<0.001$.
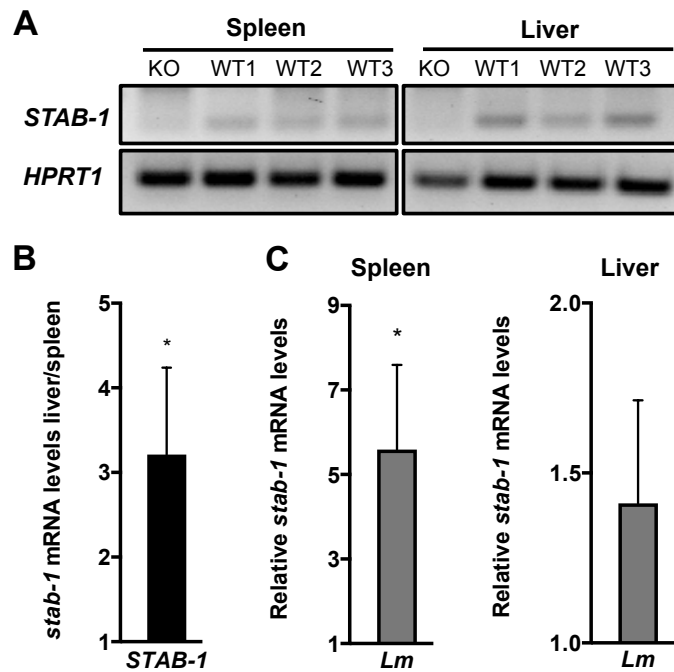
**Figure 3. *STAB-1* expression is increased in mouse organs upon *Lm* infection.** (A) RT-PCRs were achieved on total RNAs from spleen and liver of three non-infected WT mice. Control PCRs were performed on RNAs from STAB-1 KO mice organs and *HPRT1* was used as reference gene. (B) qRT-PCR was performed on RNAs extracted from spleen and liver of non-infected WT mice. Gene expression levels of *STAB-1* in the liver are presented normalized to those in the spleen, arbitrarily fixed to 1. (C) WT mice were left uninfected or intravenously infected with $5 \times 10^5$ CFU of *Lm.* Three days post-infection, *STAB-1* expression was quantified by qRT-PCR on RNAs from the spleen and liver of *Lm*-infected mice, and normalized to expression in non-infected mice organs, arbitrarily fixed to 1. (B-C) Data are presented as an average of five animals per condition. Statistical significance is indicated as compared to NI mice organs. *$p < 0.05$.
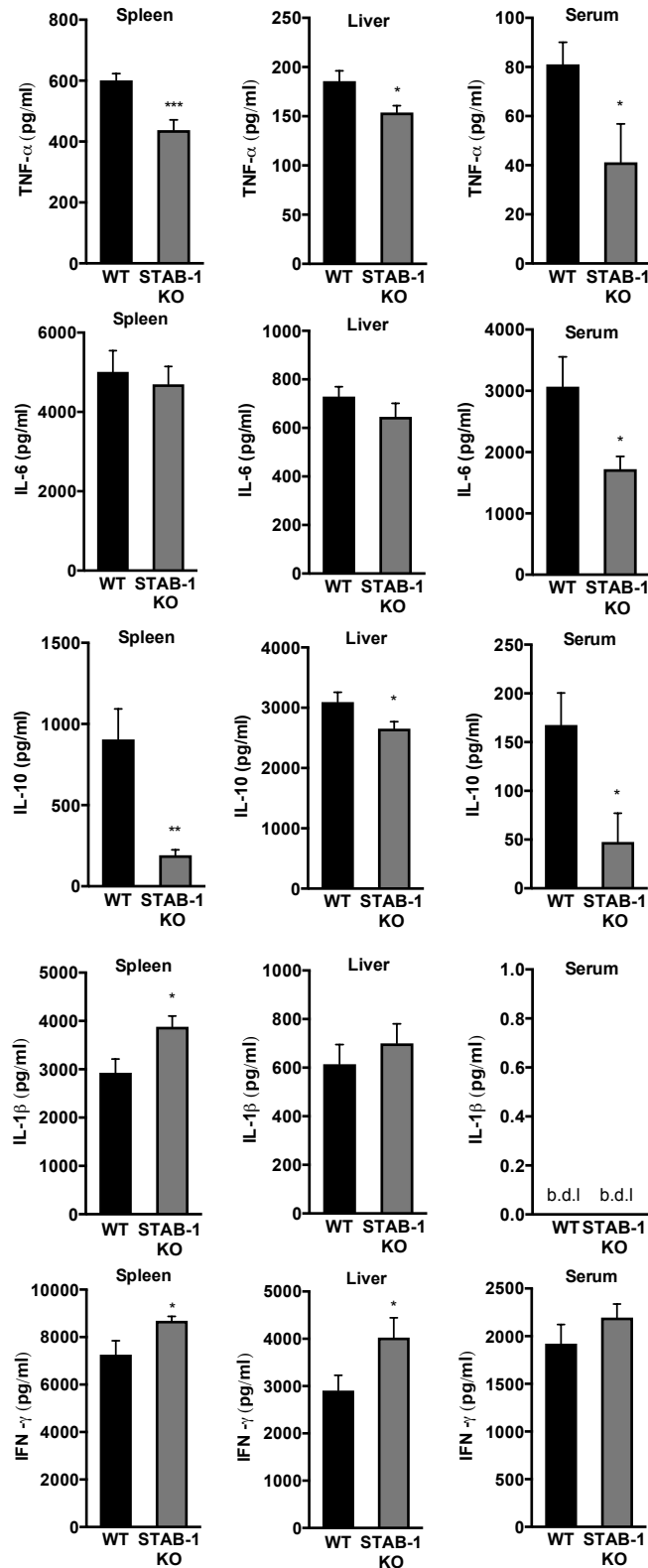
**Figure 4. STAB-1 regulates inflammatory cytokine production upon *Lm* infection.** WT and STAB-1 KO mice were intravenously infected with 5x10⁵ CFU of *Lm.* Mice were sacrificed at day three post-infection and spleen, liver and serum were collected. Levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6, interleukin (IL)-10, interleukin (IL)-1β and interferon (IFN)-γ were measured by ELISA (b.d.l: bellow detection level). Data are represented as an average of two independent experiments, each with five mice per group. *$p$<0.05; **$p$<0.01; ***$p$<0.001.
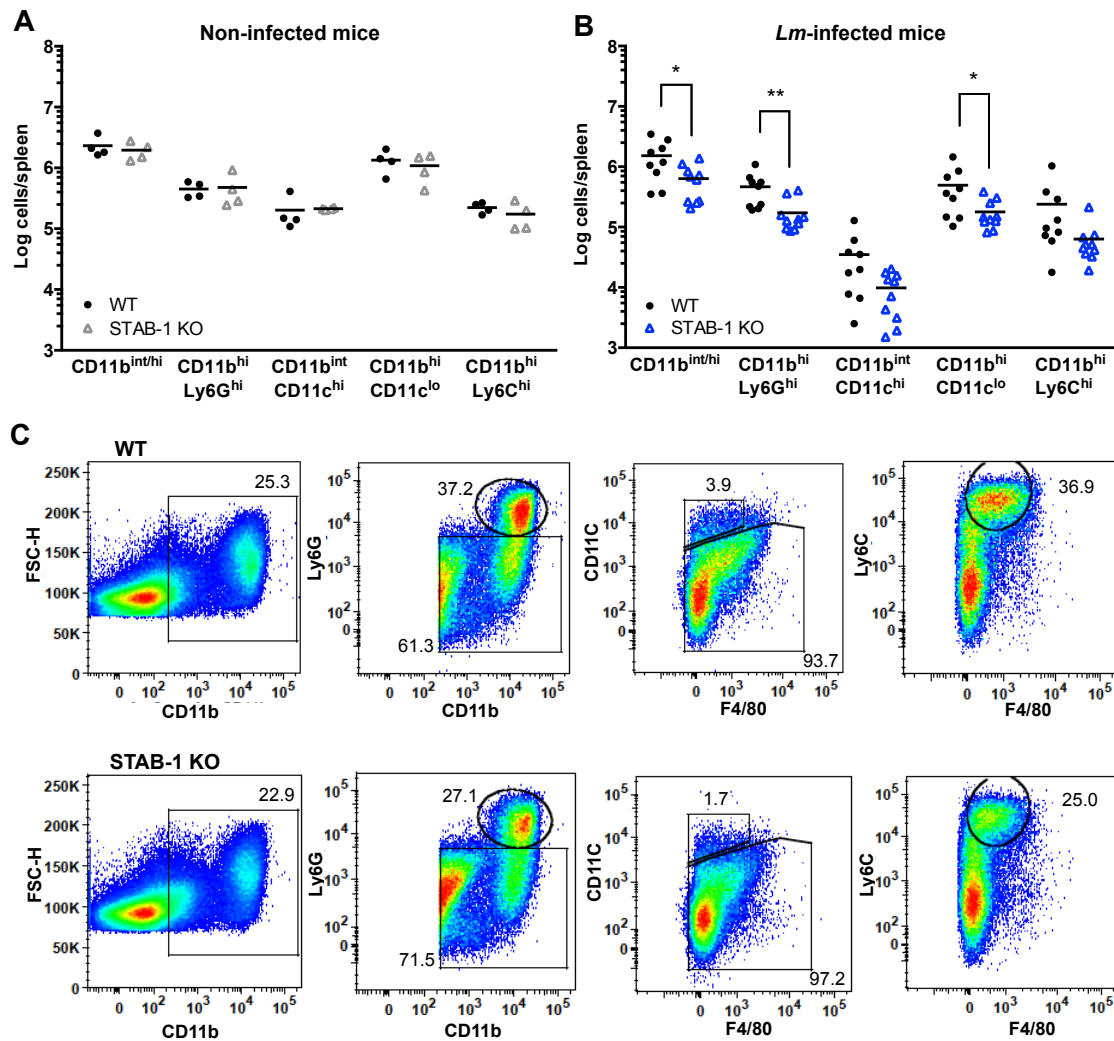
**Figure 5. STAB-1 controls immune cell accumulation in *Lm*-infected spleen.** Spleen cells from (A) non-infected mice and (B-C) *Lm* (5x10$^5$ CFU) infected WT and STAB-1 KO mice were isolated and analyzed by flow cytometry. Total numbers of myeloid cells (CD11b$^{int/hi}$), neutrophils (CD11b$^{hi}$Ly6G$^{hi}$), dendritic cells (CD11b$^{int}$CD11c$^{hi}$), macrophages (CD11b$^{hi}$ CD11c$^{lo}$) and inflammatory macrophages (CD11b$^{hi}$Ly6C$^{hi}$) are shown. Data are presented as scatter plots, with each animal represented by a dot and the mean indicated by a horizontal line. *$p<0.05$; **$p<0.01$. (C) Histograms representative of spleen cells distribution of *Lm* infected WT and STAB-1 KO mice.
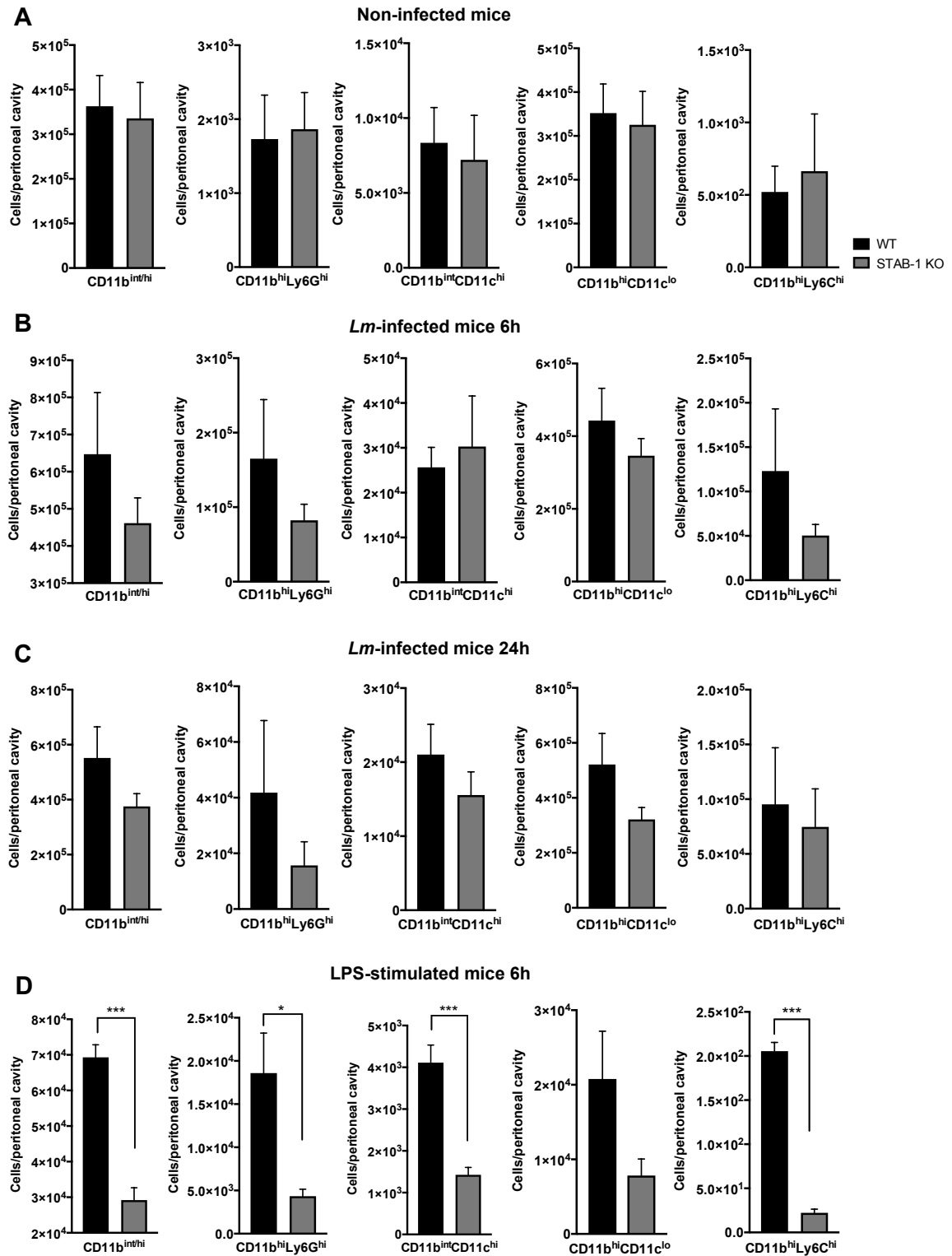
**Figure 6. STAB-1 promotes myeloid cells recruitment.** Single-cell suspensions recovered from the peritoneal cavity of WT and STAB-1 KO mice were analyzed by flow cytometry to evaluate cell population (A) Non-infected mice, (B-C) mice intraperitoneally infected with $10^5$ CFU of *Lm* for 6 h (B) or 24 h (C) and (D) mice intraperitoneally injected with purified LPS (15mg/mL) for 6 h. Data are represented as an averag of two independent experiments, with at least six mice per group. *$p<0.05$; ***$p<0.001$
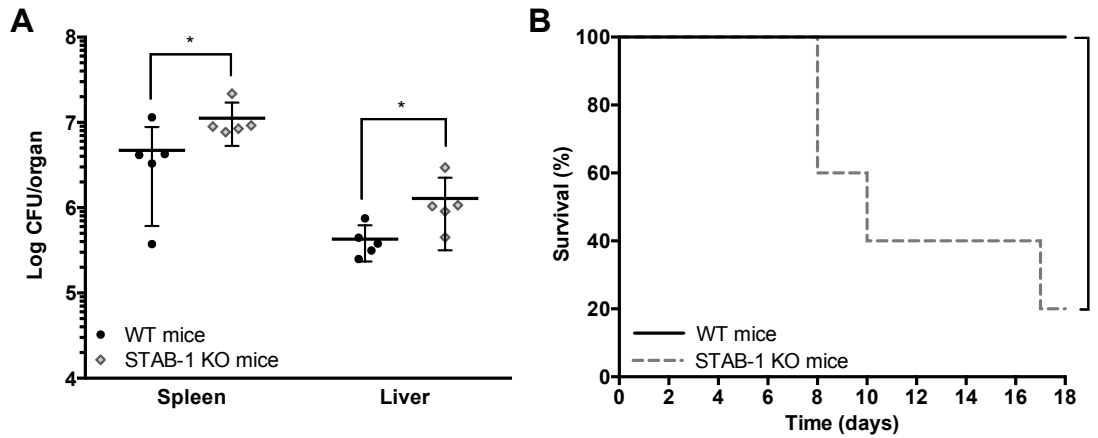
**Figure 7. STAB-1 limits *Lm* infection.** (A) Quantification of viable bacteria in spleens and livers recovered from WT and STAB-1 KO mice, three days after intravenous infection of $5x10^5$ CFU of *Lm.* Data are presented as scatter plots, each animal is represented by a dot and the mean is indicated by a horizontal line. (B) WT and STAB-1 KO mice survival after intravenous inoculation of $10^5$ CFU of *Lm* (n=5). *$p<0.05$.
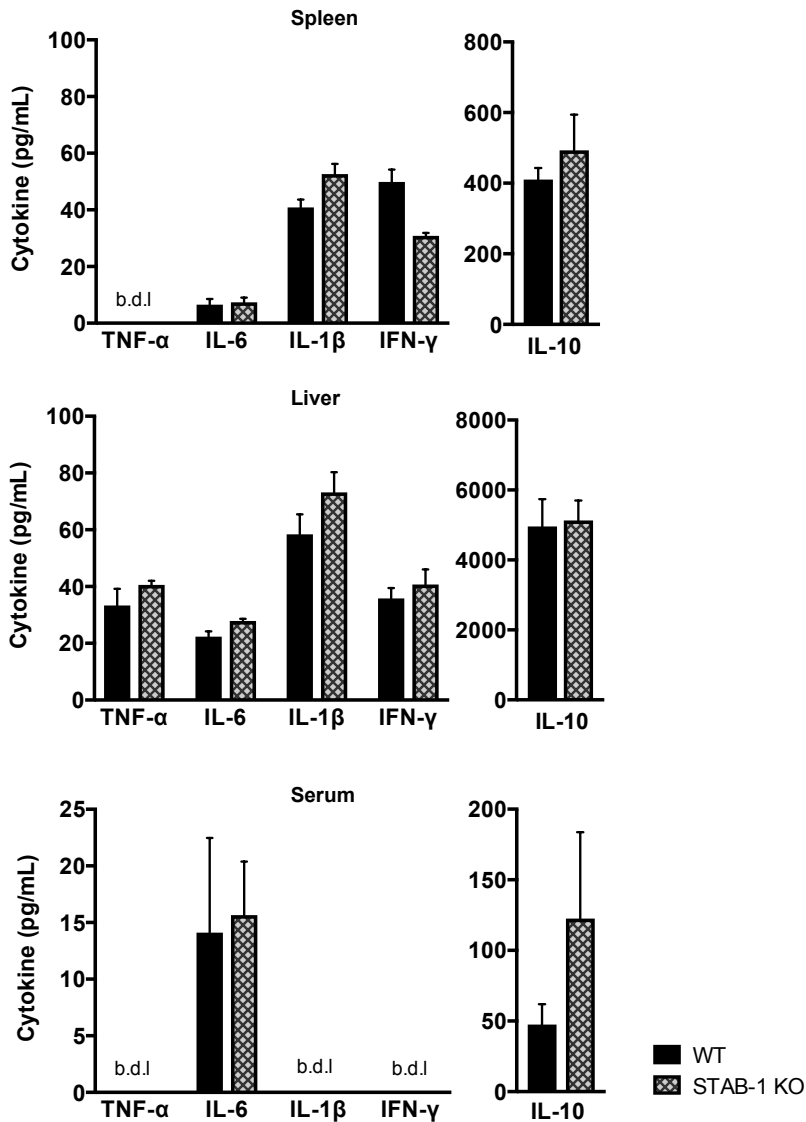
**Figure S1. STAB-1 KO mice do not have any defect on cytokine production.** Cytokine production (TNF-α, IL-6, IL-10, IL-1β, IFN-γ) in spleen, liver and serum of non-infected WT and STAB-1 KO mice quantified by ELISA (n=3).
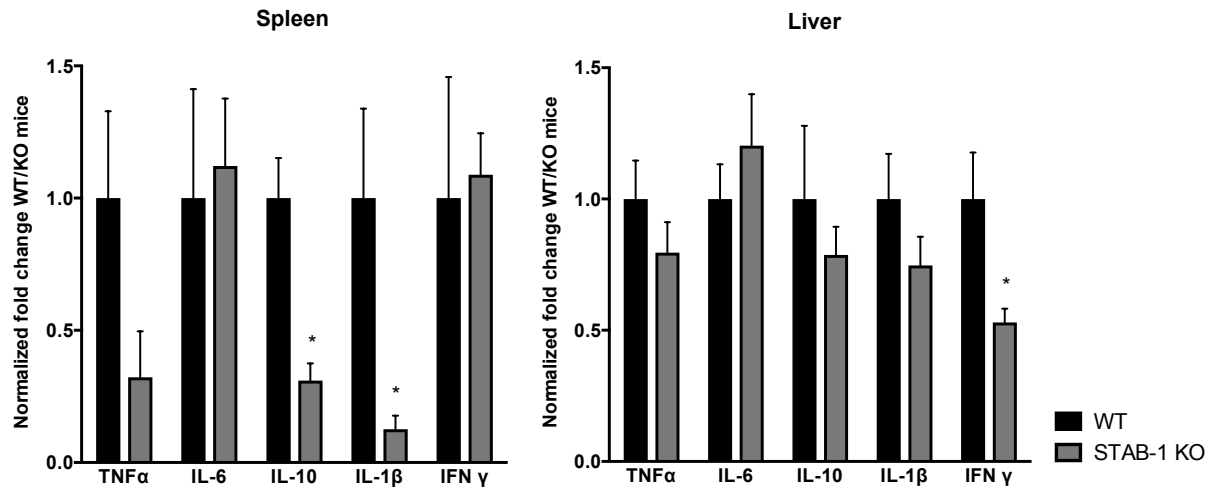
**Figure S2. STAB-1 regulates inflammatory cytokine expression upon *Lm* infection.** Cytokine expression of TNF-α, IL-6, IL-10, IL-1β and IFN-γ measured by qRT-PCR on total RNAs extracted from the organs of WT and STAB-1 KO mice intravenously infected with $5x10^5$ CFU of *Lm* (n=5). *$p<0.05$.
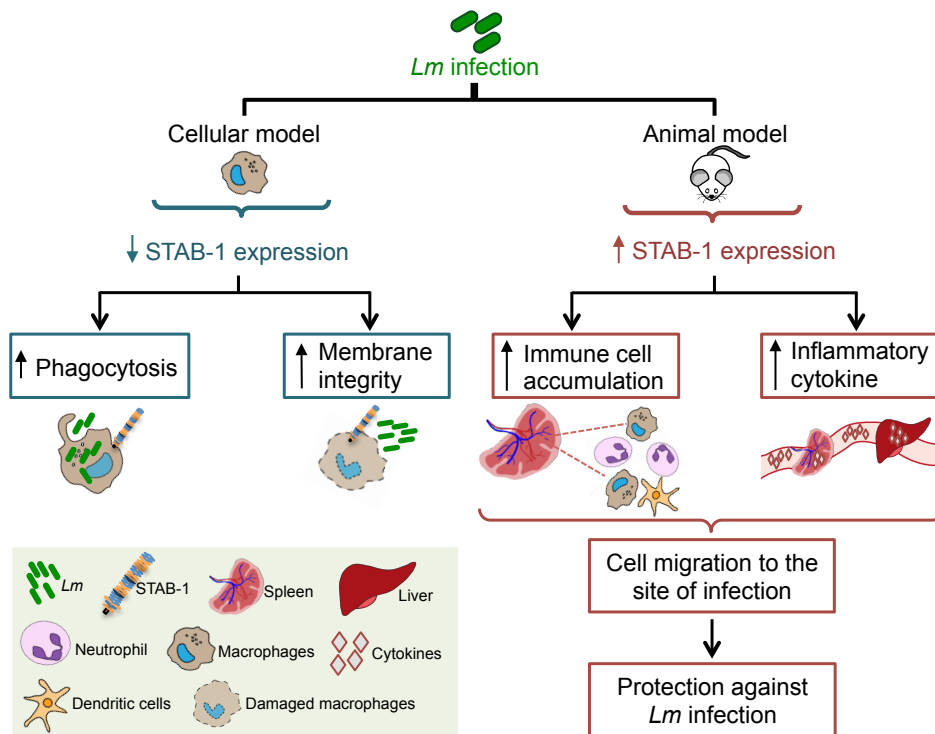
**Fig. S3. STAB-1 function as a new SR with a protective role against *Lm* infection.** In a cellular model of infection, *STAB-1* expression is down-regulated upon *Lm* infection, promotes bacterial uptake into macrophages and preserves macrophage membrane integrity. In an animal model of infection, *STAB-1* expression is increased upon infection, inducing myeloid cell accumulation in the spleen and increased production of cytokines by liver, spleen and serum. Thus, STAB-1 induces cell migration to the site of infection that in turn confers protection against *Lm* infection.

# PART I.2.

## Unravelling the Role of Scavenger Receptors in *Listeria monocytogenes* Infection

Scavenger Receptors are Required for Epithelial Cell Infection by *Listeria monocytogenes*

## I.2.1. RESULTS

### Scavenger Receptors are required for bacteria internalization into epithelial cells

The potential of *Lm* to infect different tissues is related with its ability to invade and replicate within both phagocytic and non-phagocytic host cells (Freitag *et al.* 2009). SRs are usually expressed on cells patrolling potential portals of pathogen entry, including macrophages, neutrophils, dendritic cells and microglia. Nonetheless, there are also evidences that they are expressed in several endothelial and epithelial cells (Murphy *et al.* 2005). Adhesion and invasion are decisive steps for successful colonization of host cells by *Lm*. Aiming to evaluate the role for SRs on the cellular infectious process of *Lm*, we analyzed whether SRs chemical inhibition affected *Lm* adhesion and invasion of eukaryotic cells. For this purpose, SRs were chemically saturated with different compounds known to inhibit SRs, before bacteria infection. We observed that the adhesion capacity of *Lm* was not affected by drug-cell treatment (Figure 1A). However, we found that pre-treatment of HeLa cells with three structurally different inhibitors for SRs severely impaired *Lm* entry when compared to non-treated cells (Figure 1B).
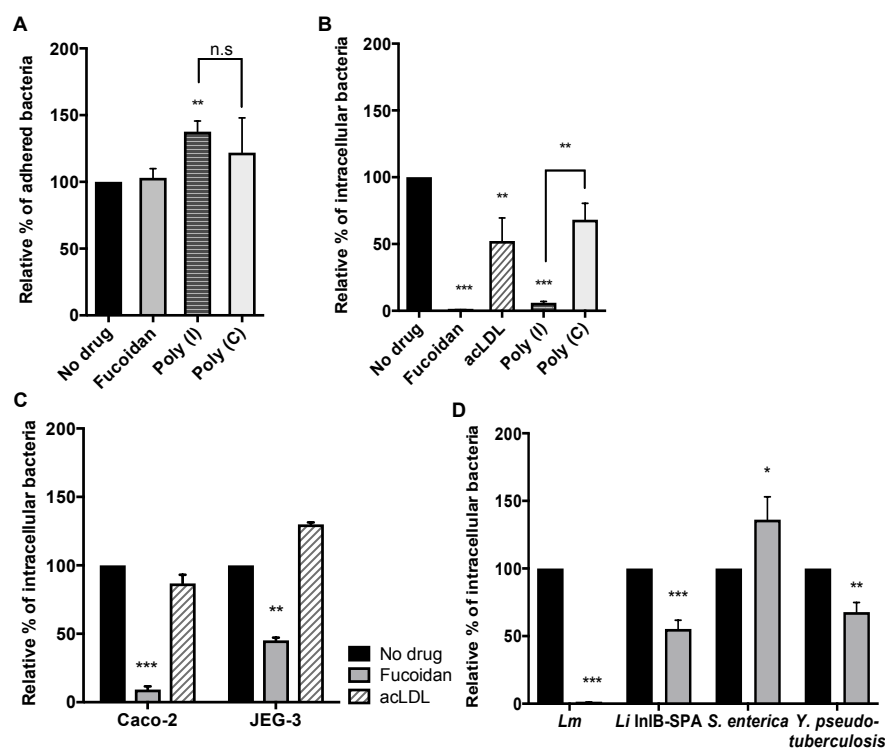


**Fig. 1. SRs are required for bacteria internalization into epithelial cells.** (A-D) Chemical saturation of SRs impairs bacteria internalization. Cells were left untreated (no drug) or pre-treated with fucoidan, acLDL, poly(I) and its control poly(C) before bacteria infection. (A) For adhesion assays, HeLa cells were infected with *Lm* for 30 min and adherent bacteria were quantified. (B-C) For invasion assays, (B) HeLa, (C) Caco-2 and JEG-3 cells were infected with *Lm* for 60 min, incubated with gentamicin for 90 min, washed and lysed to quantify the number of intracellular bacteria. (D) HeLa cells were infected with *Listeria* strains (as indicated above), *Salmonella enterica* for 15 min and *Yersinia pseudotuberculosis* for 30 min followed by 30 min with gentamicin. The number of intracellular bacteria was further quantified. Values are expressed relative to no drug values arbitrarily fixed to 100%. Values are mean ± SD of three independent assays. *$p<0.05$; **$p<0.01$; ***$p<0.001$.

Knowing that different cell lines display a specific SR expression profile, we evaluated whether SRs could play a role in *Lm* internalization of other epithelial cell lines. Pre-treatment of Caco-2 and JEG-3 cells with fucoidan also compromised *Lm* internalization (Figure 1C). In contrast, cell pre-treatment with acLDL did not affect *Lm* internalization into these epithelial cells (Figure 1C). To understand if the role of SRs was specific to *Lm* entry, HeLa cells were pre-treated with fucoidan, before being infected with *Lm* or other bacteria. The invasion capacity of the non-pathogenic *L. innocua* expressing InlB invasin was also reduced upon fucoidan-cell treatment (Figure 1D). Moreover, whereas the internalization of *Salmonella enterica* was slightly increased upon drug-cell treatment, the internalization of *Yersinia pseudotuberculosis* was impaired, although the effect was not so dramatic when compared with *Lm* infection (Figure 1D).

Altogether, these results suggest that while *Lm* adhesion to HeLa cells is independent on SRs, the *Lm* capacity to efficiently invade epithelial cells is dependent on SRs, pointing out a role for these host cell receptors in the earlier steps of *Lm* cellular infection cycle.

**Human SR-A and STABILIN-1 promote *Lm* internalization in HeLa cells**

To directly address the impact of specific SRs expressed by epithelial cells in *Lm* infection, we developed a siRNA-based approach in HeLa cells. For this purpose, we established specific depletion conditions for all the SRs and after transfection adherent and intracellular bacteria were quantified. We observed that although none of the receptors seemed to be essential for *Lm* adhesion (Figure 2A), silencing of both hSR-A and hSTAB-1 significantly diminished *Lm* internalization into HeLa cells, when compared to the entry levels of *Lm* in non-transfected (NT) cells or in cells transfected with control siRNA (scramble) (Figure 2B). The impact of SR-A in *Lm* infection was previously assessed. SR-AI/II KO mice were shown to be more susceptible to *Lm* infection and displayed increased hepatic granuloma formation (Ishiguro *et al.* 2001; Suzuki *et al.* 1997). Interestingly, STAB-1 was previously showed to bind to Gram-positive and Gram-negative bacteria *in vitro*, therefore suggesting a role for this receptor in bacterial recognition (Adachi *et al.* 2002). In order to confirm the impact of STAB-1 in *Lm* internalization, we depleted STAB-1 from HeLa cells, using two individual siRNAs complexes before *Lm* infection. As expected, the number of intracellular bacteria in STAB-1 depleted cells was significantly diminished when compared to NT cells or cells transfected with the scramble siRNA (Figure 2C). Importantly, depletion of STAB-1 was confirmed by western blot (Figure 2D).

Taken together, these data demonstrate that both hSR-A and hSTAB-1 promote *Lm* internalization into HeLa cells.
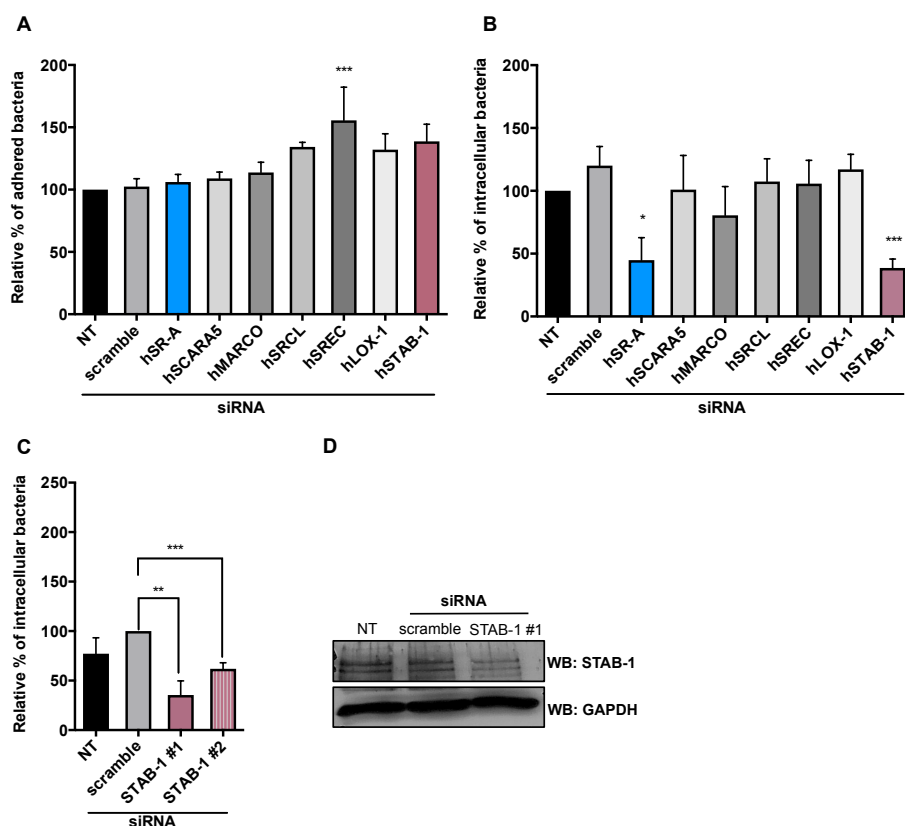
**Fig. 2. Human SR-A and STABILIN-1 promote *Lm* internalization in HeLa cells.** (A-C) Impact of SR silencing on *Lm* cellular infection. HeLa cells were left non-transfected (NT) or transfected with 10 pmol of siRNA control (scramble) or SR-specific siRNA duplexes for 48 h. (A) HeLa cells were infected with *Lm* during 30 min and adherent bacteria were quantified. (B-C) HeLa cells were infected with *Lm* for 60 min, incubated with gentamicin for 90 min, washed and lysed to quantify the number of intracellular bacteria in NT and transfected (STAB-1 #1 and #2) cells. Values are expressed relative to NT/scramble cells arbitrarily fixed to 100%. (D) Assessment of hSTAB-1 depletion efficiency by western blot, using GAPDH as a protein loading control. Values are mean ± SD of three independent experiments. Statistical significance is indicated as compared to NT/scramble cells. *$p<0.05$; **$p<0.01$; ***$p<0.001$.

## *Lm* does not interact directly with STAB-1

Having established that *Lm* internalization is reduced in STAB-1 depleted cells, we wondered whether *Lm* could benefit with the overexpression of this SR in HeLa cells. For this purpose, we engineered a cell line ectopically expressing STAB-1, using a lentiviral approach. The inducible lentiviral vector encodes a citrine fluorescent protein and it was transduced in HeLa cells, either as an empty control plasmid (mock) or fused with STAB-1 epitope-tagged with a human influenza hemagglutinin (HA) (Figure 3A). Transduction efficiency was measured by FACS, with 45% of cells being positive for citrine and HA expression (Figure 3A). By immunofluorescence staining we observed a perinuclear localization of STAB-1, although it was also expressed at the cell surface (Figure 3B). Aiming to understand if STAB-1 could work as a receptor for *Lm* and thus potentiate *Lm* adhesion, we infected both control cells (mock) and HeLa cells ectopically expressing STAB-1 with *Lm*.
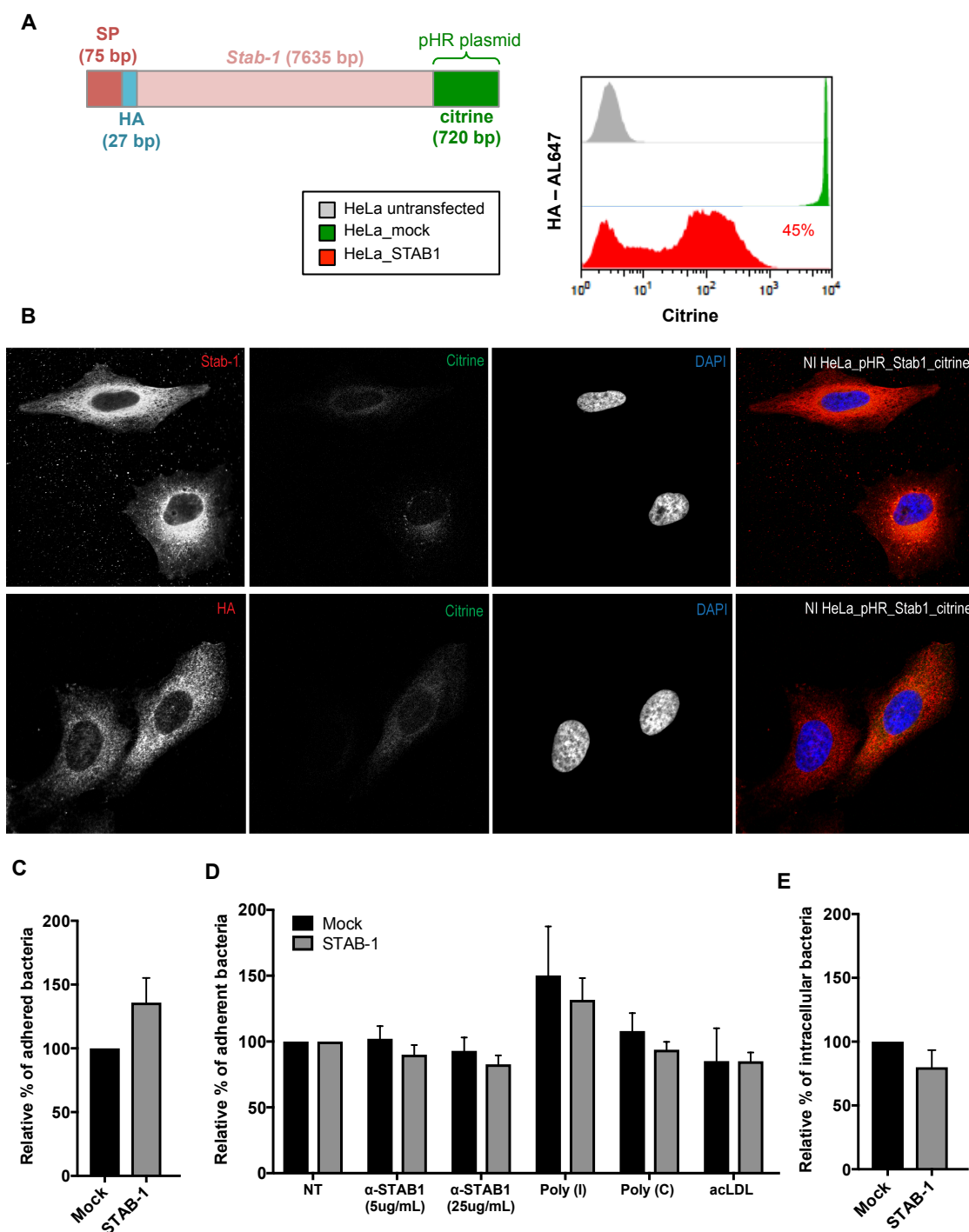
**Fig. 3. *Lm* does not interact directly with STAB-1.** (A) Schematic representation of the sequence cloned into the lentiviral expression plasmid: STAB-1 fused with a human influenza hemagglutinin (HA) in the N-terminus region, downstream its signal peptide (SP). Flow cytometry histogram representing HA- and citrine-positive HeLa cells, transduced either with an empty control vector (mock) or with a plasmid containing *STAB-1* fused with an HA (STAB-1). (B) Confocal microscopy images of HeLa cells ectopically expressing STAB-1 fused with HA, stained with an anti-STAB-1 (upper panel) or an anti-HA (bottom panel) antibodies. (C-E) Impact of STAB-1 expression on *Lm* cellular infection. (C-D) Mock and HeLa cells ectopically expressing STAB-1 were infected with *Lm* for 30 min and adherent bacteria were quantified. (D) Cells were pre-treated with an anti-STAB-1 antibody, acLDL, poly(I) and its control poly(C) before *Lm* infection. (E) Cells were infected with *Lm* for 60 min, incubated with gentamicin for 90 min, washed and lysed to quantify the number of intracellular bacteria. Values are expressed relative to mock cells arbitrarily fixed to 100%. Values are mean ± SD of three independent assays.

The percentage of bacteria adherent to STAB-1-expressing cells was increased, when compared with mock-adhered bacteria (Figure 3C). However, upon STAB-1 saturation, either with a α-STAB-1 antibody or with different SR-inhibitors, bacterial adhesion was not compromised (Figure 3D). Additionally, the percentage of intracellular bacteria was not altered by ectopically expression of STAB-1 in HeLa cells (Figure 3E).

Therefore, STAB-1 is required for *Lm* internalization although its increased expression does not favour *Lm* invasion of epithelial cells.

## I.2.2. MATERIAL AND METHODS

### Bacteria and cells

*Listeria monocytogenes* (*Lm*) EGD BUG 600 and *Listeria innocua* expressing InlB (*Li* InlB-SPA) were grown in Brain Heart Infusion (BHI) medium (BD-Difco) or BHI supplemented with 5 µg/ml erythromycin, respectively. *Salmonella enterica* serovar Typhimurium was grown in Luria Bertani (LB) medium (BD-Difco). These strains were grown at 37°C under aerobic conditions. *Yersinia pseudotuberculosis* was grown in BHI at 26°C with agitation. Bacteria are listed in Table 1. Human cervical adenocarcinoma HeLa cells (ATTC CCL-2) and human embryonic kidney epithelial HEK293T (contains the SV40 T-antigen region of replication - ATCC CRL-3216) were cultured in Dulbecco´s modified Eagle medium (DMEM) (Lonza), supplemented with 10% fetal bovine serum (FBS). Human epithelial colorectal adenocarcinoma Caco-2 cells (ATCC HTB-37) and human placental choriocarcinoma Jeg-3 cells (ATCC HTB-36) were cultured in Eagle´s minimum essential medium (EMEM) (Lonza), supplemented with 1 mM pyruvate (Lonza), 1% nonessential amino acids (Lonza) and 20% or 10% FBS, respectively. Human umbilical vein endothelial cells (HUVEC) were cultured in medium 199 (M199) (Sigma) supplemented with 10% FBS, 100 µg/ml of heparin (Sigma) and 30 µg/ml of endothelial cell growth supplement (ECGS). All cell lines were maintained at 37°C in a 7% $CO_2$ humidified atmosphere.

### Epithelial cells infection

Confluent epithelial cell lines were incubated during 30 min with: 100 µg/ml of fucoidan (Sigma-Aldrich), 10 µg/ml of acetylated low density lipoprotein (acLDL) (Invitrogen), 50 µg/ml of poly(I) or the respective control poly(C) (Santa Cruz Biotechnology). For adhesion assays, cells were infected for 30 min with exponential-phase bacteria at a multiplicity of infection (MOI) of 50, washed and lysed in 0.1% Triton X-100 for CFUs quantification. For gentamicin survival assays, cells were infected for 60 min with exponential-phase bacteria at MOI 50 and treated afterwards with 20 µg/ml of gentamicin (Lonza) for 90 min as described (Carvalho *et al.* 2015). Cells were then washed with PBS and lysed in 0.1% Triton X-100 for

CFUs quantification. HeLa cells were infected with exponential-phase *Yersinia pseudotuberculosis* (MOI 50) for 30 min followed by 30 min with gentamicin, or with exponential-phase *Salmonella enterica* Typhimurium (MOI of 100) for 15 min. Cells were then washed and lysed for CFUs quantification.

**Transfection of siRNA duplexes**

HeLa cells were transfected with 10 pmol of control siRNA-D (sc-44232 Santa Cruz Biotechnology) or with a pool of 3 individual siRNAs (20-25 nucleotides) designed to knock down SRs gene expression: hSR-A (sc-44116), hSCARA5 (sc-77423), hMARCO (sc-75747), hSRCL (sc-72913), hSREC (sc-36561), hLOX1 (sc-40185) and hSTAB-1 (#1: sc-45784) (Santa Cruz Biotechnology) or hSTAB-1 (#2: NM_015136) (Sigma). Lipofectamine RNAiMax (Invitrogen) was used as a transfection reagent following the manufacturer's instructions and assays were performed 48 h later. Depletion efficiency (STAB-1) was confirmed by western blot. Non-transfected (NT) and siRNA-transfected cells were lysed with Laemmli buffer and extracts were resolved by SDS-PAGE on a 8% gel as described (Martins *et al.* 2012). Samples were transferred onto nitrocellulose membrane and blotted with rabbit anti-STAB-1 (1:500, Millipore), followed by HRP-conjugated goat anti-rabbit IgG (1:2000, P.A.R.I.S). Signal was detected using ECL (Thermo Scientific) and digitally acquired in a ChemiDoc XRS+ system (Bio-Rad Laboratories).

**Lentiviral vector production and transduction**

Total RNA was extracted from HUVEC using TripleXtractor (GRISP), as recommended by the manufacturer. Purified RNA (5 µg) was reverse-transcribed, using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific) and oligo(dT)$_{18}$ as recommended by the manufacturer. STAB-1 DNA fragment was amplified by polymerase chain reaction (PCR) using phusion high-fidelity DNA polymerase (Thermo Scientific) and HUVEC cDNA as a template. Purified STAB-1 DNA fragment was digested and colligated in the multiple cloning site of pHR_citrine plasmid. STAB-1 signal peptide containing the HA tag (integrated DNA technologies-IDT) was annealed and fused with the STAB-1 DNA sequence previously amplified. Constructs were confirmed both by PCR and DNA sequencing. Primers and signal peptide sequences are listed in Table 2. For virus assembly, pHR plasmid containing STAB-1 fused with an HA tag, together with the envelope plasmid pMD-G and packaging plasmid pCMVR8.91 were co-transfected into HEK293T cells, using lipofectamine according the manufacturer's instructions. Viral supernatants were harvested after 72 h, filtered and incubated with target HeLa cells (supplemented with 1% of penicillin/streptomycin) for 72 h.

**Flow cytometry and cells sorting**

Ectopic expression of STAB-1 was confirmed by flow cytometry. Transduced HeLa cells were collected in ice-cold storage solution (PBS supplemented with 2% FBS and 0.1% azide), pelleted by centrifugation, washed and labelled with mouse anti-HA (1:50, abcam) during 30 min, followed by 20 min incubation with Alexa Fluor 647-conjugated anti-mouse IgG (1:500, Molecular Probes). Data were acquired using FACSCanto II flow cytometer with FACSDiva software (BD Biosciences). Data were analyzed using FlowJo software (TreeStar Inc.) and the percentage of transduced cells (positive for both citrine and HA expression), was determined. Cells were ressuspended in basic sorting buffer (PBS supplemented with 2% FBS, 1nM EDTA, 25mM HEPES) and were sorted to select the cell population that highly express HA (5.3%). Sorted cells were collected, cultured and expanded.

**Immunofluorescence**

HeLa cells ectopically expressing STAB-1 were fixed in 3% paraformaldehyde (15 min), quenched with 20 mM $NH_4Cl$ (1 h) and blocked with 10% BSA (sigma) in PBS (30 min). Antibodies were diluted in 0.2% saponin (Merck) supplemented with 1% BSA. Coverslips were incubated for 1 h with primary antibodies, rabbit anti-STAB-1 (1:100, Millipore) or mouse anti-HA (1:50, abcam). Cells were washed in 0.2% saponin containing 1% BSA and incubated 45 min with secondary Cy3-conjugated anti-mouse/rabbit (Jackson ImmunoResearch) antibodies. DNA was counterstained with DAPI (Sigma). Cells were washed and slide preparations were mounted and dried at room temperature. Images were collected with a confocal laser-scanning microscope (Leica SP5II) and processed using ImageJ.

**Statistics**

Statistics were carried out with Prism software (GraphPad), using unpaired two-tailed Student's *t*-test to compare means of two groups, and one-way ANOVA with Tukey's post-hoc test for pairwise comparison of means from more than two groups, or with Dunnett's post-hoc test for comparison of means relative to the mean of a control group. For statistically significant differences we considered: $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$.

## I.2.3. TABLES

**Table 1 -** Strains

| Strain | Description | Source |
|---|---|---|
| *Listeria monocytogenes* DC22 | EGD (wild type, serotype 1/2a) | Lab collection |
| *Listeria innocua* DC391 | *Li* expressing InlB covalently anchored to the bacterial surface by an SPA motif (InlB-SPA) | (Khelef et al. 2006) |
| *Salmonella enterica* DC534 | Serovar Typhimurium (wild-type, 12023) | (NTCC, Colindale, UK) |
| *Yersinia pseudotuberculosis* DC321 | IP2666 | (Isberg et al. 1985) |

**Table 2 –** Primers and signal peptide sequence

| Primers | Sequence (5´to 3´) | Purpose |
|---|---|---|
| STAB-1_HPaI F | CTCGTTAACCAGGTGCTGTTCAAA | STAB-1 amplification |
| STAB-1_MluI R | TAGACGCGTCTTGACTGTGAGGAT | |
| FEEL-1 SP-HA | ACGCGTGCGGCCGCATGGCGGGGCCCCGGGGCCTCCTCCCACTCTGCCTCCTGGCCTTCTGCCTGGCAGGCTTCAGCTTCGTCAGGGGGTACCCATACGATGTTCCAGATTACGCT | Signal peptide fused to the HA tag |
| FEEL-1 SP-HA_Complement | AGCGTAATCTGGAACATCGTATGGGTACCCCCTGACGAAGCTGAAGCCTGCCAGGCAGAAGGCCAGGAGGCAGAGTGGGAGGAGGCCCCGGGGCCCCGCCATGCGGCCGCACGCGT | |
| FEEL-1_F2 | TGCACGGAGTGTGCAACCAT | Sequecing |
| FEEL-1_ F3 | CTGCCGGGAAATCCTTACCACA | |
| FEEL-1_ F4 | CCTGGCTGTGAACATTTCTGAG | |
| FEEL-1_ F5 | CGGTTCTGCAACGAGTCCAT | |
| FEEL-1_ F6 | CCGAGGACCGAGCTTTCTGG | |
| FEEL-1_ F7 | TCAAGTCGCTGCCTGCATAG | |
| FEEL-1_ F8 | CACCTGCCAGGATGGCTACA | |
| FEEL-1_ F9 | GCACTTCATTGACCGTGTCCT | |
| FEEL-1_ F10 | TCTTTGGGATTACGCAGCGTCT | |
| FEEL-1_ F11 | GAGCACGCCAACTGCTTGAG | |
| FEEL-1_ F12 | CGTCCCTGTCAATGAAGGCTTT | |
| FEEL-1_ R2 | CAGCAGTGAGCAGGGTGATG | |

## I.2.4. DISCUSSION

Host cells deploy multiple transmembrane and cytosolic sensors to detect microbial pathogens and arm signalling pathways that activate innate immune responses. Currently, the involvement of SRs in the recognition of different microbial structures has been of major interest. During infection, SRs represent an important part of the innate immune defence by acting as PRRs. They are implicated in adhesion, antigen presentation, apoptotic-cell clearance, endocytosis and phagocytosis (Areschoug *et al.* 2009). SRs are expressed by several endothelial and epithelial cells (Murphy *et al.* 2005). Importantly, adhesion and internalization are hallmark steps to establish infection. We found that *Lm* capacity to

efficiently invade epithelial cells is dependent on SRs. It was previously described that fucoidan nearly completely inhibited SR-A dependent binding and internalization of *Francisella tularensis* (LVS) by J774 and THP-1 cells (Pierini 2006). *In vitro* studies revealed that the addition of poly (I) before infection resulted in a specific inhibition of adenovirus sequestration by J774 macrophages and primary Kupffer cells (Haisma *et al.* 2008). Moreover, the recognition of *Cryptococcus neoformans* by stable Chinese hamster ovary (CHO) cell line expressing SREC was found to be inhibited by acLDL, poly(I) and fucoidan (Means *et al.* 2009). Each cell line displays a specific SR expression profile. The diversity of host cell receptors and their broad expression allow bacterial tropism for a panoply of different cells and tissues. On the other hand, bacteria employ different internalization mechanisms that trigger distinct signalling pathways. The invasion capacity of the non-pathogenic *Listeria* expressing InlB was diminished upon SRs inhibition, which reinforces the particular role of SRs during bacterial invasion. *Li* InlB-SPA is only capable to induce its own uptake by hijacking cellular c-Met, but therefore remains entrapped in the vacuole. The mechanisms used by different bacteria to evade host cells could account for the impact of SRs in pathogen internalization (Isberg *et al.* 1990; Schulze-Koops *et al.* 1993; Suarez *et al.* 1998). *Yersinia* proliferates mainly as an extracellular pathogen, once it injects T3SS (type III secretion system) effectors that actively inhibit cellular uptake by blocking actin cytoskeleton rearrangements. However, the interaction of surface bacterial proteins with host cell receptors mediates its own internalization through a zipper mechanism (Pizarro-Cerda *et al.* 2016). In contrast, *Salmonella* induces its internalization into non-phagocytic cells mainly via a trigger mechanism. It injects directly into the host cell cytoplasm, via the T3SS, a number of bacterial effectors that trigger cellular responses. These effectors activate host cell GTPases that spatiotemporally stimulate actin cytoskeleton rearrangements and allow membrane ruffling (Pizarro-Cerda *et al.* 2006). More recently, it was described that *Salmonella* Rck outer membrane protein binds to the cell surface, leading to bacterial internalization via a zipper mechanism, independently of T3SS-1 (Wiedemann *et al.* 2016). The role of SRs in the context of these pathogens infection is poorly unknown. It was previously shown that CD36 works as a phagocytic receptor for a number of pathogenic bacteria, including *Salmonella* (Baranova *et al.* 2008). Moreover, DMBT1 was shown to inhibit cytoinvasion of *Salmonella enterica* and cytokine secretion *in vitro*, thus revealing that NOD2 and TLR4 regulate DMTB1 in epithelial cells to modulate bacterial recognition and invasion (Rosenstiel *et al.* 2007). Interestingly, among the SRs that we tested, SR-A and STAB-1 were found to be important for *Lm* internalization into epithelial cells. However, it seemed that STAB-1 does not interact directly with *Lm* at the surface of epithelial cells. While the bacteria internalization by a phagocytic cell is mostly driven by the cell itself, the invasion of non-professional phagocytes is highly dependent on several *Lm* factors. Having this in mind, it would be

interesting to find new bacterial proteins that account for this internalization defect. Here, we found that STAB-1 promotes *Lm* internalization in non-phagocytic cells. In an epithelial cell model, STAB-1 may work as a co-receptor of an important PRR in *Lm* internalization or may down-regulate the expression or alter the localization of certain host cell receptors crucial for *Lm* internalization. Further work will be necessary to understand the molecular details governing STAB-1-*Lm* interaction during cell invasion.

# PART II.1.

## Role of CadC in *Listeria monocytogenes* Resistance to Cadmium and in Bacterial Pathogenicity

*Listeria monocytogenes* CadC Regulates Cadmium Efflux and Fine-tunes Lipoprotein Localization to Escape the Host Immune Response and Promote Infection

# Listeria monocytogenes CadC Regulates Cadmium Efflux and Fine-tunes Lipoprotein Localization to Escape the Host Immune Response and Promote Infection

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Listeria monocytogenes is a major intracellular human foodborne bacterial pathogen. We previously revealed L. monocytogenes cadC as highly expressed during mouse infection. Here we show that L. monocytogenes CadC is a sequence-specific, DNA-binding and cadmium-dependent regulator of CadA, an efflux pump conferring cadmium resistance. CadC but not CadA is required for L. monocytogenes infection in vivo. Interestingly, CadC also directly represses lspB, a gene encoding a lipoprotein signal peptidase whose expression appears detrimental for infection. lspB overexpression promotes the release of the LpeA lipoprotein to the extracellular medium, inducing tumor necrosis factor α and interleukin 6 expression, thus impairing L. monocytogenes survival in macrophages. We propose that L. monocytogenes uses CadC to repress lspB expression during infection to avoid LpeA exposure to the host immune system, diminishing inflammatory cytokine expression and promoting intramacrophagic survival and virulence. CadC appears as the first metal efflux pump regulator repurposed during infection to fine-tune lipoprotein processing and host responses.

**Keywords.** Listeria/virulence factor; CadAC; gram-positive; host-response; pathogen.

Despite their toxicity at high concentration, some heavy metals are required as cofactors for enzymatic reactions or as structural components of bacterial proteins. Therefore, their intracellular concentration needs to be finely tuned to maintain metal homeostasis. Efflux pumps are usually substrate specific and control intracellular metal concentrations conferring heavy metal resistance [1, 2]. In its ionized form, cadmium (Cd) is toxic for many organisms, including bacteria. Erosion, forest fires, and volcanic eruptions are natural sources of Cd, which is dispersed into air, water, soils, and foodstuffs. Cd resistance systems are commonly composed of a metal-responsive transcriptional repressor (CadC) belonging to the ArsR-SmtB family [3–5] and a P1-type ATPase (CadA) that extrudes heavy metals from the cell [6].

Listeria monocytogenes is a major intracellular foodborne bacterial pathogen causing listeriosis, a systemic infection in humans [7]. Among zoonotic diseases under European Union surveillance, listeriosis is the most severe, with 99.1% of cases requiring hospitalization and a case-fatality rate of 15.6% [8].

Listeriosis is clinically characterized by septicemia and dissemination to the nervous system and fetal-placental unit [9]. As a foodborne pathogen, L. monocytogenes has the capacity to colonize various niches, ranging from inert and organic matrixes to the intestinal lumen, where it competes with resident microbiota, translocates across the epithelium, multiplies in phagocytic and nonphagocytic cells, disseminates via the blood, and evades the immune response [7]. A functional CadAC system was previously identified in L. monocytogenes Lm74 on the Tn5422 transposable element harbored by the pLm74 plasmid [10, 11] and was shown to be induced by and to confer resistance to Cd [11]. We previously showed that, in L. monocytogenes EGDe, cadC is highly expressed during infection and required for L. monocytogenes virulence [12]. Here we characterize the L. monocytogenes Cd resistance system and discover an unexpected role of CadC in bacterial pathogenicity.

## METHODS

### Bacteria and Cells

L. monocytogenes strain EGDe (ATCC-BAA-679) [13] was grown in brain heart infusion broth (BHI; BD-Difco) at 37°C with erythromycin (5 μg/mL) and chloramphenicol (7 μg/mL). Escherichia coli was grown in Luria-Bertani broth at 37°C with ampicillin (100 μg/mL), erythromycin (300 μg/mL), and kanamycin (30 μg/mL). RAW-264.7 macrophages (ATCC-TIB-71) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS; Lonza), and bone marrow–derived macrophages (BMDMs) were cultured in DMEM with

10 mM HEPES, 10% FBS, and 10% L929-conditioned medium at 37°C in 5% $CO_2$.

Deletions ($\Delta cadA$, $\Delta cadC$, $\Delta cadAC$, $\Delta lspB$, and $\Delta cadC\Delta lspB$), insertion ($\Delta lpeA$), and complementation ($\Delta cadC+cadC$) were performed as previously described [14, 15]. Overexpression (wild type [WT]+$lspB$) was performed using a pMK4 vector [16] carrying the strong constitutive Pprot promoter [17, 18]. Primers are listed in Supplementary Table 1. Constructs were confirmed by sequencing.

### Toxicity Assays

For Cd challenge, 1/100-diluted overnight cultures were challenged after exposure to 384 μM cadmium chloride ($CdCl_2$) for 210 minutes. Growth ($OD_{600}$) was measured every 45 minutes. In disk diffusion assays, plated bacterial lawns were overlaid with a 6-mm paper disk soaked with 10 μL of metal salt solution (100 mM). The growth inhibition zone diameter was measured after overnight incubation at 37°C. For minimum inhibitory concentrations, 96-wells microtiter plates containing BHI/metal-salt solutions (100 μL/100 μL) were inoculated with 1 μL of overnight cultures. Growth was assessed (at $OD_{600}$) after incubation at 37°C for 24 hours.

### Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

Bacterial cultures ($OD_{600} = 0.6$) were supplemented with 384 μM $CdCl_2$ for 15 minutes, centrifuged, washed with phosphate-buffered saline, and lyophilized. Dried samples were digested with $HNO_3$, suspended in 4 N $HNO_3$, diluted in water, and analyzed by ICP-MS.

### Proteins

*cadC* was cloned into pET28b, sequenced, and transformed in *E. coli* BL21(DE3). CadC-His$_6$ production was induced with 0.1 mM IPTG at 37°C for 3 hours. Cells were resuspended, sonicated, cleared by centrifugation, and subjected to soluble fraction purification by Ni-NTA-agarose chromatography (Qiagen). Lipoproteins were recovered by the Triton X-114 phase-partitioning method [19], and culture supernatant proteins were recovered by trichloroacetic acid precipitation [20]. Sodium dodecyl sulfate–polyacrylamide gel electrophoretic protein bands were excised, reduced with DTT, alkylated with iodoacetamide, and in-gel digested with trypsin. Peptide identification was performed by matrix-assisted laser desorption/ionization time of flight/time of flight mass spectrometry [21].

### Electrophoretic Mobility Shift Assays (EMSAs)

CadC DNA binding assays were performed in 50 mM Tris-HCl (pH 7.4), 6 mM $MgCl_2$, 100 mM NaCl, 50 mM KCl, 100 ng of DNA, and purified CadC/GFP. After 20 minutes at room temperature, samples were resolved in 6% polyacrylamide gel and visualized by DNA staining.

### Chromatin Immunoprecipitation (ChIP) and Quantitative Polymerase Chain Reaction (qPCR) Analyses

ChIP assays were performed as described elsewhere [22], using anti-CadC polyclonal rabbit serum generated through CadC-His$_6$ as previously described [15]. A total of 1–10 ng of ChIP-purified DNA (NZYGelpure) was analyzed by qPCR. Fold enrichment is shown normalized to an unrelated promoter region (*inlA*) and as compared to mock-IP (IP without anti-CadC).

### Macrophage Infection

Bone marrow cells were collected from C57BL/6 mouse femurs and differentiated for 10 days. BMDMs or RAW macrophages were exposed for 20 or 30 minutes, respectively, to *L. monocytogenes* ($OD_{600} = 0.8$) at a multiplicity of infection of 10. Macrophages were treated for 10 minutes–4.5 hours with 20 μg/mL gentamicin, washed, and lysed (0.1% Triton X-100), and intracellular bacteria were enumerated by plating.

### Transmission Electron Microscopy

*L. monocytogenes* ($OD_{600} = 0.8$) was fixed for 1 hour at room temperature (in 4% paraformaldehyde, 2.5% glutaraldehyde, and 0.1 M sodium-cacodylate [pH 7.2]), stained for 2 hours with 1% osmium tetroxide, and compacted in 30% bovine serum albumin. Bacterial pellets were fixed overnight in 1% glutaraldehyde, dehydrated in ethanol, and embedded in Epon-812. Ultrathin sections (40–50 nm) were placed on 400-mesh copper grids and visualized (Jeol JEM-1400).

### RNA Techniques

RNA was extracted from *L. monocytogenes* ($OD_{600} = 0.8$) and RAW macrophages (5 hours after infection), treated (TURBO-DNA-free, Ambion), checked for quality (Experion, Bio-Rad Laboratories), reverse transcribed (iScript, Bio-Rad Laboratories), and analyzed by real-time qPCR as previously described [12]. Gene expression data were analyzed by the comparative cycle threshold method [23], normalized to relative reference gene expression (*L. monocytogenes* 16S ribosomal RNA or *Mus musculus hprt1*).

### Mouse Infections

Intravenous and oral inoculations were performed as described elsewhere (n = 5) [24]. Animal procedures were in agreement with European Commission (directive 2010/63/EU) and Portuguese (Decreto-Lei 113/2013) guidelines, approved by the Instituto de Biologia Molecular e Celular Ethics Committee and the Direcção Geral Veterinária (license PTDC/SAU-MIC/111581/2009).

### Enzyme-Linked Immunosorbent Assay (ELISA)

Cytokine levels released into infected RAW macrophage supernatant were measured using a murine ELISA kit (eBioscience).

**Statistical Analyses**

Statistics were performed with Prism (GraphPad), using the unpaired 2-tailed Student *t* test to compare mean values of 2 groups and 1-way analysis of variance with either the Tukey post hoc test for pair-wise comparison of mean values from >2 groups or the Dunnett post hoc test for comparison of mean values relative to the mean value for a control group.

## RESULTS

### *L. monocytogenes* Chromosomally Encodes a Cd Efflux System Expressed in the Presence of Cd and Independently of PrfA

*L. monocytogenes cadAC* encodes proteins with high level of identity to CadAC Cd efflux systems from several species (Figure 1A and 1B and Supplementary Figure 1*A*). *L. monocytogenes* CadC displays a classic DNA-binding helix-turn-helix motif and type 1 metal-binding site composed of 4 critical cysteines [25] (Figure 1A). The predicted *L. monocytogenes* CadC structure is close to that of *Staphylococcus aureus* CadC (Supplementary Figure 1*B*). However, *L. monocytogenes* CadC lacks the type 2 metal-binding site present in *S. aureus* CadC but dispensable for metal binding [26] (Figure 1A). *L. monocytogenes* CadA exhibits a metal-binding domain and motifs conserved in P1-type ATPases and is predicted to be a membrane protein with 8 transmembrane domains (Supplementary Figure 1*C*) [27]. Whereas *cadA* and *cadC* are harbored by plasmids in different *Listeria* strains [10, 11, 28], they are located on the *L. monocytogenes* chromosome. *L. monocytogenes cadA* is found downstream of *cadC*, with an oppositely oriented gene in between (*lspB*) encoding a putative lipoprotein signal peptidase (Lsp) type II [29, 30] (Figure 1B). *cadA-lspB-cadC* are flanked by an integrase-encoding gene (*lmo1097*) and 12 Tn916-like genes (*lmo1103–lmo1114*). The average GC content percentage (32%) of the *lmo1097–lmo1102* locus is notably lower than that of surrounding regions (39%–43%; Figure 1B).

We first analyzed whether *cadA, lspB*, and *cadC* are transcribed from a single promoter. Although independent transcripts were detected for the 3 genes in the presence of Cd, no cotranscript was observed (Figure 1C), indicating that in the conditions tested there is no *cadA-lspB-cadC* cotranscription.

PrfA controls the expression of major *L. monocytogenes* virulence genes [31, 32]. We found a palindromic sequence, TTAACAgaTTTCAA, bearing 2 mismatches with the consensus PrfA box (TTAACAttTGTTAA), 661-bp upstream from the *cadC* start codon. PrfA-dependent *cadC* transcription was assessed on wild-type (WT) and Δ*prfA* strains grown in the presence of Cd in either BHI broth or glycerol-supplemented minimal medium. PrfA is fully active in minimal medium [33]. Levels of *cadC* transcripts were similar in both strains under both conditions (Figure 1D),

demonstrating that PrfA does not control *cadC* expression. Thus, *L. monocytogenes* encodes a chromosomal putative Cd efflux system whose expression is Cd dependent and PrfA independent.

### CadA Is a Functional Cd Efflux Pump Required for *L. monocytogenes* Resistance to Cd

Growth rates of single-deletion (Δ*cadA* and Δ*cadC*) and double-deletion (Δ*cadAC*) mutants were comparable to that of the WT strain (Figure 2A), indicating that none of the Cad proteins is essential for viability and growth in rich medium. Addition of Cd to mid-exponential-phase cultures induced a slight decrease in the growth of WT and Δ*cadC* strains, whereas it notably impaired the growth of Δ*cadA* and Δ*cadAC* strains, revealing the role of CadA in *L. monocytogenes* resistance to Cd. While the growth inhibition zones observed when *L. monocytogenes* lawns were grown on BHI plates overlaid with disks saturated with $CdCl_2$ were equivalent for WT and Δ*cadC* strains, Δ*cadA* displayed increased Cd susceptibility (Figure 2B), confirming the role of CadA in Cd resistance and demonstrating that CadC is not required for Cd resistance. Similar areas of growth inhibition were observed with disks saturated either with $CdCl_2$ or cadmium sulfate (Figure 2B), showing that Cd is the cause of toxicity. WT and Δ*cadA* strains showed similar growth inhibition in response to all other metal salts tested (Figure 2B), indicating that CadA mainly confers resistance to Cd. In agreement with this finding, compared with the WT strain, the Δ*cadA* strain displayed a 10-fold decrease in the minimum inhibitory concentration of Cd, while it remained unchanged for zinc, cobalt, copper, and nickel (Figure 2C). We measured intracellular levels of Cd, zinc, and lead in WT and Δ*cadA* strains. Whereas zinc and lead levels were equivalent in both strains, the Δ*cadA* strain accumulated nearly 6-fold more intracellular Cd (Figure 2D), demonstrating that CadA is required to maintain homeostatic intracellular Cd concentrations. These results confirm that *L. monocytogenes* CadA is a functional Cd efflux pump required to confer resistance to Cd-induced toxicity.

### CadC Directly Regulates *cadA*, *cadC*, and *lspB* Expression in Response to Cd

We assessed the role of CadC and Cd in *cadA, cadC*, and *lspB* transcription. In absence of CadC, *cadA* and *lspB* transcript levels were significantly increased, and *cadA*, *cadC* and *lspB* transcript levels rose in response to Cd (Figure 3A), showing that CadC actively represses *cadA* and *lspB* transcription, whereas Cd activates *cadA, cadC*, and *lspB* expression.

We identified conserved CadC boxes (Cx) exclusively in the promoter regions of *cadA* (*cadA* Cx), *cadC* (*cadC* Cx), and *lspB* (*lspB* Cx), similar to the well-characterized *S. aureus* Cx (Figure 3B). *L. monocytogenes* CadC was produced, purified (Supplementary Figure 2*A*), and used in EMSAs with DNA fragments containing each Cx. CadC appeared capable of delaying
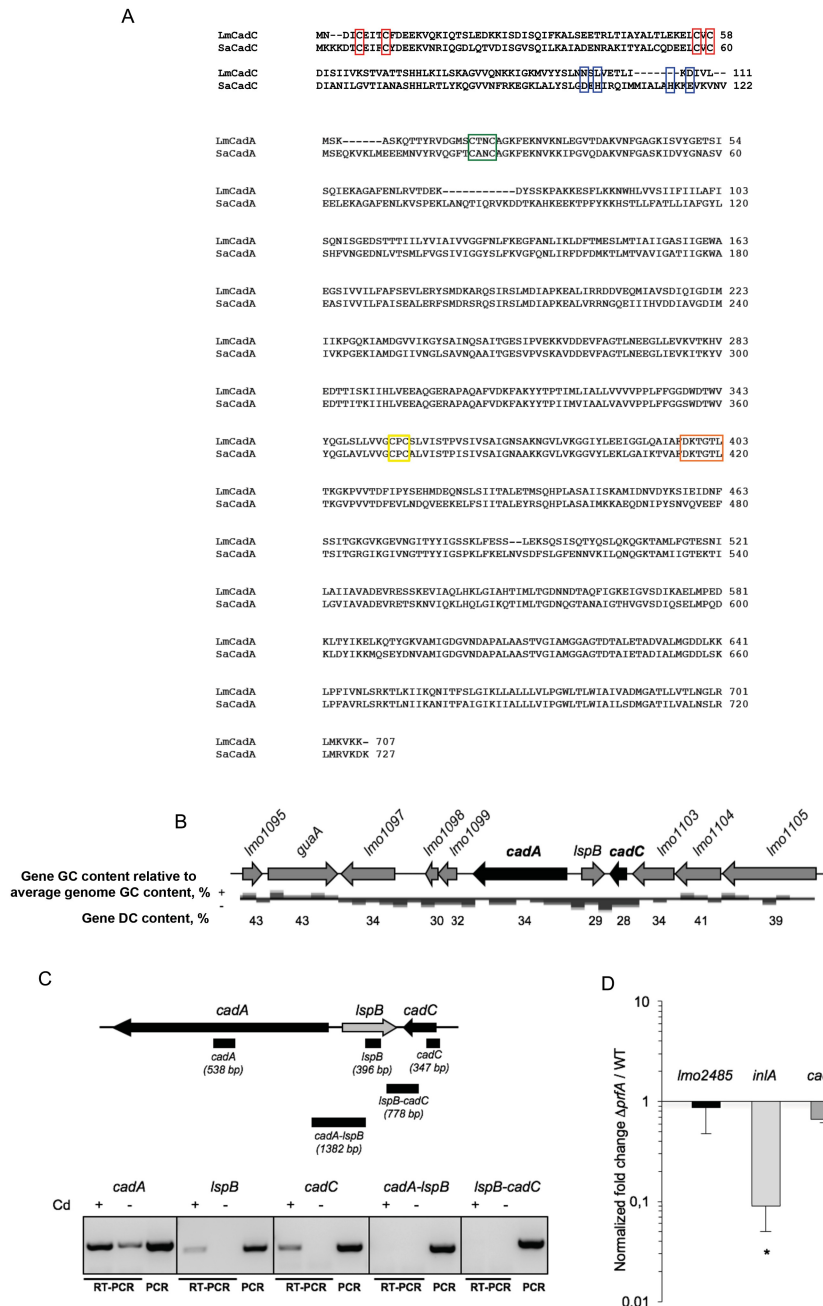
**Figure 1.** Identification of a cadmium resistance system in the genome of *Listeria monocytogenes*. *A*, Alignment of the CadC and CadA protein sequences of *L. monocytogenes* and *Staphylococcus aureus* pI258. Metal-binding sites 1 (red boxes) and 2 (blue boxes) are indicated. The green box indicates a CXXC metal-binding site. The yellow box shows a CPC motif, and the orange box indicates a DKTGTLT sequence. *B*, *L. monocytogenes* genomic organization and GC content of the region encompassing *cadAC*. Variations in the DNA GC content relative to the average GC content of the whole genome are indicated by bars. Numbers correspond to the percentage of the DNA content composed of GC for each gene. *C*, Transcriptional analysis of the *cadA-lspB-cadC* region by reverse transcription–polymerase chain reaction (RT-PCR). Predicted fragments (*cadA*, *cadA-lspB*, *lspB*, *lspB-cadC*, and *cadC*) amplified with the different primer sets are indicated on the schematic representation of the *cadA-lspB-cadC* locus. RT-PCR analyses were performed on RNAs from logarithmic-phase cultures of *L. monocytogenes* growing in brain heart infusion broth at 37°C, in the absence (-) or presence (+) of cadmium (Cd). Control PCR analyses were performed on genomic DNA. *D*, Analysis of PrfA regulation of *cadC* transcription. Quantitative real-time PCR was performed on RNAs extracted from logarithmic-phase cultures of wild-type (WT) and Δ*prfA* strains grown in brain heart infusion agar at 37°C. *inlA* and *lmo2845* were used as PrfA-dependent and PrfA-independent control genes, respectively [47, 15]. Gene expression levels in the Δ*prfA* mutant are presented normalized to those in the WT strain (set at 1). Values are mean ± SD from 3 independent experiments. *$P < .05$, compared with the WT strain.

the migration of *cadA* Cx, *cadC* Cx, and *lspB* Cx (Figure 3B) but not of a negative control promoter DNA (*inlA*). Similarly, an unrelated protein (GFP) did not delay *cadC* Cx migration. Thus, direct CadC binding to Cx appears to be sequence and protein specific. To confirm that *L. monocytogenes* CadC binds to Cx in vivo, CadC was immunoprecipitated from *L. monocytogenes* extracts, using an anti-CadC antibody, and coprecipitated DNA was analyzed by qPCR, using primers specific for
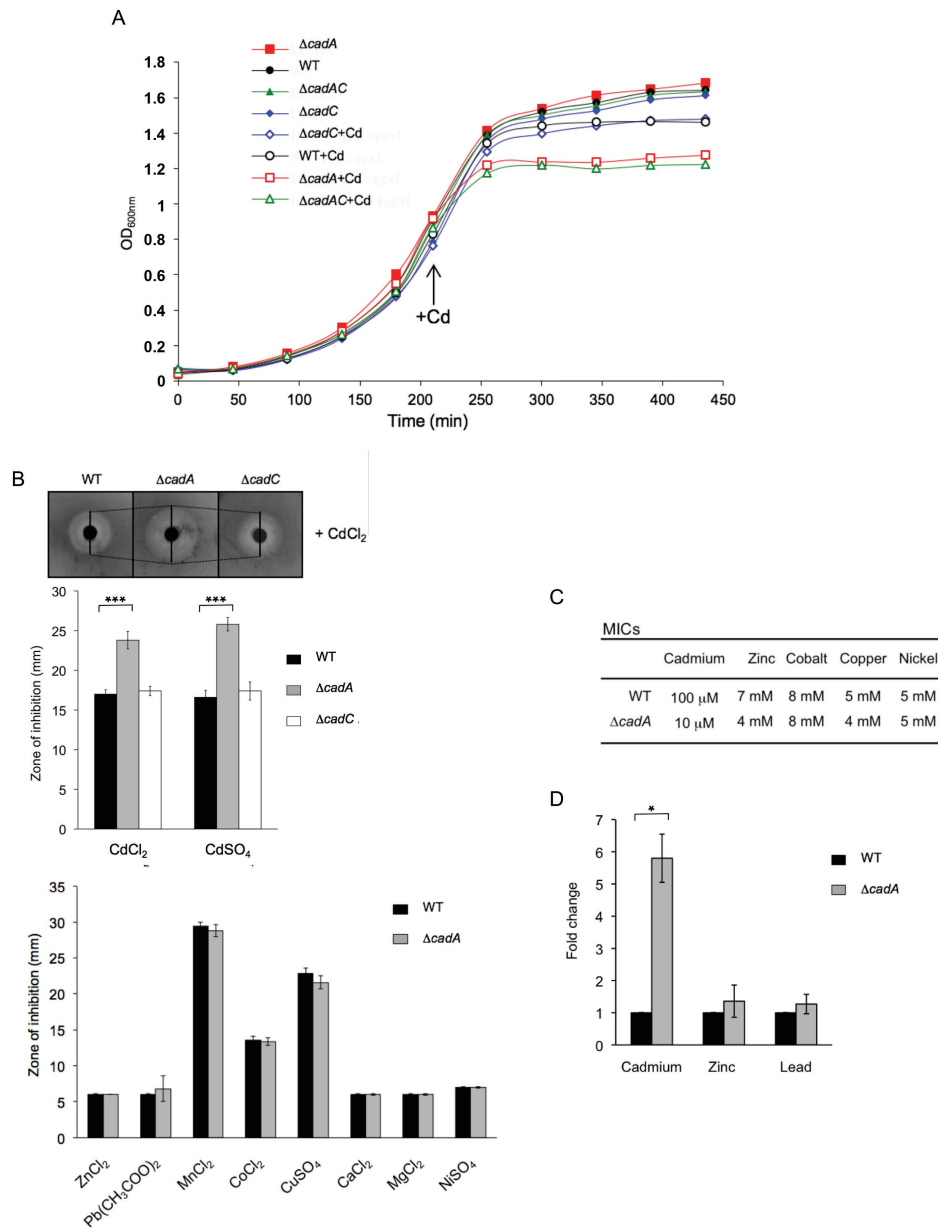
**Figure 2.** CadA behaves as a cadmium efflux pump required for *Listeria monocytogenes* resistance to cadmium. *A*, Growth curves of wild-type (WT), Δ*cadA*, Δ*cadC*, and Δ*cadAC* strains in brain heart infusion agar at 37°C and challenged after 210 minutes with 384 μM cadmium chloride (CdCl₂). Representative results from 3 independent experiments. *B*, Growth inhibition of WT, Δ*cadA* and Δ*cadC* strains in agar medium overlaid with disks saturated with 100 mM CdCl₂ or cadmium sulfate (CdSO₄) or with 100 mM of zinc chloride (ZnCl₂), lead acetate (Pb[CH₃COO]₂), manganese chloride (MnCl₂), cobalt chloride (CoCl₂), cupric sulfate (CuSO₄), calcium chloride (CaCl₂), magnesium chloride (MgCl₂), or nickel sulfate (NiSO₄). Values are mean ± SD from 5 independent experiments. *C*, Minimum inhibitory concentrations (MICs) of cadmium, zinc, cobalt, copper, and nickel for the WT and Δ*cadA* strains. *D*, Intracellular levels of cadmium, zinc, and lead measured by inductively coupled plasma mass spectrometry in the WT and Δ*cadA* strains. Values are mean ± SD from 3 independent experiments. *$P < .05$ and ***$P < .001$.

the different Cx (ChIP-qPCR). Enrichment was observed for all Cx tested (Figure 3B) and was shown to be specific by normalization to a negative control DNA and to mock IP, demonstrating the CadC binds specifically to *L. monocytogenes* Cx in vivo.

EMSAs were also performed with DNA fragments containing WT *cadC* Cx sequence (Cx) or with point (Cx–M1-6) or transversed (Cx-T) mutations (Figure 3C). Whereas CadC altered the migration of native Cx, with the exception of the M2 substitution (T→G), every other mutation abrogated the mobility shift. The interaction between CadC and Cx thus appears highly specific and dependent on the conserved palindromic sequence.

In the presence of increased concentrations of Cd, CadC was released from Cx (Figure 3D). In addition, the Cd concentration necessary to abrogate CadC binding to Cx was 10-fold
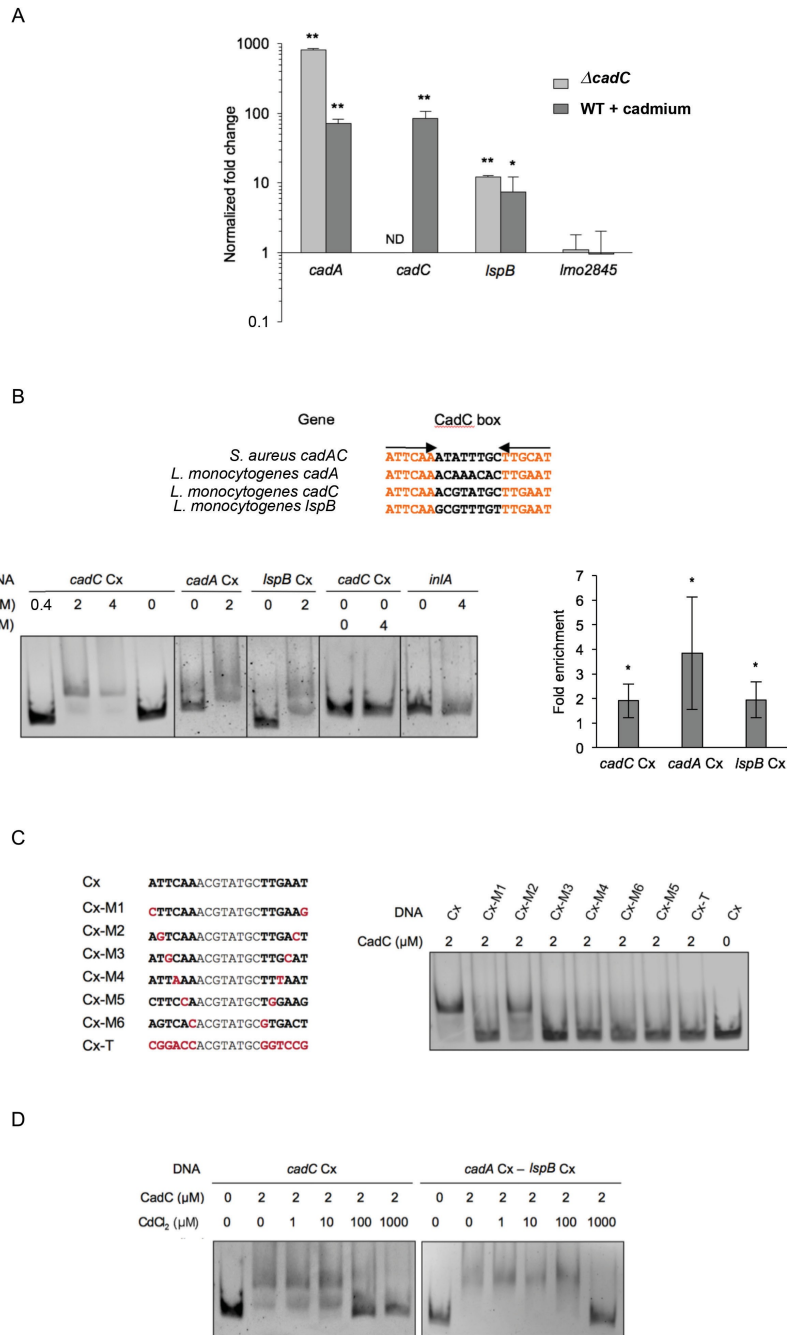
**Figure 3.** CadC directly regulates *cadA, cadC*, and *lspB* expression in response to cadmium. *A, cadA, cadC*, and *lspB* transcription is dependent on CadC and the cadmium concentration. Real-time quantitative polymerase chain reaction (qPCR) analysis of RNAs extracted from logarithmic-phase cultures of wild-type (WT) and Δ*cadC* strains grown in brain heart infusion (BHI) broth at 37°C and WT grown in BHI supplemented with Cd (WT+Cd). *lmo2845* was used as a CadC- and cadmium-independent control gene. Gene expression levels are shown normalized to those in the WT grown in BHI agar in the absence of cadmium (set at 1). Values are mean ± SD from 3 independent experiments. ND, no data. *$P < .05$ and **$P < .01$, compared with the WT strain. *B*, CadC binds directly to *cadA, cadC*, and *lspB* CadC boxes. Upper panel, Alignment of CadC boxes upstream of *Staphylococcus aureus* pI258 *cadC, Listeria monocytogenes cadA, L. monocytogenes cadC*, and *L. monocytogenes lspB*. Palindromes are indicated by arrows. Bottom left panel, Increasing amounts of purified CadC were used in electrophoretic mobility shift assays (EMSAs) with DNA fragments containing the *cadA, cadC*, or *lspB* CadC box (Cx) generated by PCR, using primers listed in Supplementary Table 1. An unrelated promoter region (*inlA*) and an unrelated protein (GFP) were used as negative controls. Bottom right panel, chromatin immunoprecipitation qPCR was conducted to quantify the capacity of CadC to bind Cx in vivo. Fold enrichment is shown normalized to an unrelated promoter region (*inlA*) and as compared to mock-IP. Values are mean ± SD from 5 independent experiments. *$P < .05$. *C*, Specificity of the interaction between CadC and Cx, using Cx-containing DNA fragments in which the palindromic sequence was either present in its unaltered form (Cx) or contained point (Cx-M1-5) or transversed (Cx-T) mutations (indicated in red). *D*, In the presence of cadmium, CadC fails to bind *cadA, cadC*, and *lspB* Cx. Increasing amounts of cadmium chloride (CdCl₂) were used in EMSAs with purified CadC and *cadA* Cx, *cadA* Cx-*lspB* Cx, and *cadC* Cx DNA fragments. Experiments yielding data shown in panels *B–D* were performed at least twice, and representative results are shown.

lower than that required to prevent CadC binding to a DNA fragment containing 2 Cx (*cadA* Cx–*lspB* Cx).

These results demonstrate that, in the absence of Cd, CadC represses the expression of *cadAC* by directly binding conserved Cx present in *cadA* and *cadC* promoters. In the presence of Cd, CadC cannot bind to or is detached from Cx, allowing CadA expression, thus inducing Cd resistance. The Cd concentration required to prevent CadC binding depends on the number of Cx. The expression of *lspB* is also subjected to Cd-dependent CadC-mediated regulation. However, *lspB* appears to be unnecessary for Cd resistance (Supplementary Figure 2*B*).

### CadC Is Required for Efficient *L. monocytogenes* Infection In Vivo

We evaluated the role of CadAC during *L. monocytogenes* infection in vivo by determining bacterial loads in the liver and spleen of intravenously inoculated mice. Seventy-two

hours after infection, bacterial counts for the WT and Δ*cadA* strains were similar in both organs (Figure 4A). However, they appeared to be significantly lower for Δ*cadC* and Δ*cadAC* strains. Complementation of the Δ*cadC* mutant (Δ*cadC*+*cadC*) restored bacterial loads to WT levels. Oral inoculation confirmed the impaired colonization of mouse organs by the Δ*cadC* and Δ*cadAC* strains as compared to the WT and Δ*cadA* strains (Figure 4B). CadC thus plays a role in *L. monocytogenes* in vivo infection, independent of *cadA* expression.

### In the Absence of CadC Repression, *lspB* Expression Is Deleterious for *L. monocytogenes* Infection

Because CadC is overexpressed in mouse organs [12], we postulated that CadC-dependent *lspB* repression would be necessary for efficient *L. monocytogenes* infection. We constructed Δ*lspB* and Δ*cadC*Δ*lspB* mutants and an *lspB*-overexpressing
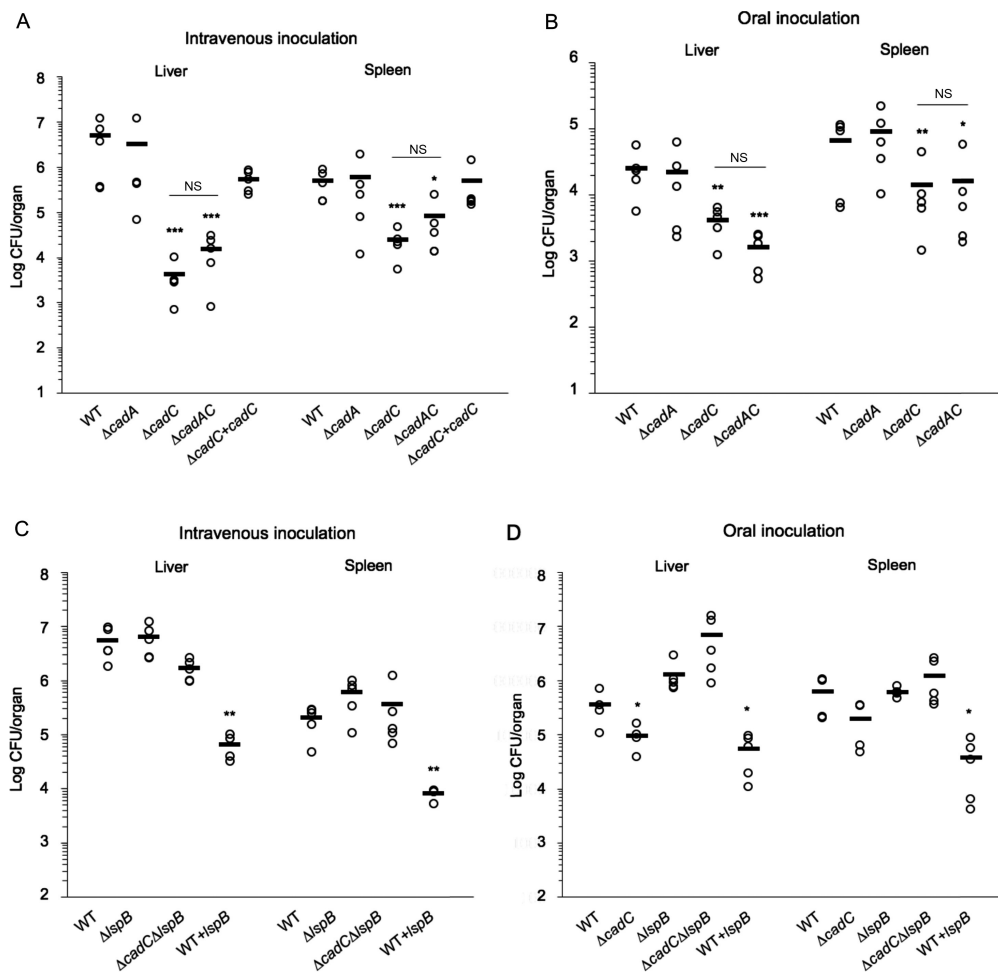


**Figure 4.** In the absence of CadC repression, *lspB* expression is deleterious for *Listeria monocytogenes* infectious capacity. *A*, Bacterial counts of the wild-type (WT), Δ*cadA*, Δ*cadC*, Δ*cadAC*, and Δ*cadC*+*cadC* strains in the liver and spleen of C57BL/6 mice 72 hours after intravenous inoculation of $10^5$ bacteria per animal. *B*, Bacterial counts of the WT, Δ*cadA*, Δ*cadC*, and Δ*cadAC* strains in the liver and spleen of C57BL/6 mice 72 hours after oral inoculation of $10^9$ bacteria per animal. *C*, Bacterial counts of the WT, Δ*lspB*, Δ*cadC*Δ*lspB*, and WT+*lspB* strains in the liver and spleen of C57BL/6 mice 72 hours after intravenous inoculation of $10^5$ bacteria per animal. *D*, Bacterial counts of the WT, Δ*lspB*, Δ*cadC*Δ*lspB*, and WT+*lspB* strains in the liver and spleen of BALB/c mice 72 hours after oral inoculation of $10^9$ bacteria per animal. Data are presented as scatter plots, with each animal indicated by an empty circle and mean values indicated by a horizontal line (n = 5). CFU, colony-forming units; NS, not significant. \**P* < .05, \*\**P* < .01, and \*\*\**P* < .001.

strain (WT+*lspB*). Analysis of growth rates and *lspB* transcription indicated that neither the absence nor the overexpression of *lspB* have a significant impact on *L. monocytogenes* viability or replication in rich medium (Supplementary Figure 2*D* and 2*E*). Transmission electron microscopy revealed no difference regarding overall and cell wall morphology of *lspB*-overexpressing strains (Supplementary Figure 2F). A total of 72 hours after intravenous (Figure 4C) or oral (Figure 4D) inoculation of mice, bacterial counts for the WT and Δ*lspB* strains were not significantly different, indicating that LspB is not required for *L. monocytogenes* infection. Interestingly, whereas Δ*cadC* bacteria were attenuated (Figure 4A and 4D), the Δ*cadC*Δ*lspB* strain behaved like the WT strain (Figure 4C and 4D), suggesting that the Δ*cadC* phenotype is associated with *lspB* expression levels. In agreement with this, the *lspB*-overexpressing strain appeared to be significantly attenuated after intravenous (Figure 4C) or oral (Figure 4D) inoculation. Increased *lspB* expression, either through the absence of CadC repression or through overexpression, thus appears to be detrimental to the infectious capacity of *L. monocytogenes*.

## LspB Controls LpeA Extracellular Release

LspB has a high degree of identity with known type II signal peptidases (SPase II) of other *Listeria* strains and bacterial species (Figure 5A), particularly the *Streptococcus thermophilus* SPase II (75% identity). LspB harbors the 5 highly conserved SPase II domains and the 6 residues critical for SPase II activity [34]. The predicted LspB topology shows the presence of 4 transmembrane domains, suggesting membrane localization (Supplementary Figure 1*D*). SPase II enzymes were shown to be involved in lipoprotein membrane insertion and release to the extracellular medium [35]. We thus hypothesized that differential *lspB* expression could result in changes in the repertoire of *L. monocytogenes* surface-exposed and/or released lipoproteins. Whereas no difference was observed in membrane lipoprotein extracts, a band was detected between 25–37 kDa, with increased intensity in culture supernatants from *lspB*-overexpressing strains (Δ*cadC* and WT+*lspB*; Figure 5B and 5C). The protein present in this band was identified by mass spectrometry as the
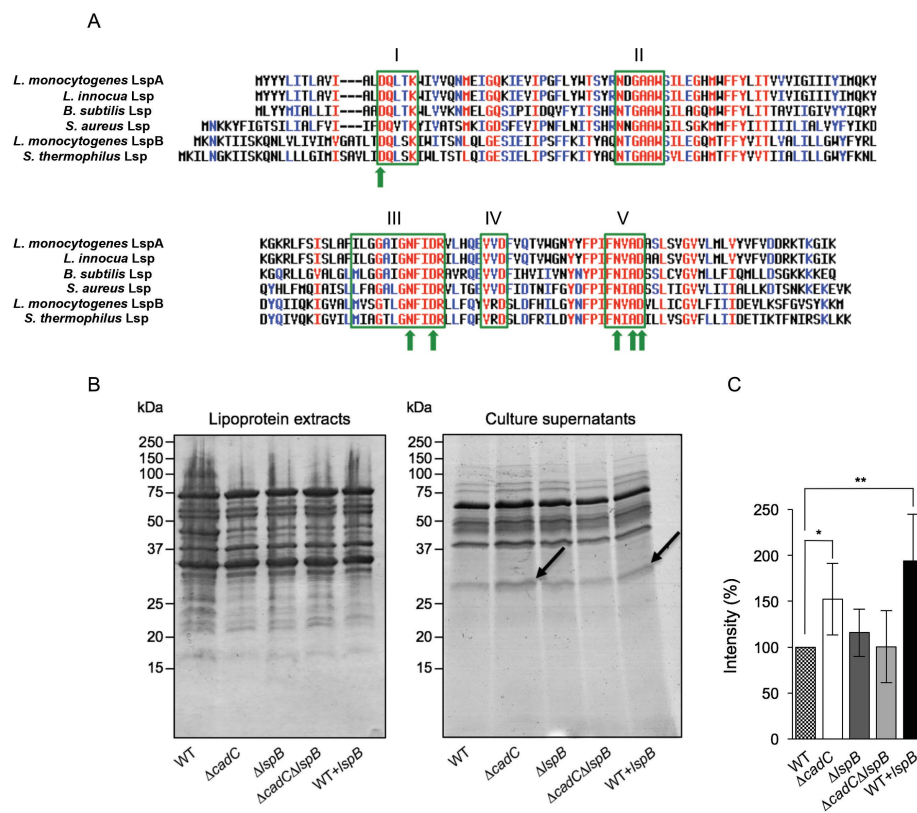


**Figure 5.** LspB controls LpeA release in the extracellular medium. *A*, Alignment of the protein sequence of type II signal peptidases (SPase II) from *Listeria monocytogenes* LspA and LspB, *Listeria innocua* Lsp, *Bacillus subtilis* Lsp, *S. aureus* Lsp and *Streptococcus thermophilus* Lsp. Residues present in at least 3 SPase II enzymes are in blue, while residues conserved in all SPase II enzymes are in red. Conserved domains (I–V) as defined by Tjalsma et al [34] are highlighted in green boxes. Residues important for activity/stability are indicated by green arrows. *B*, Lipoprotein membrane extracts and culture supernatants from the wild-type (WT), Δ*cadC*, Δ*lspB*, Δ*cadC*Δ*lspB*, and WT+*lspB* strains separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and stained with Coomassie blue. Arrows indicate a band between 25 and 37 kDa with increased intensity in the culture supernatants from Δ*cadC* and WT+*lspB* strains. *C*, Quantifications of LpeA in culture supernatants (ImageJ). Values are mean ± SD from 3 independent experiments and are presented normalized to the loading control and as a percentage relative to the mean value of the band intensity for the WT strain (set at 100). *P < .05 and **P < .01.

*L. monocytogenes* lipoprotein LpeA (lipoprotein promoting entry A) [36] (Supplementary Table 3). LspA was the first SPase II identified in *L. monocytogenes* [44]. We analyzed *lspA* expression levels and showed that they were similar in the different strains (Supplementary Figure 3*A*), indicating that increased LpeA levels in culture supernatants from *lspB*-overexpressing strains are unrelated to a differential *lspA* expression. Thus, *lspB* encodes a secondary *L. monocytogenes* SPase II, which promotes the release of the LpeA lipoprotein to the extracellular medium.

### *lspB* Derepression Induces Expression of Inflammatory Cytokines Limiting Intramacrophagic Survival

LpeA was shown to be required for entry into intestinal and hepatic cells [36]. In addition, an LpeA-deficient mutant survives longer in macrophages and is slightly more virulent than WT bacteria in mice [36]. We thus hypothesized that, in the presence of high LspB levels, more LpeA would be found in the extracellular medium, which would decrease *L. monocytogenes* survival in macrophages. We analyzed the capacity of *L. monocytogenes* strains to survive in mouse BMDMs. Whereas no significant difference was observed at 30 minutes after infection (Figure 6A), intramacrophagic survival of Δ*cadC*, Δ*cadAC,* and WT+*lspB* strains was significantly decreased 5 hours after infection, compared with findings for WT bacteria (Figure 6B). Strains Δ*cadA,* Δ*cadC*+cadC, Δ*lspB*, and Δ*cadC*Δ*lspB* behaved similarly to WT. These results indicate that *L. monocytogenes* phagocytosis is not dependent on CadA, CadC, or LspB; that high *lspB* expression is detrimental for *L. monocytogenes*
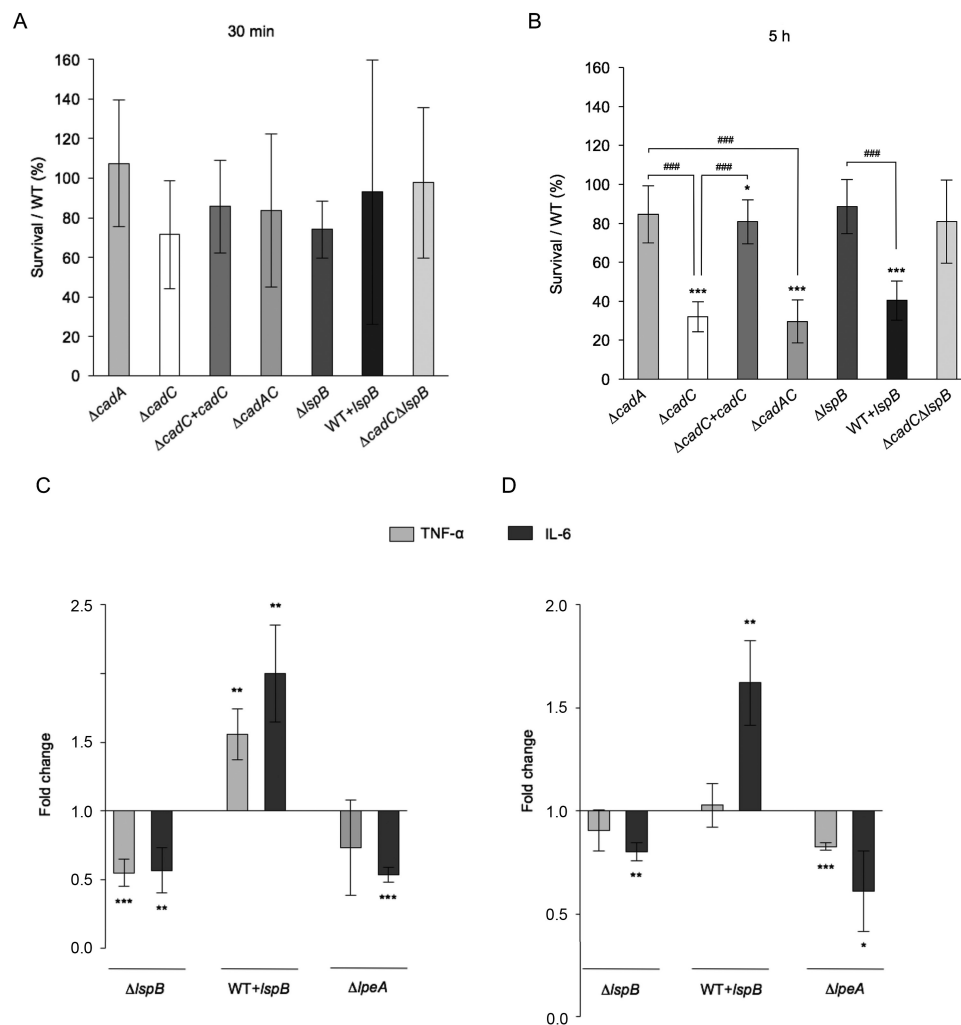


**Figure 6.** In the absence of CadC repression, *lspB* expression decreases *Listeria monocytogenes* survival in macrophages and induces inflammatory cytokine expression. *A* and *B*, Intracellular counts of the wild-type (WT), Δ*cadA*, Δ*cadC*, Δ*cadC*+cadC, Δ*cadAC*, Δ*lspB*, WT+*lspB*, and Δ*cadC*Δ*lspB* strains in mouse bone marrow-derived macrophages (BMDMs) 30 minutes (*A*) and 5 hours (*B*) after infection. Values are mean ± SD from 3 independent experiments and are presented as percentages relative to the mean counts of the WT strain (set at 100). *$P < .05$, **$P < .01$, and ***$P < .001$, compared with the WT strain; ###$P < .001$, compared with the indicated strains. *C* and *D*, Quantification of tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6) expression levels by real-time quantitative polymerase chain reaction (*C*) and secretion levels by enzyme-linked immunosorbent assay (*D*) 5 hours after infection of RAW macrophages with either WT, Δ*lspB,* WT+*lspB*, or Δ*lpeA* strains. Values are mean ± SD from 3 independent experiments. *$P < .05$, **$P < .01$, and ***$P < .001$, compared with the WT strain.

intramacrophagic survival; that CadC-mediated repression avoids LspB-disruptive effects on *L. monocytogenes* infection; and that the ΔcadC phenotype in vivo is not related to *cadA* derepression.

Secreted *L. monocytogenes* lipoproteins were shown to induce inflammatory cytokines (tumor necrosis factor α [TNF-α] and interleukin 6 [IL-6]) in a Toll-like receptor 2 (TLR2)–dependent manner during infection [37]. Given that *lspB* overexpression results in higher LpeA levels in culture supernatants, we tested whether this increased lipoprotein release could promote inflammatory cytokine expression. RAW macrophages were infected, and TNF-α and IL-6 expression and secretion levels were assessed 5 hours after infection. In Δ*lspB*- and Δ*lpeA*-infected RAW macrophages, TNF-á and IL-6 levels were significantly reduced. Inversely, infection by *lspB*-overexpressing bacteria resulted in increased cytokine expression and secretion (Figure 6C and 6D). As observed in BMDMs, the WT+*lspB* strain showed a significantly reduced capacity to survive in RAW macrophages (Supplementary Figure 3*B*).

*L. monocytogenes* thus uses CadC to repress *lspB* expression during infection, avoiding excessive LpeA exposure to the host immune system, reducing inflammatory response, and promoting intramacrophagic survival and virulence.

## DISCUSSION

CadA is an efflux pump required for Cd resistance, but in contrast to its homologues in *S. aureus* [38] and *S. thermophilus* [39], it is not essential for zinc and lead efflux and does not significantly contribute to resistance against these or other metals. Whereas *L. monocytogenes* CadA was proposed to alternatively transport zinc [40], resistance to zinc was previously shown to be independent of *L. monocytogenes* CadAC [11]. Although we cannot exclude that *L. monocytogenes* CadA might participate in detoxification of high levels of zinc, it mainly acts as a Cd efflux pump.

*L. monocytogenes* CadC appears as a *trans*-acting, sequence-specific, DNA-binding, and Cd-dependent regulator of *cadA, cadC,* and *lspB* expression. We are the first to show that the conservation of almost every nucleotide within the Cx palindrome is crucial for CadC binding. As previously suggested [39], we also show that the Cd concentration necessary to release CadC from DNA is proportional to the number of Cx. Whereas *cadAC* are generally part of an operon under the control of a unique promoter containing 2 Cx, in *L. monocytogenes* these 2 genes are noncontiguous and controlled by 2 different Cx-containing promoters. They are separated by *lspB* with the opposite orientation and 2 Cx between *cadA* and *lspB*. This suggests that *L. monocytogenes* evolved an additional regulation level, allowing a differential regulation of *cadA* and *cadC*. *L. monocytogenes* also appears to be the first bacterium shown to use a Cd efflux pump repressor to control genes unrelated

to Cd resistance. This atypical organization of the *cadAC* locus (ie, it is split by an SPase-encoding gene) is only found with a remarkable conservation in a *cis*-mobilizable element of *S. thermophilus* [41]. The chromosomal *L. monocytogenes cadAC* locus is predicted to be part of a *L. monocytogenes* integrative and conjugative element [41], and its GC content is markedly lower than that of the surrounding regions, suggesting that *L. monocytogenes* could have acquired this locus by horizontal gene transfer.

We demonstrate that, during infection, *L. monocytogenes* represses *lspB* via CadC to ensure maximal infection efficiency. LspB, as for other gram-positive SPase II enzymes [42], is dispensable for bacterial growth in vitro and for virulence in vivo. SPase II enzymes specifically process the N-terminal signal peptide of prolipoproteins translocated through the Sec system and lipidated by a diacylglyceryl transferase (Lgt). Lipoproteins are ultimately chained to the membrane via a lipid moiety covalently bound to an N-terminal conserved cysteine [43]. In addition to the presence in its sequence of all the conserved domains/residues critical for SPase II activity [34] and its predicted membrane localization, our results point to LspB as a secondary *L. monocytogenes* SPase II involved in the processing of LpeA. However, we cannot exclude that LspB could act upon other lipoproteins. LspA, the first SPase II identified in *L. monocytogenes,* is involved in lipoprotein processing, including LpeA, and in macrophage phagosome escape [44]. LspA is also involved in lipoprotein release to the extracellular medium, a process also dependent on Lgt. The retention and release of lipoproteins appears thus as a complex process in *L. monocytogenes,* cocontrolled by Lgt and SPases.

LpeA can be secreted, particularly in the absence of Lgt [35, 45]. In a *lgt* mutant, soluble lipoproteins induce the secretion of inflammatory cytokines in a TLR2-dependent manner during infection [37]. *lspB* derepression and overexpression leads to increased LpeA release and inflammatory cytokines secretion and correlates with decreased intramacrophagic survival and virulence in mice. Interestingly, an *lpeA* mutant survives better inside macrophages and induced early mouse mortality [36]. Group B *Streptococcus*–secreted lipoproteins also activate host inflammatory response through TLR2 signaling [42]. In this model, the absence of *lgt* and/or *lsp* leads to decreased TLR2-mediated recognition, reduced inflammatory response, and increased lethality [42]. Secreted mycoplasma lipoproteins also have the ability to modulate the host immune system in a TLR2-dependent manner [46]. Bacteria thus use different lipoprotein-processing enzymes (Lgt and Lsp) to control lipoprotein exposure to host immune recognition mechanisms. While LpeA is required for host cell invasion [36], it also activates host-protective inflammatory responses. To reach a midpoint, *L. monocytogenes* developed an original strategy to spatially and temporally regulate LpeA exposure at the bacterial surface and to the host immune system. Interestingly, *L. monocytogenes*

downregulates *lgt*, *lspA*, and *lspB* and upregulates *cadC* during mouse infection (Supplementary Table 2) [12], suggesting that the localization of *L. monocytogenes* lipoproteins is tightly regulated during infection to promote virulence.

We propose that *L. monocytogenes* acquired a mobile element containing the *cadA-lspB-cadC* locus to control lipoprotein localization via CadC-dependent *lspB* regulation. During infection, this process minimizes lipoprotein exposure to the host immune system, diminishing inflammatory cytokine expression and promoting intramacrophagic survival and infection. This constitutes the first example of a heavy metal efflux pump regulator repurposed by a bacterial pathogen to fine-tune lipoprotein localization and host immune responses during infection. Being nonessential for bacterial growth, these transcriptional repressors could represent new targets for innovative antibacterial strategies.

## Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

## Notes

*Potential conflicts of interest.* All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

## References

1. Saier MH Jr, Paulsen IT, Sliwinski MK, Pao SS, Skurray RA, Nikaido H. Evolutionary origins of multidrug and drug-specific efflux pumps in bacteria. FASEB J **1998**; 12:265–74.
2. Nies DH. Efflux-mediated heavy metal resistance in prokaryotes. FEMS Microbiol Rev **2003**; 27:313–39.
3. Endo G, Silver S. CadC, the transcriptional regulatory protein of the cadmium resistance system of *Staphylococcus aureus* plasmid pI258. J Bacteriol **1995**; 177:4437–41.
4. Osman D, Cavet JS. Bacterial metal-sensing proteins exemplified by ArsR-SmtB family repressors. Nat Prod Rep **2010**; 27:668–80.
5. Busenlehner LS, Weng TC, Penner-Hahn JE, Giedroc DP. Elucidation of primary (alpha(3)N) and vestigial (alpha(5)) heavy metal-binding sites in *Staphylococcus aureus* pI258 CadC: evolutionary implications for metal ion selectivity of ArsR/SmtB metal sensor proteins. J Mol Biol **2002**; 319:685–701.
6. Nucifora G, Chu L, Misra TK, Silver S. Cadmium resistance from *Staphylococcus aureus* plasmid pI258 cadA gene results from a cadmium-efflux ATPase. Proc Natl Acad Sci U S A **1989**; 86:3544–8.
7. Cossart P. Illuminating the landscape of host-pathogen interactions with the bacterium *Listeria monocytogenes*. Proc Natl Acad Sci U S A **2011**; 108:19484–91.
8. European Food Safety Authority, European Centre for Disease Prevention and Control. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2013. EFSA J **2015**; 13:3991.
9. Swaminathan B, Gerner-Smidt P. The epidemiology of human listeriosis. Microbes Infect **2007**; 9:1236–43.
10. Lebrun M, Audurier A, Cossart P. Plasmid-borne cadmium resistance genes in *Listeria monocytogenes* are present on Tn5422, a novel transposon closely related to Tn917. J Bacteriol **1994**; 176:3049–61.
11. Lebrun M, Audurier A, Cossart P. Plasmid-borne cadmium resistance genes in *Listeria monocytogenes* are similar to cadA and cadC of *Staphylococcus aureus* and are induced by cadmium. J Bacteriol **1994**; 176:3040–8.
12. Camejo A, Buchrieser C, Couvé E, et al. In vivo transcriptional profiling of *Listeria monocytogenes* and mutagenesis identify new virulence factors involved in infection. PLoS Pathog **2009**; 5:e1000449.
13. Glaser P, Frangeul L, Buchrieser C, et al. Comparative genomics of Listeria species. Science **2001**; 294:849–52.
14. Lauer P, Chow MY, Loessner MJ, Portnoy DA, Calendar R. Construction, characterization, and use of two *Listeria monocytogenes* site-specific phage integration vectors. J Bacteriol **2002**; 184:4177–86.
15. Reis O, Sousa S, Camejo A, et al. LapB, a novel *Listeria monocytogenes* LPXTG surface adhesin, required for entry into eukaryotic cells and virulence. J Infect Dis **2010**; 202:551–62.
16. Sullivan MA, Yasbin RE, Young FE. New shuttle vectors for *Bacillus subtilis* and *Escherichia coli* which allow rapid detection of inserted fragments. Gene **1984**; 29:21–6.
17. Archambaud C, Gouin E, Pizarro-Cerda J, Cossart P, Dussurget O. Translation elongation factor EF-Tu is a target for Stp, a serine-threonine phosphatase involved in virulence of *Listeria monocytogenes*. Mol Microbiol **2005**; 56:383–96.
18. Leimeister-Wächter M, Domann E, Chakraborty T. The expression of virulence genes in *Listeria monocytogenes* is thermoregulated. J Bacteriol **1992**; 174:947–52.
19. Kurokawa K, Lee H, Roh KB, et al. The triacylated ATP binding cluster transporter substrate-binding lipoprotein of *Staphylococcus aureus* functions as a native ligand for toll-like receptor 2. J Biol Chem **2009**; 284:8406–11.
20. Pinheiro J, Reis O, Vieira A, et al. *Listeria monocytogenes* encodes a functional ESX-1 secretion system whose expression is detrimental to in vivo infection. Virulence **2016**:0.
21. Almeida MT, Mesquita FS, Cruz R, et al. Src-dependent tyrosine phosphorylation of non-muscle myosin heavy chain-IIA restricts *Listeria monocytogenes* cellular infection. J Biol Chem **2015**; 290:8383–95.
22. Lobel L, Sigal N, Borovok I, Belitsky BR, Sonenshein AL, Herskovits AA. The metabolic regulator CodY links *Listeria monocytogenes* metabolism to virulence by directly activating the virulence regulatory gene prfA. Mol Microbiol **2015**; 95:624–44.
23. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods **2001**; 25:402–8.
24. Cabanes D, Lecuit M, Cossart P. Animal models of Listeria infection. Curr Protoc Microbiol **2008**; Chapter 9:Unit9B.1.
25. Sun Y, Wong MD, Rosen BP. Role of cysteinyl residues in sensing Pb(II), Cd(II), and Zn(II) by the plasmid pI258 CadC repressor. J Biol Chem **2001**; 276:14955–60.
26. Kandegedara A, Thiyagarajan S, Kondapalli KC, Stemmler TL, Rosen BP. Role of bound Zn(II) in the CadC Cd(II)/Pb(II)/Zn(II)-responsive repressor. J Biol Chem **2009**; 284:14958–65.
27. Kühlbrandt W. Biology, structure and mechanism of P-type ATPases. Nat Rev Mol Cell Biol **2004**; 5:282–95.
28. Kuenne C, Voget S, Pischimarov J, et al. Comparative analysis of plasmids in the genus Listeria. PLoS One **2010**; 5: pii: e12511.
29. Bierne H, Cossart P. *Listeria monocytogenes* surface proteins: from genome predictions to function. Microbiol Mol Biol Rev **2007**; 71:377–97.
30. Desvaux M, Hébraud M. The protein secretion systems in Listeria: inside out bacterial virulence. FEMS Microbiol Rev **2006**; 30:774–805.
31. Milohanic E, Glaser P, Coppée JY, et al. Transcriptome analysis of *Listeria monocytogenes* identifies three groups of genes differently regulated by PrfA. Mol Microbiol **2003**; 47:1613–25.
32. Scortti M, Monzó HJ, Lacharme-Lora L, Lewis DA, Vázquez-Boland JA. The PrfA virulence regulon. Microbes Infect **2007**; 9:1196–207.
33. Stoll R, Mertins S, Joseph B, Müller-Altrock S, Goebel W. Modulation of PrfA activity in *Listeria monocytogenes* upon growth in different culture media. Microbiology **2008**; 154:3856–76.

34. Tjalsma H, Zanen G, Venema G, Bron S, van Dijl JM. The potential active site of the lipoprotein-specific (type II) signal peptidase of *Bacillus subtilis*. J Biol Chem **1999**; 274:28191–7.

35. Baumgärtner M, Kärst U, Gerstel B, Loessner M, Wehland J, Jänsch L. Inactivation of Lgt allows systematic characterization of lipoproteins from *Listeria monocytogenes*. J Bacteriol **2007**; 189:313–24.

36. Réglier-Poupet H, Pellegrini E, Charbit A, Berche P. Identification of LpeA, a PsaA-like membrane protein that promotes cell entry by *Listeria monocytogenes*. Infect Immun **2003**; 71:474–82.

37. Machata S, Tchatalbachev S, Mohamed W, Jänsch L, Hain T, Chakraborty T. Lipoproteins of *Listeria monocytogenes* are critical for virulence and TLR2-mediated immune activation. J Immunol **2008**; 181:2028–35.

38. Yoon KP, Silver S. A second gene in the *Staphylococcus aureus* cadA cadmium resistance determinant of plasmid pI258. J Bacteriol **1991**; 173: 7636–42.

39. Schirawski J, Hagens W, Fitzgerald GF, Van Sinderen D. Molecular characterization of cadmium resistance in *Streptococcus thermophilus* strain 4134: an example of lateral gene transfer. Appl Environ Microbiol **2002**; 68:5508–16.

40. Banci L, Bertini I, Ciofi-Baffoni S, et al. Structural basis for metal binding specificity: the N-terminal cadmium binding domain of the P1-type ATPase CadA. J Mol Biol **2006**; 356:638–50.

41. Pavlovic G, Burrus V, Gintz B, Decaris B, Guédon G. Evolution of genomic islands by deletion and tandem accretion by site-specific recombination: ICESt1-related elements from *Streptococcus thermophilus*. Microbiology **2004**; 150:759–74.

42. Henneke P, Dramsi S, Mancuso G, et al. Lipoproteins are critical TLR2 activating toxins in group B streptococcal sepsis. J Immunol **2008**; 180:6149–58.

43. Sankaran K, Wu HC. Lipid modification of bacterial prolipoprotein. Transfer of diacylglyceryl moiety from phosphatidylglycerol. J Biol Chem **1994**; 269:19701–6.

44. Réglier-Poupet H, Frehel C, Dubail I, et al. Maturation of lipoproteins by type II signal peptidase is required for phagosomal escape of *Listeria monocytogenes*. J Biol Chem **2003**; 278:49469–77.

45. Trost M, Wehmhöner D, Kärst U, Dieterich G, Wehland J, Jänsch L. Comparative proteome analysis of secretory proteins from pathogenic and nonpathogenic Listeria species. Proteomics **2005**; 5:1544–57.

46. Seya T, Matsumoto M. A lipoprotein family from Mycoplasma fermentans confers host immune activation through Toll-like receptor 2. Int J Biochem Cell Biol **2002**; 34:901–6.

47. Dramsi S, Kocks C, Forestier C, Cossart P. Internalin-mediated invasion of epithelial cells by *Listeria monocytogenes* is regulated by the bacterial growth state, temperature and the pleiotropic activator prfA. Mol Microbiol **1993**; 9:931–41.

48. Claros MG, von Heijne G. TopPred II: an improved software for membrane protein structure predictions. Comput Appl Biosci **1994**; 10:685–6.

# PART II.2.

**Role of CadC in *Listeria monocytogenes* Resistance to Cadmium and in Bacterial Pathogenicity**

Negative Regulation of *Listeria monocytogenes* Bile Salt Hydrolase is Required to Promote Systemic Infection

# Negative regulation of *Listeria monocytogenes* bile salt hydrolase is required to promote systemic infection

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Running title: *Listeria* CadC fine-tunes *bsh* expression

## ABSTRACT

Bacterial tolerance to bile salts is closely related to the expression of bile salt hydrolases (BSH) that catalyze the hydrolysis of the conjugated bile salts, rendering bile less toxic to the bacterial cells. The *Listeria monocytogenes* (*Lm*) *bsh* gene encodes *Lm* BSH shown to be required for resistance to bile and virulence. We previously revealed CadC as a metal efflux pump regulator repurposed during infection to fine-tune lipoprotein processing and host responses. Here, we investigated the potential involvement of CadC in the *Lm* resistance to different environmental and host-related stresses. We found that CadC controls BSH activity and *Lm* resistance to bile by repressing *bsh* expression. Whereas CadC appeared dispensable to *Lm* survival in the gastrointestinal tract, we demonstrated that CadC-independent expression of *bsh* induces the expression of the cholic acid efflux pump MdrT, restricting *Lm* virulence. By RNAseq, we showed that CadC regulates additional genes, in particular σ$^B$-activated genes during colonization of the host intestinal lumen including virulence genes. Altogether these data point out CadC as a new general repressor repurposed to fine-tune virulence gene expression over the *Listeria* infectious process.

Keywords: *Listeria*; CadC; BSH, Gram-positive; pathogen

## BACKGROUND

*Listeria monocytogenes (Lm)* is a major intracellular foodborne bacterial pathogen that causes a human systemic infection named listeriosis [1]. Among zoonotic diseases under EU-surveillance, listeriosis is the most severe (hospitalization 99%; case fatality rate 16%) [2]. It is clinically characterized by septicemia and dissemination to the central nervous system and fetoplacental unit [3]. *Lm* has the capacity to colonize various niches, from inert and organic matrixes to the intestinal lumen where it competes with resident microbiota, translocates across the epithelium, multiplies in phagocytic and non-phagocytic cells and disseminates *via* the blood [1, 4]. *Lm* can grow at temperatures ranging from 0 to 45ºC, under acidic conditions (pH 2.5) and high osmolarity (20% NaCl). *Lm* may encounter such conditions in nature, at various stages of food processing, but also during host infection being exposed to proteolytic enzymes, acidic environment, high osmolarity and bile salts [5].

Bile acids are amphipathic molecules synthesized in the liver from cholesterol, conjugated with either glycine or taurine and secreted into the small intestine from the gall bladder [6]. They are major components of bile capable to degrade lipid-containing membranes and represent a key challenge to bacterial survival in the human gastrointestinal tract [7]. Bacterial tolerance to bile salts is closely related to the expression of bile salt hydrolases (BSH) that catalyze the hydrolysis of the conjugated bile salts, undermining the toxicity of bile to the bacterial cells. The *bsh* gene encodes the *Lm* BSH shown to be required for *Lm* resistance to bile and virulence [8]. PrfA, the major *Lm* virulence regulator, and σB, the general stress responsive alternative sigma factor, were shown to control *bsh* expression and *Lm* tolerance to bile [8-10].

To adapt and resist to the host environment, *Lm* evolved a myriad of mechanisms that should be spatially and timely regulated [11]. CadC is the transcriptional regulator of CadA, an efflux pump conferring cadmium resistance [12]. We recently showed that during *in vivo* infection, *Lm* uses CadC to directly repress the expression of the LspB lipoprotein signal peptidase avoiding the exposure of the LpeA lipoprotein to the host immune system, thus diminishing inflammatory cytokine expression and promoting intramacrophage survival and virulence [12]. CadC appears thus to be repurposed during infection to fine-tune lipoprotein processing and host responses. Here we show that CadC is also involved in the negative regulation of the *Lm* bile salt hydrolase required to promote systemic infection.

## METHODS

### Bacterial strains

*L. monocytogenes* EGDe (*Lm*) was grown in Brain Heart Infusion (BHI) medium (BD-Difco) at 37ºC with shaking. Construction of the P*iap*-*bsh* strain was performed using the splicing-by-overlap-extension (SOE) procedure. Two pairs of primers were used (*lmo2068MA-MB* and *catMA-MB*) (Table S1) to

amplify respectively a 556-bp fragment from the upstream region of *bsh* and the 684-bp *cat* gene respectively. Resulting products were mixed in a 1:1 ratio and re-amplified using primers *lmo2068MA* and *catMB*. The final product was digested and cloned into pMAD. Two other primer pairs were used (*iapMA-MB* and *bshMC-MD*) (Table S1) to amplify respectively the 226-bp promoter region of *iap* and the first 583-bp of *bsh*. Resulting products were mixed and re-amplified using primers *iapMA* and *bshMD*. The final product was digested and cloned into pMAD already containing the first fragment, and electroporated into *Lm* WT and Δ*cadC* to generate respectively $P_{iap}$-*bsh* and Δ*cadC*-$P_{iap}$-*bsh* strains. Deletions (Δ*cadC* and Δ*pgdA*) were available [12, 13]. Plasmid constructions and complementations were verified by PCR and DNA sequencing.

### Resistance to pH 5.5, salt stress and lysozyme

Growth under stressful stimuli was monitored by optical density measurement at 600 nm as described [14]. For comparative analysis of *Lm* resistance to pH 5.5 and salt stress, bacterial cultures were diluted 100-fold in BHI alone (control) or BHI pH 5.5 or containing 5% NaCl. For lysozyme resistance, exponential-phase cultures were challenged with different doses of chicken egg white lysozyme (Sigma).

### BSH activity assays

Stationary cultures were dropped (10 µl) in MRS (Man, Rogosa and Sharpe) agar plates supplemented with increasing concentrations of purified glycochenodeoxycholic acid (GDCA, Merck Millipore 361311) or taurochenodeoxycholic acid (TDCA, Santa Cruz Biotechnologies). Plates were incubated anaerobically for 72h at 37°C (GENbox, Biomérieux).

### Sensitivity to bile salts

*Listeria* were grown to log phase in BHI broth at 37 ºC. Cultures were diluted in BHI and $5 \times 10^3$ bacteria/ml were challenged with increasing concentrations of GDCA in a 24-well plate. Plates were then incubated with agitation at 37ºC in aerobic conditions. After 16h, CFUs were assessed by bacterial enumeration of serial dilutions on BHI agar.

### RNA techniques

RNAs were extracted from *Lm* ($OD_{600}$=0.8), treated (TURBO-DNA-free, Ambion), quality-checked (Experion, Bio-Rad-Laboratories), reverse-transcribed (iScript, Bio-Rad-Laboratories) and analyzed by qRT-PCR as described [15] using primers Table S1. Gene expression data were analyzed by comparative cycle threshold method normalized to relative reference gene expression (*Lm* 16S rRNA).

**Electrophoretic mobility shift assays (EMSAs)**

CadC-DNA binding assays were performed as previously described [12] in 50 mM Tris-HCl pH7.4, 6 mM $MgCl_2$, 100 mM NaCl, 50 mM KCl, 100 ng DNA and increasing concentrations of CadC/GFP. After 20 min at RT samples were resolved in 6% polyacrylamide gel and visualized by DNA staining.

*In vivo* **infection studies**

Infections were performed in six-to-eight week-old specific-pathogen-free females C57BL/6 mice (Charles River) as described [16]. Briefly, for oral infections, 12 h starved animals (n=5 per strain) were inoculated by gavage with $2x10^9$ CFU in PBS containing 150 mg/ml $CaCO_3$. Intravenous infections were performed through the tail vein with $10^5$ CFU in PBS. At indicated time points animals were euthanatized by general anesthesia. The stomach, spleen and liver were aseptically collected, homogenized in sterile PBS, and serial dilutions of the organ homogenates plated in BHI agar. For analysis of *Lm* fecal carriage, total feces produced by each infected animal (n=5 per strain) up to a given time-point were collected, homogenized in PBS and serial dilutions plated in *Listeria* selective media (Oxoid) for bacterial enumeration. 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*. Animal procedures followed European Commission (directive 2010/63/EU) and Portuguese (Decreto-Lei 113/2013) guidelines, approved by IBMC Ethics Committee and Direcção Geral Veterinária (ref. 015302).

**Expression tiling arrays**

ListIP Tiling Arrays were used [17]. RNAs were reverse-transcribed using SuperScript II reverse transcriptase (Life Technologies). cDNA was digested by DNase I and the size of digestion products was analyzed in the Agilent Bioanalyser 2100. Sample preparation for each chip was then processed following the Affymetrix GeneChip Expression Analysis Technical Manual (P/N 702232 Rev. 2) as previously described [17]. Scanning of the arrays was then performed using the GeneChip scanner 3000. Intensity signals of each probe cells were computed by the GeneChip operating software (GCOS). Data analysis of the tiling sub-array was performed using the Bioconductor software (http://www.bioconductor.org) based on R package as described in [17].

**Statistics**

Statistics were performed with Prism (GraphPad), using unpaired two-tailed Student's *t*-test to compare means of two groups, and one-way ANOVA with Tukey's post-hoc test for pairwise comparison of

means from more than two groups, or with Dunnett's post-hoc test for comparison of means relative to the mean of a control group.

## RESULTS

### CadC controls BSH activity and *Lm* resistance to bile salts

We investigated the potential involvement of CadC in *Lm* resistance to different stresses that *Lm* could encounter in environmental and host conditions. In particular, we assessed the growth of the Δ*cadC* mutant at pH 5.5 or in the presence of high concentrations of either NaCl or lysozyme. As shown in Fig 1A, no significant difference was observed concerning the growth of the wild type and mutant strains in BHI broth at pH 5.5 or containing 5% NaCl. Similarly, no difference was detected after the addition of lysozyme (200 μg/ml) to bacterial cultures in exponential growth phase (Fig 1B). As expected, we observed an immediate and significant decrease in the survival of the lysozyme-hypersensitive Δ*pgdA* mutant [13] (Fig 1B). These data demonstrate that CadC has no role in the growth of *Lm* under pH 5.5 or in presence of salt, and do not confer resistance to the cell wall degrading activity of lysozyme.

During infection, *Lm* has to resist to host bile [7]. This resistance is promoted by the BSH that catalyzes the deconjugation of glyco- (GDCA) and tauro- (TDCA) conjugated bile salts under low oxygen levels [8, 9]. We analyzed the impact of *cadC* deletion on BSH activity and resistance to bile acids. The BSH activity of the WT, Δ*cadC* and the complemented Δ*cadC*+*cadC* strains was evaluated by patch inoculation onto Man-Rogosa-Sharpe (MRS) medium supplemented with increasing concentrations of GDCA or TDCA and grown under microaerophilic conditions. The WT strain exhibited the formation of a classical white area of precipitated bile acids [18], confirming the presence of BSH activity (Fig 1C-D), whereas the non-pathogenic *L. innocua*, that lacks *bsh* [8], was mostly incapable of precipitating GDCA (Fig 1C). As compared to the WT strain, the Δ*cadC* mutant displayed a more pronounced precipitate, a phenotype reverted in the Δ*cadC*+*cadC* strain. In addition, the Δ*cadC* strain was able to precipitate bile acids at lower concentrations where the WT was unable to deconjugate them (Fig 1C-D). The Δ*cadAC* double mutant behaved as the Δ*cadC* single mutant (Fig 1C), indicating that CadC plays a role in *Lm* BSH activity independent of CadA. These results indicate that in absence of CadC, *Lm* exhibits a higher BSH activity.

To correlate increased BSH activity and resistance to bile toxicity in the Δ*cadC* mutant, the survival of WT and Δ*cadC* strains was evaluated in BHI broth supplemented with increasing concentrations of GDCA. As previously observed [9], GDCA inhibited the growth of WT cells in a dose-dependent manner, starting from 0.05%. The Δ*cadC* mutant appeared significantly more resistant to GDCA than the WT strain (Fig 1E).

Altogether these results demonstrate that CadC negatively impacts *Lm* BSH activity and resistance to bile salts.

**CadC represses *bsh* expression**

CadC is a transcriptional repressor previously shown to control *cadAC* and *lspB* expression [12]. CadC-dependent *bsh* transcription was thus assessed by qRT-PCR on RNAs extracted from WT and Δ*cadC* strains grown in BHI. CadA was used as control gene directly repressed by CadC [12]. As expected, *cadA* was highly expressed in the Δ*cadC* mutant as compared to the WT strain (Fig 2A). In the same way, *bsh* appeared significantly upregulated in absence of CadC (Fig 2A), indicating that *bsh* transcription is dependent on CadC.

*bsh* expression was previously shown to be dependent on PrfA and σ$^B$ regulation [8-10]. In addition, we previously showed that PrfA does not control *cadC* expression [12]. To determine if the observed CadC-dependent repression of *bsh* could be due to an indirect regulation through these two regulators, *prfA* and *sigB* expression was assessed by qRT-PCR on RNAs extracted from WT and Δ*cadC* strains. Both *prfA* and *sigB* appeared to be expressed independently on CadC (Fig 2A), thus indicating that the CadC-dependent repression of *bsh* is not dependent on PrfA or σ$^B$ regulation.

CadC represses the expression of target genes by directly binding to conserved CadC boxes present in their promoter [12]. Even in absence of a CadC box in the promoter region of *bsh* [12], we analyzed whether *bsh* expression could be controlled by the direct binding of CadC to its promoter. Increasing amounts of purified CadC were used in EMSA with a DNA fragment containing the *bsh* promoter region (Fig 2B). The promoter region of *cadA* was used as positive control. At the CadC concentration that is sufficient to delay the *cadA* promoter mobility, no shift was observed for the promoter regions of *bsh*, this being also observed using higher CadC concentartion (Fig 2B). An unrelated protein (GFP) was used to verify the specificity of the *cadA*-CadC box delayed migration.

Altogether, these data suggest that CadC represses indirectly *bsh* expression.

**CadC is not required for *Lm* survival in the gastrointestinal tract**

BSH was previously shown to play an important role in *Lm* persistence within the gastrointestinal tract [8]. In addition, preliminary results seemed to indicate that an overexpression of *bsh* could generate an increased *Lm* intestinal multiplication [8]. As *bsh* is repressed by CadC, we analyzed the possible role of CadC in the gastrointestinal phase of listeriosis, i.e. in the earliest stage of the infectious process. We first assessed the survival/multiplication over 12 hours post inoculation of the WT and Δ*cadC* strains in the stomach of mice intragastrically inoculated with a sub-lethal bacterial dose (2x10$^9$ CFUs). For the WT strain, the number of bacteria in mouse stomachs was around 10$^6$ one hour post-inoculation (p.i.),

and increased to reach $10^7$ CFUs at 12 h, demonstrating the survival and multiplication of *Lm* in the mouse stomach environment (Fig 3A). The Δ*cadC* mutant behaved as the WT strain. In addition, the persistence of the Δ*cadC* mutant was studied and compared with the parental strain in stools of mice after intragastric injection of a sub-lethal inoculum over four days after inoculation. We observed for both strains a regular and similar decrease of the number of viable *Lm* in mouse stools over time (Fig 3B). Altogether, these results demonstrate that CadC is dispensable for *Lm* persistence within the gastrointestinal tract.

**Optimal CadC-dependent regulation of *bsh* expression is required to confer full *Lm* virulence**

We next evaluated the importance of the fine regulation of *bsh* expression during infection and addressed the role of CadC in this process. For this purpose, we constructed a strain in which *bsh* would escape the control by CadC. We replaced the *bsh* promoter by the promoter of the *iap* gene on *Lm* chromosome ($P_{iap}$-*bsh*). *iap* was previously shown to have an expression pattern similar to *cadC* and reverse to *bsh*, i.e. highly expressed in infected mouse spleens as compared to growth in the intestinal lumen or in rich medium (BHI) (Tab 1) [17, 19]. We verified by EMSA the absence of binding of CadC to a DNA fragment corresponding to the *iap* promoter (Fig 4A). We confirmed that the *in vi*tro growth of the $P_{iap}$-*bsh* strain was comparable to that of the WT (Fig 4B), and further validated the absence of control of *bsh* by CadC in this strain by analysing *bsh* expression, BSH activity and resistance to bile toxicity. As expected, *cadA* transcript levels increased in the Δ*cadC*-$P_{iap}$-*bsh* as compared to the $P_{iap}$-*bsh* strain, while *bsh* expression was not significantly different in both strains (Fig 4C), confirming the CadC-independent expression of *bsh* in the $P_{iap}$-*bsh* strain. In addition, whereas the Δ*cadC* generated a more pronounced bile acid precipitate than the WT, no differences were detectable between the Δ*cadC* and $P_{iap}$-*bsh* strains (Fig 4D). In accordance, as compared to *Lm* WT, both strains revealed the same phenotype regarding resistance to bile toxicity (Fig 4E). Altogether, these results confirm the high and CadC-independent expression of *bsh* in the $P_{iap}$-*bsh* strain.

We then tested the effect of *bsh* misregulation on virulence by intravenous infection of mice with the WT or $P_{iap}$-*bsh* strains ($10^5$ CFUs). Three days p.i., bacterial counts for $P_{iap}$-*bsh* were significantly lower than those for the WT in both spleens and livers, mimicking the phenotype of the Δ*cadC* mutant (Fig 5A). These results strongly suggest that the CadC-independent expression of *bsh* is unfavorable for infection and highlight the importance of the fine-tuning of *bsh* expression for *Lm* pathogenicity.

**Uncontrolled *bsh* expression induces an overexpression of *mdrT* in presence of bile salts**

BSH is able to hydrolyse conjugated glycodeoxycholic and taurodeoxycholic acids, leading to the deconjugation of glyco- and tauro-bile acids, and the release of free cholic acids [8, 20]. Host cholic acids were shown to be exported by *Lm* through the MdrT efflux pump [21]. *mdrT* is controlled by BrtA, a bile sensor which loses the ability to bind to and repress the *mdrT* promoter in the presence of cholic acid [21, 22]. In addition to be a cholic acid efflux pump, MdrT also transports cyclic di-AMP (c-di-AMP), a small non-DNA nucleic acid capable of eliciting robust host innate immune responses during infection [23, 24]. The unregulated expression of MdrT was shown to significantly restrict virulence *in vivo* [23, 25]. We thus hypothesized that an uncontrolled expression of *bsh in vivo* could induce an overproduction of cholic acid, in turn promoting a stronger *mdrT* expression and restricting *Lm* virulence. To test this hypothesis, we analysed by qRT-PCR the expression of *bsh* and *mdrT* in the WT and $P_{iap}$-*bsh* strains, in absence or presence of bile salts (GDCA). We first observed that *bsh* is more expressed in the $P_{iap}$-*bsh* strain as compared to the WT, independently of the presence of GDCA (Fig 5B). Whereas the overexpression of *bsh* in the $P_{iap}$-*bsh* strain had no effect on the *mdrT* expression in absence of bile salts, the presence of GDCA induced an increased *mdrT* expression in the $P_{iap}$-*bsh* strain as compared to WT (Fig 5B). This result indicates that, in presence of bile salts, an overexpression of *bsh* engenders an upregulation of *mdrT*, most probably through the generation of increased levels of cholic acid.

**CadC regulates additional genes, including virulence genes**

We postulated that CadC could be a more global virulence regulator. To assess this, we performed a whole genome transcriptional analysis to search for genes potentially regulated by CadC. The expression profile of ∆*cadC* was compared to that of WT during exponential growth in BHI at 37ºC, using *Lm* tiling arrays [17]. Genes showing at least a two-fold change in their level of expression are listed in Figure 6A. We found 53 genes differentially regulated in the ∆*cadC* mutant as compared to the WT strain, 45 of which appearing more expressed in absence of *cadC*. We further confirmed our results by qRT-PCR. We selected the 8 down-regulated genes and a subset of 8 genes up-regulated in ∆*cadC* and performed qPCR on cDNA from bacteria grown to exponential phase. qRT-PCR results and array data exhibited a very strong correlation coefficient ($R^2$=0.96) (Fig 6B), validating the differential expression levels detected by transcriptomics.

A large number of differentially expressed genes appeared to encode nutrient transport systems (Fig 6A and 6C). In particular, 8 genes are implicated in inorganic ion transport and metabolism and are up-regulated in ∆*cadC*. This group includes *cadA* and *lspB* previously shown to be directly repressed by CadC [12], and *bsh*. Remarkably, the LPXTG surface protein-encoding genes *inlH* and *lmo0610*, both

previously implicated in *Lm* virulence [26, 27], appeared also up-regulated in Δ*cadC*. Interestingly, 29 of the 45 genes more expressed in absence of CadC were previously shown to be activated during survival in the mouse intestinal lumen [17] (Fig 6D). In addition, of these 45 CadC-repressed genes, 21 were shown to be activated by σ$^B$, the master regulator of class II stress genes [28] particularly important for regulating transcription during the gastrointestinal stages of *Lm* infection [29].

To unravel a potential mechanism of CadC transcriptional regulation, we searched for a conserved motif in the promoter region of the 53 genes. Despite an exhaustive bioinformatic analysis, we were unable to detect any common regulatory sequence. To determine whether the expression of some of these genes could be controlled by the direct binding of CadC to their promoter region, as previously described for *cadA* and *lspB* [12], increasing amounts of purified CadC were used in EMSA with DNA fragments containing the promoter region of genes up- (*lmo0019*) or down- (*lmo2002* and *lmo2336*) regulated in the Δ*cadC* mutant. At the CadC concentration that is sufficient to delay the *cadA* promoter mobility (Fig 2B), and even at higher amounts, no shift was detected for the promoter regions of the two genes tested (Fig 6E).

Altogether, these data suggest that CadC might act indirectly as a new regulator of virulence gene expression.


## DISCUSSION

To optimize interactions with the host and establish an effective infection, *Lm* varies its phenotypic properties in response to environmental signals [17, 19]. One of the best examples is the critical regulatory switch that occurs during the *Lm* transition from the host cell vacuole to cytosol, when *Lm* remodels its transcriptional profile by activating the master virulence regulator PrfA [30, 31]. Bacterial adaptation to life inside the host is, however, more complex than simply expressing genes as their products are needed. The presence of unrequired factors at inappropriate times during bacterial colonization can be detrimental to a successful survival strategy [32]. We previously showed that, during *in vivo* infection, *Lm* repurposed the cadmium efflux pump regulator CadC to directly repress the expression of the LspB lipoprotein signal peptidase avoiding the exposure of the LpeA lipoprotein to the host immune system, diminishing inflammatory cytokine expression and promoting intramacrophage survival and virulence [12]. Here we show that *Lm* also uses CadC to modulate BSH activity and *Lm* resistance to bile by repressing *bsh* expression. However, whereas CadC appears to be dispensable for *Lm* survival in the gastrointestinal tract, CadC-independent expression of *bsh* is unfavorable for *Lm* host infection, strongly suggesting that the optimal CadC-dependent regulation of *bsh* expression is required to confer full *Lm* virulence. The deconjugation of bile salts by BSH produces the release of free cholic acids [8, 20], which are exported by *Lm* through the MdrT efflux pump [21]. We reveal that, in presence

of bile salts, an overexpression of *bsh* induces an upregulation of *mdrT*, most probably through BrtA, a bile sensor, which loses the ability to bind to and repress the *mdrT* promoter in the presence of cholic acid [21, 22]. Whereas MdrT protects *Lm* from the bactericidal effects of bile [21], its unregulated expression was shown to significantly restrict virulence *in vivo* [23, 25]. This strongly suggests that the uncontrolled expression of *bsh in vivo* induces an overproduction of cholic acid, in turn promoting a stronger *mdrT* expression and restricting *Lm* virulence. We thus propose that *Lm* uses CadC to repress *bsh* expression during phases of the infection where an over-expression of *bsh* would be deleterious for infection.

MdrT can also transport c-di-AMP, which is sensed by the host cytosolic innate immune receptor STING [33], activating a strong type I interferon (IFN-β) response [23, 24]. Paradoxically, IFN-β production was shown to increase the burden and lethality of *Lm* [34, 35]. The diversity of physiologically relevant substrates transported by MdrT reinforces the need for its tight regulation *in vivo*, in particular through CadC repression.

We then postulated that CadC could play a more general role as virulence regulator and found 53 genes differentially regulated in the *cadC* mutant. We were unable to detect any common regulatory motif or CadC binding on the promoter sequence of regulated genes other than *cadAC* and *lspB*, suggesting that CadC might act indirectly to control expression of other genes. CadC appears mostly as a repressor, 45 of the 53 CadC-controlled genes being more expressed in absence of CadC, ion transport and metabolism genes constituting the largest group. *lmo0153-lmo0155* encode a putative zinc ABC transporter, *lmo2494* encodes a putative regulatory protein of phosphate transport, *lmo2230* is annotated as an arsenate reductase and *lmo2231* encodes a protein of the cation efflux superfamily. These data could suggest a peripheral function for CadC in controlling arsenic, zinc and phosphate homeostasis. Most importantly, two genes known to be required for *Lm* virulence, *inlH* and *lmo0610*, appear also repressed by CadC, suggesting that the *in vivo* regulation of these genes by CadC could be crucial for *Lm* at specific stages of the infectious process. Among the few genes that appear activated by CadC, *lmo2000-lmo2002* encode components of a mannose/fructose/sorbose family PTS system, and *lmo1998-lmo1999* encode two sugar isomerases putatively implicated in hexosamine metabolism, converting fructose-6-phosphate into glucosamine-6-phosphate. The end product of this pathway is N-acetylglucosamine, a component of *Lm* peptidoglycan that is also used for teichoic acids decoration [36, 37]. CadC could be thus involved in the regulation of the composition and/or structure of the cell wall. However, following an HPLC analysis, we were unable to detect any defect in the cell wall content of the ΔcadC strain (our unpublished data). *lmo2335-lmo2336* (*fruA-fruB*) encode components that participate to the transport and conversion of fructose to fructose-1,6-bisphosphate, and *lmo0278* is a putative glycerol-3-phosphate ABC transporter. Fructose-1,6-bisphosphate and glycerol-3-phosphate are central

intermediates for the cell metabolism and can be used for glycolysis or deviated to other metabolic pathways. The CadC regulation of these carbon source transporters might be important for the growth and adaptability of *Lm* to the host environment.

Adaptability of pathogens to the host environment is crucial for the success of the infectious process and requires the tight and coordinated regulation of virulence factor expression. We and others previously showed that the *in vivo* differential expression of the *Lm* genome is coordinated by a complex regulatory network [17, 19]. During infection, *Lm* up-regulates the expression of the major virulence regulators, PrfA and VirR, and that of the master regulator of class II stress genes, $\sigma^B$. In particular, the fine regulation of the PrfA regulon through complex PrfA-SigB interactions appears essential for the success of the infection. Indeed, bacteria need to ensure the rapid increase in the expression of virulence genes and their subsequent down-regulation to avoid overexpression and irreversible host cell damages [38, 39]. *cadC* is highly expressed during infection [19], and we observed here that a high proportion of CadC repressed genes were previously shown to be activated during survival in the mouse intestinal lumen [17], and controlled by $\sigma^B$, the master transcriptional regulator during the gastrointestinal stages of *Lm* infection [29]. We thus propose that CadC represses during systemic infection $\sigma^B$-controlled genes important for the gastrointestinal stages of *Lm* infection, revealing CadC as a crucial new player of the complex network of transcriptional regulators that contributes to fine-tune virulence gene expression over the *Listeria* infectious process.

## ACKNOWLEDGEMENTS

## REFERENCES

1. Cossart P. Illuminating the landscape of host-pathogen interactions with the bacterium Listeria monocytogenes. Proc Natl Acad Sci U S A **2011**; 108:19484-91.

2. European Food Safety Authority ECfDPaC. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2013. European Food Safety Authority, European Centre for Disease Prevention and Control EFSA Journal **2015**; 13:3991.

3. Swaminathan B, Gerner-Smidt P. The epidemiology of human listeriosis. Microbes Infect **2007**; 9:1236-43.

4. Carvalho F, Sousa S, Cabanes D. How Listeria monocytogenes organizes its surface for virulence. Frontiers in cellular and infection microbiology **2014**; 4:48.

5. NicAogain K, O'Byrne CP. The Role of Stress and Stress Adaptations in Determining the Fate of the Bacterial Pathogen Listeria monocytogenes in the Food Chain. Front Microbiol **2016**; 7:1865.

6. Chand D, Avinash VS, Yadav Y, Pundle AV, Suresh CG, Ramasamy S. Molecular features of bile salt hydrolases and relevance in human health. Biochim Biophys Acta **2017**; 1861:2981-91.

7. Begley M, Gahan CG, Hill C. The interaction between bacteria and bile. FEMS Microbiol Rev **2005**; 29:625-51.

8. Dussurget O, Cabanes D, Dehoux P, et al. Listeria monocytogenes bile salt hydrolase is a PrfA-regulated virulence factor involved in the intestinal and hepatic phases of listeriosis. Mol Microbiol **2002**; 45:1095-106.

9. Begley M, Sleator RD, Gahan CG, Hill C. Contribution of three bile-associated loci, bsh, pva, and btlB, to gastrointestinal persistence and bile tolerance of Listeria monocytogenes. Infect Immun **2005**; 73:894-904.

10. Zhang Q, Feng Y, Deng L, et al. SigB plays a major role in Listeria monocytogenes tolerance to bile stress. Int J Food Microbiol **2011**; 145:238-43.

11. Camejo A, Carvalho F, Reis O, Leitao E, Sousa S, Cabanes D. The arsenal of virulence factors deployed by Listeria monocytogenes to promote its cell infection cycle. Virulence **2011**; 2:379-94.

12. Pombinho R, Camejo A, Vieira A, et al. Listeria monocytogenes CadC regulates cadmium efflux and fine-tunes lipoprotein localization to escape the host immune response and promote infection. J Infect Dis **2017**.

13. Boneca IG, Dussurget O, Cabanes D, et al. A critical role for peptidoglycan N-deacetylation in Listeria evasion from the host innate immune system. Proc Natl Acad Sci U S A **2007**; 104:997-1002.

14. Carvalho F, Atilano ML, Pombinho R, et al. L-Rhamnosylation of Listeria monocytogenes Wall Teichoic Acids Promotes Resistance to Antimicrobial Peptides by Delaying Interaction with the Membrane. PLoS Pathog **2015**; 11:e1004919.

15. Pinheiro J, Reis O, Vieira A, et al. Listeria monocytogenes encodes a functional ESX-1 secretion system whose expression is detrimental to in vivo infection. Virulence **2016**:1-12.

16. Cabanes D, Lecuit M, Cossart P. Animal models of Listeria infection. Curr Protoc Microbiol **2008**; Chapter 9:Unit9B 1.

17. Toledo-Arana A, Dussurget O, Nikitas G, et al. The Listeria transcriptional landscape from saprophytism to virulence. Nature **2009**; 459:950-6.

18. Dashkevicz MP, Feighner SD. Development of a differential medium for bile salt hydrolase-active Lactobacillus spp. Appl Environ Microbiol **1989**; 55:11-6.

19. Camejo A, Buchrieser C, Couve E, et al. In vivo transcriptional profiling of Listeria monocytogenes and mutagenesis identify new virulence factors involved in infection. PLoS Pathog **2009**; 5:e1000449.

20. Ooi LG, Liong MT. Cholesterol-lowering effects of probiotics and prebiotics: a review of in vivo and in vitro findings. Int J Mol Sci **2010**; 11:2499-522.

21. Quillin SJ, Schwartz KT, Leber JH. The novel Listeria monocytogenes bile sensor BrtA controls expression of the cholic acid efflux pump MdrT. Mol Microbiol **2011**; 81:129-42.

22. Yamamoto T, Hara H, Tsuchiya K, et al. Listeria monocytogenes strain-specific impairment of the TetR regulator underlies the drastic increase in cyclic di-AMP secretion and beta interferon-inducing ability. Infect Immun **2012**; 80:2323-32.

23. Crimmins GT, Herskovits AA, Rehder K, et al. Listeria monocytogenes multidrug resistance transporters activate a cytosolic surveillance pathway of innate immunity. Proc Natl Acad Sci U S A **2008**; 105:10191-6.

24. Woodward JJ, Iavarone AT, Portnoy DA. c-di-AMP secreted by intracellular Listeria monocytogenes activates a host type I interferon response. Science **2010**; 328:1703-5.

25. Schwartz KT, Carleton JD, Quillin SJ, Rollins SD, Portnoy DA, Leber JH. Hyperinduction of host beta interferon by a Listeria monocytogenes strain naturally overexpressing the multidrug efflux pump MdrT. Infect Immun **2012**; 80:1537-45.

26. Personnic N, Bruck S, Nahori MA, et al. The stress-induced virulence protein InlH controls interleukin-6 production during murine listeriosis. Infect Immun **2010**; 78:1979-89.

27. Cummins J, Casey PG, Joyce SA, Gahan CG. A mariner transposon-based signature-tagged mutagenesis system for the analysis of oral infection by Listeria monocytogenes. PLoS One **2013**; 8:e75437.

28. Hain T, Hossain H, Chatterjee SS, et al. Temporal transcriptomic analysis of the Listeria monocytogenes EGD-e sigmaB regulon. BMC Microbiol **2008**; 8:20.

29. Kazmierczak MJ, Wiedmann M, Boor KJ. Contributions of Listeria monocytogenes sigmaB and PrfA to expression of virulence and stress response genes during extra- and intracellular growth. Microbiology **2006**; 152:1827-38.

30. Freitag NE, Port GC, Miner MD. Listeria monocytogenes - from saprophyte to intracellular pathogen. Nat Rev Microbiol **2009**; 7:623-8.

31. de las Heras A, Cain RJ, Bielecka MK, Vazquez-Boland JA. Regulation of Listeria virulence: PrfA master and commander. Curr Opin Microbiol **2011**; 14:118-27.

32. Gray MJ, Freitag NE, Boor KJ. How the bacterial pathogen Listeria monocytogenes mediates the switch from environmental Dr. Jekyll to pathogenic Mr. Hyde. Infect Immun **2006**; 74:2505-12.

33. Sauer JD, Sotelo-Troha K, von Moltke J, et al. The N-ethyl-N-nitrosourea-induced Goldenticket mouse mutant reveals an essential function of Sting in the in vivo interferon response to Listeria monocytogenes and cyclic dinucleotides. Infection and Immunity **2011**; 79:688-94.

34. Auerbuch V, Brockstedt DG, Meyer-Morse N, O'Riordan M, Portnoy DA. Mice lacking the type I interferon receptor are resistant to Listeria monocytogenes. J Exp Med **2004**; 200:527-33.

35. O'Connell RM, Saha SK, Vaidya SA, et al. Type I interferon production enhances susceptibility to Listeria monocytogenes infection. J Exp Med **2004**; 200:437-45.

36. Kamisango K, Saiki I, Tanio Y, et al. Structures and biological activities of peptidoglycans of Listeria monocytogenes and Propionibacterium acnes. J Biochem (Tokyo) **1982**; 92:23-33.

37. Kamisango K, Fujii H, Okumura H, et al. Structural and immunochemical studies of teichoic acid of Listeria monocytogenes. J Biochem **1983**; 93:1401-9.

38. Decatur AL, Portnoy DA. A PEST-like sequence in listeriolysin O essential for Listeria monocytogenes pathogenicity. Science **2000**; 290:992-5.

39. Schnupf P, Hofmann J, Norseen J, Glomski IJ, Schwartzstein H, Decatur AL. Regulated translation of listeriolysin O controls virulence of Listeria monocytogenes. Mol Microbiol **2006**; 61:999-1012.

**Table S1 - Primers**

| Primer name | Sequence (5' to 3') |
| --- | --- |
| Primers used for mutagenesis | |
| catMA | GACGAGTGTACTTATAAAAGCCAGTCATTAGGCCTATCTG |
| catMB | ATGACGCGTGGAGGCATATCAAATGAAC |
| iapMA | ATACGCGTGCCCTAAGAATACACGTTCGC |
| iapMB | GACGTACACATATTCATAAAACTCCTCTC |
| bshMC | TATGAATATGTGTACGTCAATAACTTATACAAC |
| bshMD | ATGAATTCCACTCGAAAGAACGCGATAATTG |
| Primers used for Real Time RT-PCR | |
| 16SqF | CTCGTGTCGTGAGATGTTGG |
| 16SqR | CGTGTGTAGCCCAGGTCATA |
| inlHqF | AGTGGGACAGTTACACAACC |
| inlHqR | TTTTCGCATCATACCAGCC |
| bshqF | GGTATTGCTGCTGTGAT |
| bshqR | CGCATTGACCAAGAATCC |
| prfAqF | TCATTAGCGAGCAGGCTACC |
| prfAqR | GCAAATAGAGCCAAGCTTCC |
| sigBqF | AGAAACGGGTGAACTACTCG |
| sigBqR | CTTCCTCATTCTGCAACGCC |
| cadAqF | TGAACGAGCACCAGCACAAGCG |
| cadAqR | CCCATGTGTCCCAATCACCACC |
| lmo0153qF | ACCGTGATTGATGCGAGTA |
| lmo0153qR | TGTAAGACCGTTCTGGATATTAGT |
| lmo0278qF | CGGTATTCGTCCAGAAGATATTCA |
| lmo0278qR | GTTCCGAGCGAGCATCAA |
| lmo0794qF | CGCCTTACTATCCAACTG |
| lmo0794qR | AACTATTGCCATCGCTAC |
| lmo1998qF | CGACCTATTCATACGCAATGTGTT |
| lmo1998qR | AACGCTGGCTATCTTCTGTCA |
| lmo1999qF | CCACCATCCATCTCGTAA |
| lmo1999qR | ATAGCCAAGCGTGACATA |
| lmo2000qF | CTTCGGTTATCGGTCTGG |
| lmo2000qR | AGAAGGTGAAGCCAAGTG |
| lmo2001qF | GTCAACAAGAAGAATGCGGTATT |
| lmo2001qR | CAAGTGCTGGCTCTGTATTAGTA |
| lmo2002qF | GGCAAATGATGGGGTAGCA |
| lmo2002qR | TGTAAGCGTCTTCAACCGATT |
| lmo2230qF | CGAAGTGCCATTGCTGAAG |
| lmo2230qR | CTGCCAGTAATTCTGAACTAGGT |
| lmo2269qF | TCGTTGATGAATCAGGTGGGTGTC |
| lmo2269qR | AGGAGCATCGTCAGGAAGCATT |
| lmo2335qF | AAGCAGAGGAAGGTAGTCAAG |
| lmo2335qR | GCGATGGCGATAGCGATA |
| lmo2336qF | CGTAAGGTGCTGGAGACTCA |
| lmo2336qR | TTGGTTATCTTCACTTGCGGTAAT |
| lmo2646qF | AGAAGAAGCCGCAAATGTG |

| Primer name | Sequence (5' to 3') |
|---|---|
| *lmo2646qR* | GGAGCCAGACGAGCAATA |
| Primers used for EMSA | |
| *bshEF* | TCCTGATATGGGAATGGGTGGC |
| *bshER* | GTGATCCTTCGTTGTATAAGTT |
| *cadAEF* | CATTTACTTACCTTAAGACAAG |
| *cadAER* | CGTACAAGACATACCGTCTAC |
| *lmo0019EF* | ATAGCGTATTTTGGTTCCGCCA |
| *lmo0019ER* | CACTGAACACTAATCCAAGGAC |
| *lmo2336EF* | CTGGATTTTCACGCCATTTTCG |
| *lmo2336ER* | CCAAGTTTCTCAATACTCTCCA |
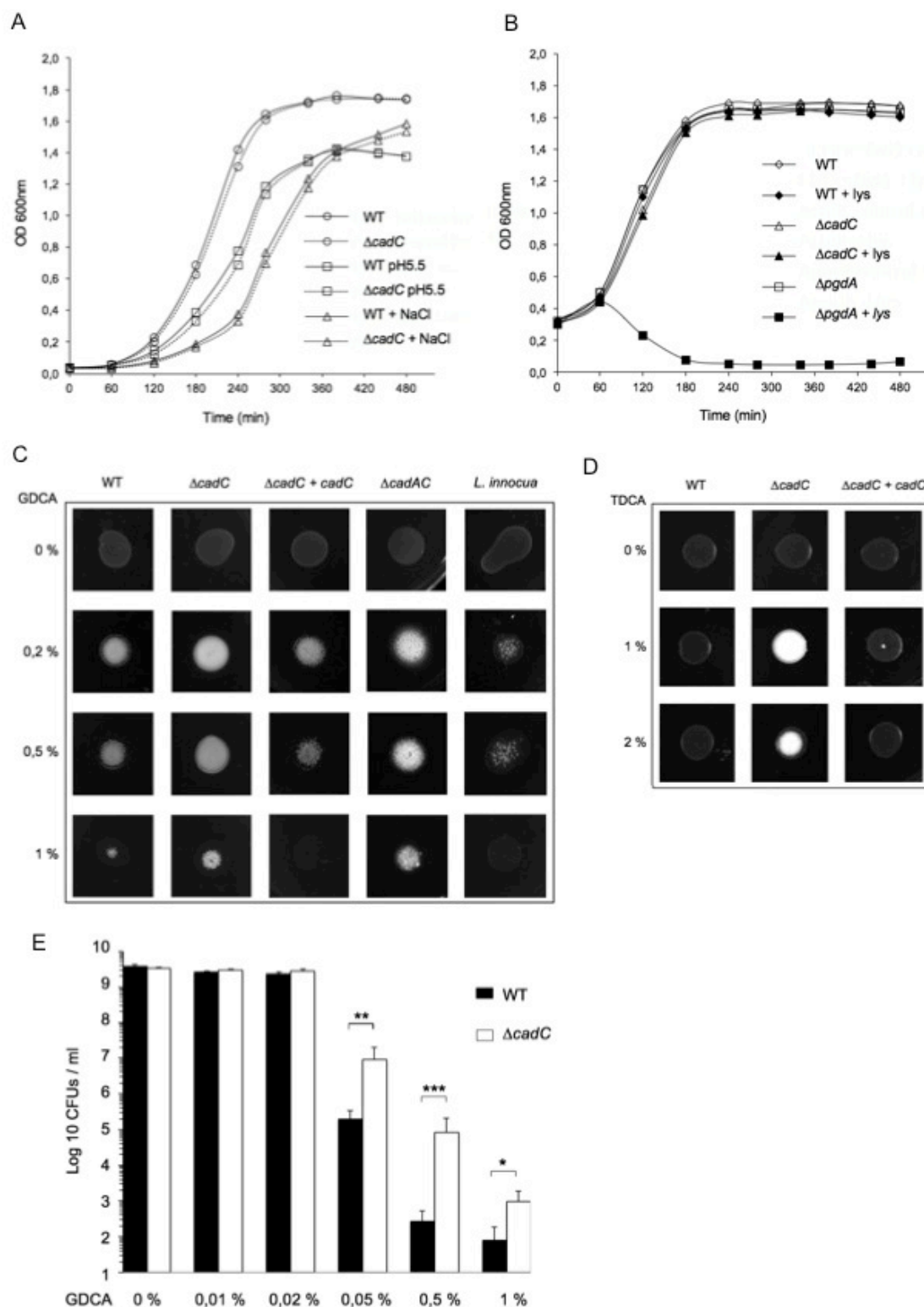
**Figure 1. CadC controls BSH activity and *Lm* resistance to bile salts.** (A) Growth curves of wild-type (WT) and Δ*cadC* in BHI, BHI pH 5.5 or BHI containing 5% NaCl. (B) Growth curves of WT, Δ*cadC* and Δ*pgdA* in BHI or BHI supplemented with lysozyme (200 μg/ml). Representative results from 3 independent experiments. (C-E) Effect of CadC deletion on BSH activity. (C-D) WT, Δ*cadC*, Δ*cadC*+*cadC*, Δ*cadAC* and *L. innocua* were patch inoculated onto MRS agar supplemented with increasing concentration of (C) GDCA or (D) TDCA and incubated in microaerophilic conditions at 37°C for 72h. Experiments were performed at least twice, and representative results are shown. (E) Overnight cultures of WT and Δ*cadC* were inoculated into BHI supplemented with increasing concentrations of GDCA and CFUs were quantified 16 h later. Values are mean ± SD (n=3).
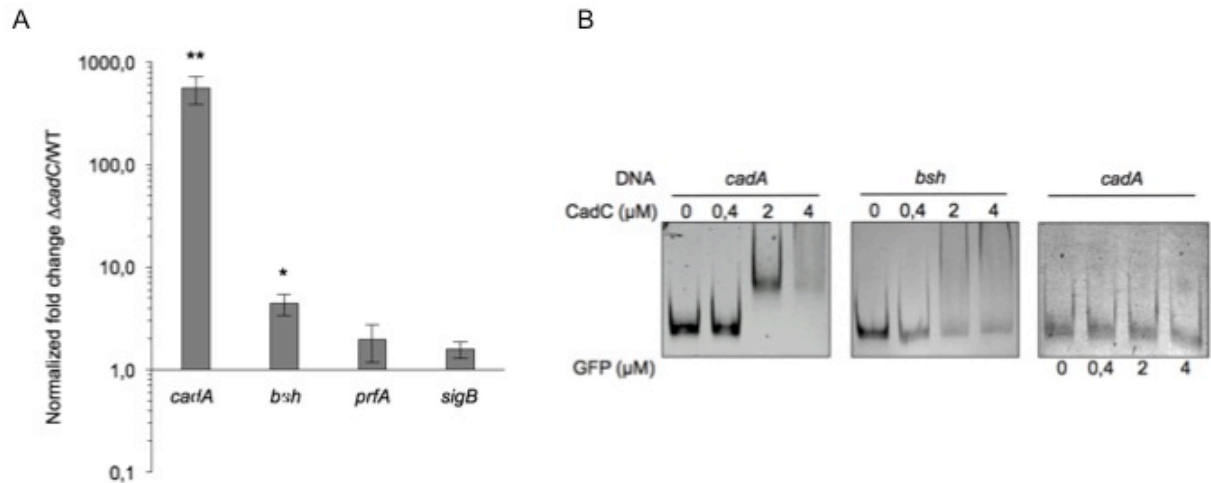
**Figure 2. CadC represses *bsh* expression.** (A) *cadA, bsh, prfA* and *sigB* expression determined by qRT-PCR on RNAs extracted from logarithmic cultures of WT and Δ*cadC* strains grown in BHI at 37 ℃. *cadA* was used as CadC-dependent control gene and gene expression levels in the Δ*cadC* mutant are presented normalized to those in the WT strain (set at 1). Values are mean ± SD (n=3). *$P<0.05$ and **$P<0.01$. (B) CadC does not bind directly to *bsh* promoter region. Increasing amounts of purified CadC were used in electrophoretic mobility shift assays (EMSAs) with DNA fragments containing the *cadA* or *bsh* promoter regions generated by PCR, using primers listed in Table S1. An unrelated protein (GFP) was used as negative control. Experiments were performed at least twice, and representative results are shown.
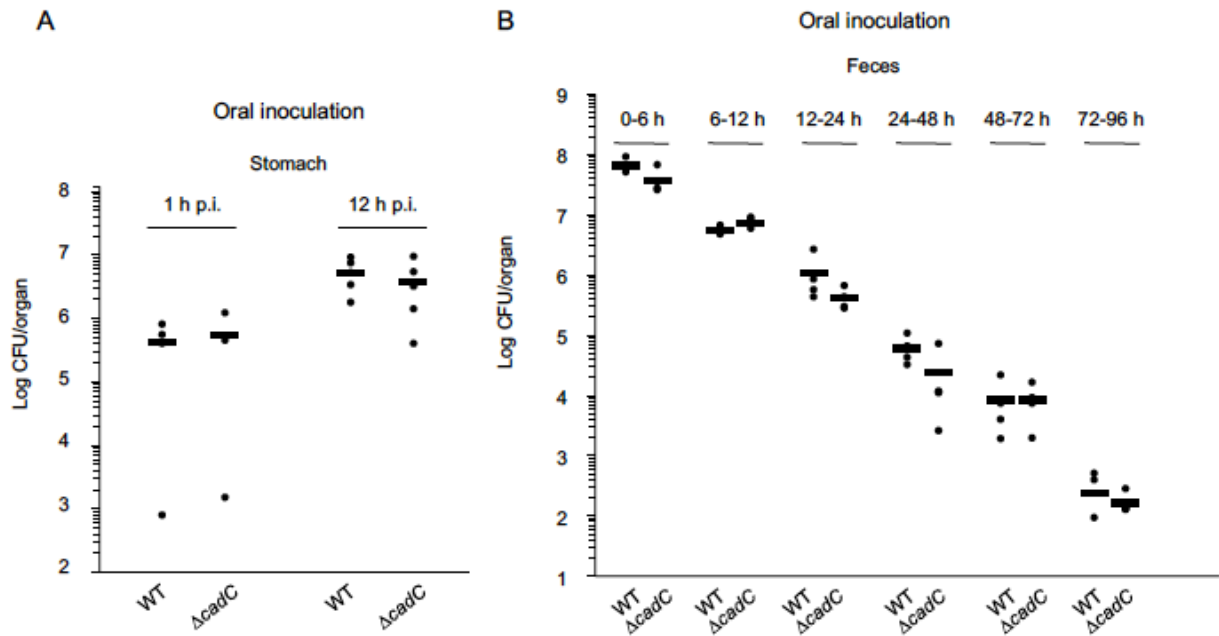
**Figure 3. CadC is not required for *Lm* survival in the gastrointestinal tract.** (A-B) Bacterial counts of the WT and Δ*cadC* strains in the (A) stomach and (B) feces at different time points after oral inoculation ($10^9$ bacteria per animal) of C57BL/6 mice.
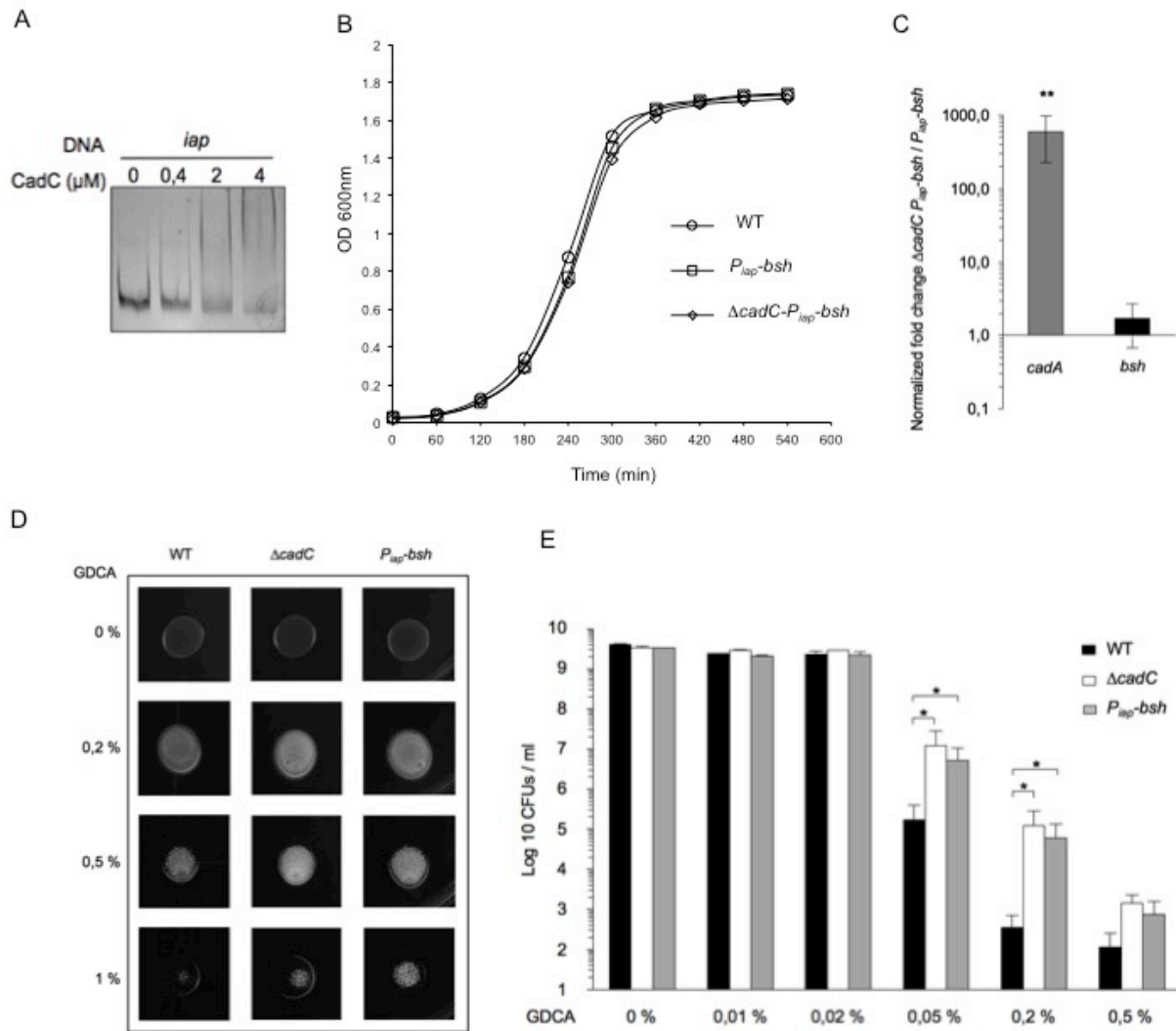
**Figure 4. CadC-independent expression of *bsh* in the *P$_{iap}$-bsh* strain.** (A) CadC does not bind to the *iap* promoter region. Increasing amounts of purified CadC were used in EMSAs with DNA fragments containing the *iap* promoter region generated by PCR. (B) Growth curves of WT, *P$_{iap}$-bsh* and Δ*cadC-P$_{iap}$-bsh* in BHI. (C) *cadA* and *bsh* expression by qRT-PCR on RNAs extracted from logarithmic cultures of *P$_{iap}$-bsh* and Δ*cadC-P$_{iap}$-bsh* strains in BHI at 37°C. Values are mean ± SD (n=3). **$P<0.01$. (D) WT, Δ*cadC* and *P$_{iap}$-bsh* strains were patch inoculated onto MRS agar supplemented or not with increasing concentrations of GDCA and incubated at 37ºC for 72h under microaerophilic conditions. Experiments were performed at least twice, and representative results are shown. (E) Overnight cultures of WT, Δ*cadC* and *P$_{iap}$-bsh* were inoculated into BHI supplemented with increasing concentrations of GDCA and after 16 h CFUs were quantified. Values are mean ± SD (n=3).
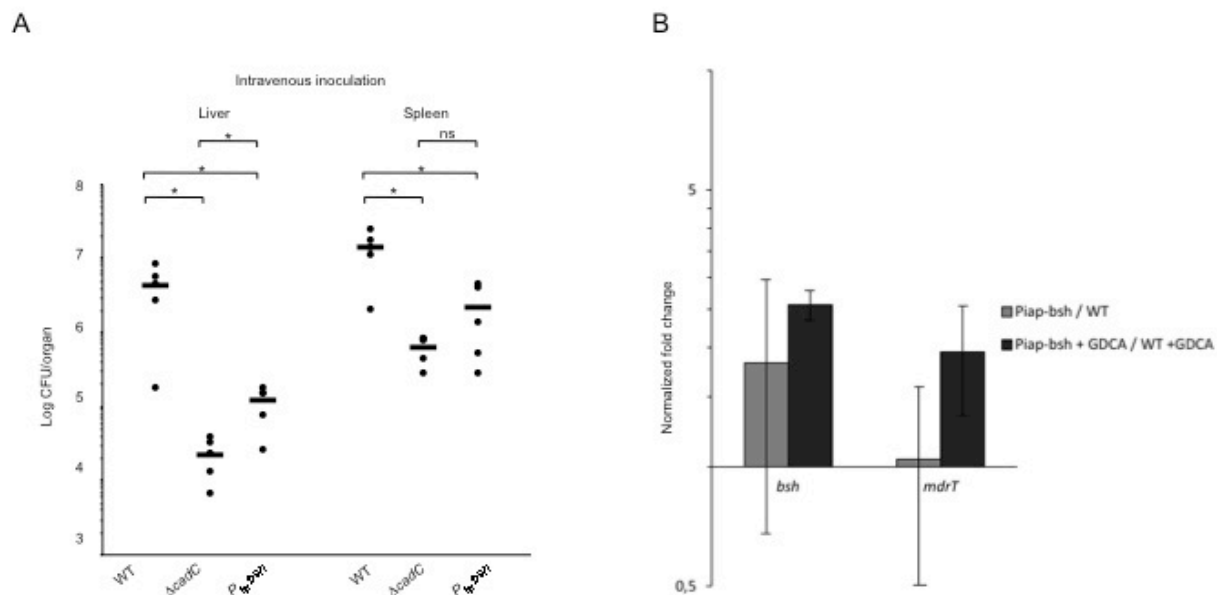
**Figure 5. Fine regulation of *bsh* expression is required to confer full *Lm* virulence.** (A) Bacterial counts of WT, Δ*cadC* and *P*$_{iap}$-*bsh* strains in the liver and spleen of C57BL/6 mice 72 hours after intravenous inoculation of 10$^5$ bacteria per animal. Data are presented as scatter plots, each point represents an animal and mean values are indicated by a horizontal line (n = 5). ns, not significant. *$P<0.05$. (B) *bsh* and *mdrT* expression by qRT-PCR on RNAs extracted from logarithmic cultures of WT and *P*$_{iap}$-*bsh* strains grown in BHI or BHI supplemented with GDCA, at 37°C. Values are mean ± SD (n=3).

**A**

| Gene | Gene designation | Annotation | Fold Change DcadC/WT | SigB regulation | Expression in intestine lumen |
|---|---|---|---|---|---|
| lmo0019 | | SH3 domain-containing protein | 6,45 | | |
| lmo0043 | arcA | arginine deiminase | 4,66 | | |
| lmo0153 | | similar to zinc-binding lipoprotein | 6,11 | | |
| lmo0154 | | similar to zinc ABC transporter, ATP-binding protein | 9,00 | | |
| lmo0155 | | similar to zinc ABC transporter, permease protein | 16,41 | | |
| lmo0263 | inlH | internalin H | 4,82 | | |
| lmo0278 | | similar to sugar ABC transporter, ATP-binding protein | -4,79 | | |
| lmo0321 | | unknown | 4,26 | | |
| lmo0322 | | unknown | 11,08 | | |
| lmo0439 | | unknown | 5,90 | | |
| lmo0445 | | similar to transcription regulator | 6,50 | | |
| lmo0554 | | similar to NADH-dependent butanol dehydrogenase | 4,53 | | |
| lmo0610 | | LPXTG-motif cell wall anchor domain protein | 5,39 | | |
| lmo0654 | | unknown | 4,41 | | |
| lmo0669 | | similar to oxidoreductase | 4,96 | | |
| lmo0670 | | unknown | 6,54 | | |
| lmo0794 | | similar to NAD-dependent epimerase/dehydratase | 5,86 | | |
| lmo0911 | | putative transcriptional activator | 4,63 | | |
| lmo1099 | | similar to a protein encoded by Tn916 | 22,47 | | |
| lmo1100 | cadA | cadmium resistance protein | 116,97 | | |
| lmo1101 | lspB | lipoprotein signal peptidase | 5,24 | | |
| lmo1113 | | similar to a protein encoded by Tn916 | 7,41 | | |
| lmo1114 | | similar to a protein encoded by Tn916 | 7,06 | | |
| lmo1241 | | unknown | 5,03 | | |
| lmo1671 | adcA | zinc-binding lipoprotein | 5,46 | | |
| lmo1789 | | similar to NADP dehydrogenase | 4,86 | | |
| lmo1882 | rpsN | 30S ribosomal protein S14 | 8,11 | | |
| lmo1998 | | similar to opine catabolism protein (sugar isomerase) | -4,29 | | |
| lmo1999 | | similar to glucosamine-fructose-6-phosphate | -11,47 | | |
| lmo2000 | | similar to PTS mannose/fructose/sorbose-specific enzyme IID | -17,51 | | |
| lmo2001 | | similar to PTS mannose/fructose/sorbose-specific enzyme IIC | -12,91 | | |
| lmo2002 | | similar to PTS mannose/fructose/sorbose-specific enzyme IIB | -7,41 | | |
| lmo2067 | bsh | bile salt hydrolase | 6,54 | | |
| lmo2157 | sepA | sepA | 4,44 | | |
| lmo2213 | | unknown | 4,63 | | |
| lmo2230 | | similar to arsenate reductase | 4,00 | | |
| lmo2231 | | similar to cation efflux superfamily protein | 6,63 | | |
| lmo2269 | | unknown | 6,92 | | |
| lmo2335 | fruA | PTS system, fructose-specific, IIABC component | -9,32 | | |
| lmo2336 | fruB | fructose-1-phosphate kinase | -13,00 | | |
| lmo2387 | | similar to chloride channel protein | 4,03 | | |
| lmo2484 | | unknown | 3,94 | | |
| lmo2494 | phoU | similar to phosphate transport system protein | 7,36 | | |
| lmo2645 | | similar to sugar isomerase domain protein | 11,08 | | |
| lmo2646 | | unknown | 14,22 | | |
| lmo2647 | | similar to creatinine amidohydrolase | 9,25 | | |
| lmo2648 | | similar to phosphotriesterase | 8,58 | | |
| lmo2649 | | similar to PTS system lactose/cellobiose specific IIC subunit | 5,54 | | |
| lmo2650 | | similar to PTS system lactose/cellobiose specific IIB subunit | 8,57 | | |
| lmo2651 | | similar to PTS system fructose/mannitol specific IIA subunit | 6,87 | | |
| lmo2673 | | similar to universal stress family protein | 3,76 | | |
| lmo2711 | | unknown | 4,86 | | |
| lmo2748 | | similar to general stress protein 26 (GSP26) | 3,68 | | |

**C**

1 Amino acid transport and metabolism
2 Carbohydrate transport and metabolism
3 Inorganic ion transport and metabolism
4 Lipid transport and metabolism
5 Secondary metabolites biosynthesis, transport and catabolism
6 Cell wall/membrane biogenesis
7 Defense mechanisms
8 Energy production and conversion
9 Signal transduction mechanisms
10 Transcription
11 Translation
12 Function unknown
13 General function prediction only
14 Not in COGs

**D**

**B**

**E**



**Figure 6**. CadC regulates additional genes, including virulence genes (A) Genes differentially expressed in the ΔcadC mutant as compared to WT as determined by tiling arrays. In red are shown genes whose expression is up-regulated and in green down-regulated in ΔcadC as compared to WT. PrfA and/or SigB regulation and expression in the intestine lumen previously described for the identified genes are indicated. (B) Validation of tiling arrays data by qRT-PCR. Fold changes in gene expression in the ΔcadC mutant compared to that in the WT, measured by tiling arrays and qRT-PCR, log transformed and compared for correlation analysis. (C) Relative abundance of categories of genes differentially expressed in the ΔcadC mutant. Colors correspond to categories in the COG database. (D) Venn diagram showing the 53 genes controlled by CadC. (E) Binding of CadC to the promoter region of regulated genes. Increasing amounts of purified CadC were used in EMSAs with PCR-generated DNA fragments containing the promoter region of genes repressed (lmo0019) or activated (lmo2002 and lmo2336) by CadC. Experiments were performed at least twice, and representative results are shown.

# CHAPTER IV:  GENERAL DISCUSSION

Thousands of years of co-evolution allowed different species to adapt within different life styles, ecological niches and host specificities. Throughout our lifetime we are permanently in contact with a multitude of microbial species, either commensal bacteria that colonize our mucosal surfaces, or pathogenic microorganisms, which are usually targeted by our immune defences to prevent infection. Exploring host-pathogen interactions is a crucial way to understand and control the fundamental paradigms of cell biology and bacterial physiology. The host develops strategies to sense pathogens therefore inducing effective immune responses, whether bacteria evolve mechanisms that interfere with host signalling pathways in order to escape the immune system and thus promote infection. These sophisticated mechanisms are in constant evolution to overcome the new challenges invariably refined by pathogens and their hosts. This work improves our understanding on the control of innate immune responses, either by the host or by bacteria, to their own profit.

One of the main questions is how the host immune system is able to sense pathogens? Host cells deploy multiple transmembrane and cytosolic sensors to detect microbial pathogens and arm signalling pathways that activate innate immune responses. These receptors belong to a family of sensors known as PRRs, which are conserved receptors able to sense invariant signature molecules named PAMPs. SRs are a class of PRRs, originally implicated on the recognition of modified lipoproteins and intimately associated with the maintenance of host homeostasis. Interestingly, it is now appreciated that they largely contribute to microbial pathogenesis, once they recognize an enormous repertoire of PAMPs. The results presented in this thesis contributed to an improved understanding on the importance of SRs for *Lm* cellular infectious process and for host innate immune responses against infection. SRs are widely expressed in cells patrolling potential portals of pathogen entry, such as macrophages, neutrophils, dendritic cells, microglia and B cells. Phagocytes are extremely dynamic and have an enormous potential to engulf, digest and kill microbial invaders. Moreover, they are among the first cells to be recruited to the site of infection. However, we cannot underrate the fact that SRs are also expressed in non-professional phagocytes, such as epithelial and endothelial cells. These cells are extremely relevant in the context of pathogen infection, mainly for a microbe such as *Lm*, which is an intracellular facultative pathogen capable to invade, infect and multiplies within both phagocytic and non-phagocytic cells. SRs have been reported to play opposite roles during bacterial infection: whereas some SRs recognize a myriad of microbial proteins activating downstream immune responses to fight and eliminate the pathogen, some pathogens hijack SR function exploiting them to bind and invade cells, thus promoting intracellular survival and proliferation.

Particularly significant are the newfound roles of STAB-1 in *Lm* phagocytosis and macrophage integrity. We show that STAB-1 KO mice display deregulated inflammatory cytokine production, impaired cellular recruitment of myeloid cells and increased

susceptibility to *Lm*, thus revealing the key role of STAB-1 in restraining bacterial infection. Our findings have expanded the current knowledge on SRs and raised the role of SRs in the overall *Lm* infectious process. STAB-1 was previously shown to support binding of tumour-infiltrating lymphocytes to tumour vasculature. The anti-STAB-1 antibody therapy has an inhibitory effect on the progression of primary and metastatic tumours, which suggests that this molecule may be a new immunomodulatory target for cancer immunotherapy (Karikoski *et al.* 2014). However, in the context of pathogenic infection STAB-1 is beneficial for host immune response against *Lm* infection. STAB-1 is important for cells recruitment. Whether in a cancer scenario STAB-1 induces leukocyte entrance into the tumours, in the case of a bacterial infection STAB-1 contributes for the recruitment of immune cells that help to fight infection. SRs have the ability to recognize not only the pathogens *per se* but also the damage they cause. STAB-1 did not trigger an immune response against the non-pathogenic *L. innocua*, suggesting that the response should be prompted by a specific *Lm* protein or by a DAMP. It would be of major interest to explore whether *Lm* displays a structural component or specific virulence factors that account for this host innate immune response against *Lm*. Considering the role of STAB-1 in the migration of T and B lymphocytes and its ability to bind both Gram-positive and Gram-negative bacteria *in vitro* (Adachi *et al.* 2002; Kzhyshkowska 2010*)*, it will be particularly important to assess the role of STAB-1 in the context of other pathogen infections. Our results with LPS suggest that the host immune response is not specific of Gram-positive bacteria. Remarkably, STAB-1 avidly binds Hsp70-peptide complexes and other chaperones, and heat shock proteins play important roles in cell signalling and immunity. Understand the impact of heat shock proteins as partners of STAB-1 immunoregulatory function would be interesting. STAB-1 is able to mediate the engulfment and clearance of apoptotic bodies, through PS recognition, thus contributing for the maintenance of tissue homeostasis. Unravel the role of STAB-1 in this process *in vivo*, in the context of pathogen infection might allow us to understand its impact on the phagocytosis of dead cells targeted by the pathogen.

STAB-1 is expressed in spleen, liver and placenta, which are major organs affected by *Lm* infection. The results of this work may pave a way to identify novel targets and pathways to interrupt or limit *Lm* infection. Augmentation of protective host cell pathways represents a novel therapeutic option to efficiently reduce invading pathogens. In particular, STAB-1 may be triggered in response to a specific *Lm* PAMP or DAMPs resultant from the infectious process, which remains yet to be identified. Administration of specific ligands known be recognized by STAB-1 could also trigger STAB-1-mediated responses.

Pathogens evolved multiple and complex factors to survive in hostile environments and thus contribute to the successful colonization of the host. *Lm* lives as a saprophyte in the extracellular environment but it can easily switch to life within human cells. To be a pathogen,

*Lm* displays a complex arsenal of virulence genes and regulatory mechanisms that cooperate for the successful survival and persistence of the pathogen within the host. The elucidation of host subversion mechanisms to promote bacterial virulence paves the way for major breakthroughs in exploring host-pathogen interaction and design new therapeutic strategies.

Despite their toxicity at high concentrations, heavy metals are required in trace amounts for enzymatic reactions or as structural components of bacterial proteins. Importantly, significant changes in metal availability may occur at different locations within the host, once it exploits the bacterial requirements for transition metals by sequestering these elements. Therefore, the intracellular concentration of metals needs to be finely tuned in order to maintain metal homeostasis. Efflux pumps are part of the complex system that confers heavy metal resistance to bacteria. CadC is a metal-responsive transcriptional regulator, highly expressed *in vivo* and required for the *Lm* infectious process. The results presented in this thesis contributed to an improved understanding on new *Lm* strategies to evade the host immune system and promote bacterial survival. We characterized the *Lm* cadmium efflux system and demonstrated the role of CadC in *Lm* immune evasion and pathogenicity (Figure 14).

The locus *cadA-lspB-cadC* has an average of GC (guanine-cytosine) content notably lower than that of surrounding regions, suggesting that *Lm* could have acquired this locus by horizontal gene transfer. This leads us to think that *Lm* incorporated this locus for a specific reason rather than only controls metal homeostasis. Indeed, we found that *Lm* acquired this locus, in part, to manipulate host innate immune responses to its own profit.

The expression of *Lm* virulence determinants requires a tight and coordinated regulation. PrfA is the major transcriptional regulator controlling the expression of several genes whose products play a critical role in different steps of *Lm* pathogenesis. Additionally, $\sigma^B$ regulates several genes that are predicted to be important in stress tolerance, carbohydrate metabolism, transport, cell envelope processes and virulence. This work largely contributes to describe CadC as a key regulator of metal homeostasis and virulence. By regulating, directly or indirectly specific *Lm* genes, CadC is so far advantageous for bacterial pathogenesis. We revealed that CadC is a general regulator of gene transcription, working both as a gene repressor and activator. It would be of major relevance to study how the regulation of these genes by CadC is orchestrated and also their impact in virulence.

We unveil that *Lm* controls lipoprotein localization via CadC-dependent *lspB* regulation. During infection, this process minimizes lipoprotein exposure to the host immune system, diminishing inflammatory cytokine expression and promoting intramacrophagic survival and infection (Figure 14).
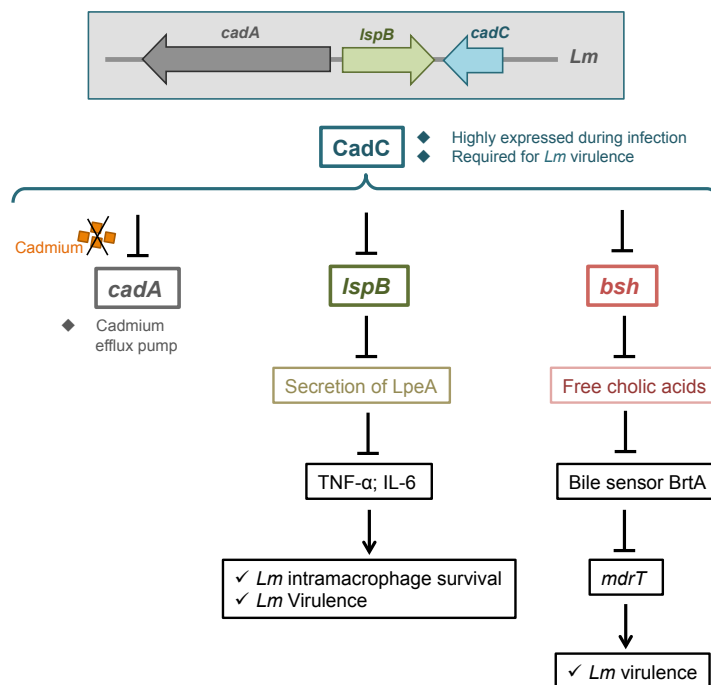
**Fig. 14.** The unexpected role for CadC in the fine control of the expression of *Lm* virulence factors. In the absence of cadmium, *Lm* CadC represses the expression of *cadA*, which encodes a cadmium efflux pump. Moreover, CadC also represses the expression of *lspB* to avoid the exposure of LpeA, therefore inhibiting cytokine expression and promoting intramacrophage survival and virulence. CadC also represses *bsh*, which catalyzes the hydrolysis of the conjugated bile salts. The result is the reduction of the free cholic acids that are less sensed by the bile sensor, BrtA, which binds and represses *mdrT* expression thus contributing to *Lm* virulence.

Bacterial lipoproteins contribute to crucial physiological roles, including substrate binding, antibiotic resistance and protein folding. Nevertheless, they also have an impact in virulence-associated processes and immunomodulation, working as PAMPs (Nakayama *et al.* 2012). The lipidation of prelipoproteins in *Lm* is required to promote NF-kβ activation via TLR2 (Machata *et al.* 2008). Group B Streptococcus secretes lipoproteins that activate host inflammatory response through TLR2 signalling. In this case, the absence of *lsp* leads to decreased TLR2-mediated recognition, reduced inflammatory response and increased mortality (Henneke *et al.* 2008). The lipoprotein Blr of GBS was shown to prevent SR-A-mediated recognition and non-opsonic phagocytosis, indicating that lipoproteins can be ligands for SRs (Areschoug *et al.* 2009). In our case, *Lm* controls lipoprotein localization through *lspB* repression to minimize exposure to the host immune system, and STAB-1 appears crucial for the activation of the innate immune response upon *Lm* infection. It would be thus interesting to analyze the potential role of STAB-1 in the activation of host defences through lipoprotein recognition.

CadC is able to control BSH activity and *Lm* resistance to bile through the repression of *bsh* expression. The optimal CadC-dependent regulation of *bsh* expression is required to confer full *Lm* virulence. Therefore, the uncontrolled expression of *bsh in vivo* induces an overproduction of cholic acid, in turn promoting a stronger *mdrT* expression and restricting

*Lm* virulence. We thus propose that *Lm* uses CadC to repress *bsh* expression during phases of the infection where an over-expression of *bsh* would be deleterious for its virulence (Figure 14). During replication within the cytosol of infected cells, *Lm* uses two multidrug efflux pumps, MdrM and MdrT to secrete the small nucleic acid second messenger cyclic-di-AMP (c-di-AMP). These molecules are sensed by the host cytosolic innate immune receptor STING (Sauer *et al.* 2011), activating a strong type I interferon (IFN-β) response and promoting *Lm* virulence. Importantly, *Lm* activation of STING down-regulates long-lived cell-mediated immunity (Archer *et al.* 2014; Schwartz *et al.* 2012). Moreover, c-di-AMP in Gram-positive live bacteria was considered a *vita*-PAMP, engaging STING to mediate endoplasmic reticulum stress (Moretti *et al.* 2017). The diversity of physiologically relevant substrates transported by MdrT reinforces the need for its tight regulation *in vivo*, in particular through CadC repression.

Thus, searching for new virulence factors could reveal new PAMPs that are recognized by the host to initiate an immune response. On the other side, the study of new PRRs could disclose innovative ways to recognize bacteria. Our findings have expanded the current knowledge on the *Lm* pathogenicity and on the regulation of immune-evasive strategies employed by *Lm*. This points out CadC as a new general repressor repurposed during infection to fine-tune virulence gene expression over the *Lm* infectious process. Interestingly, these transcriptional repressors could represent new targets for innovative antibacterial strategies.

# CHAPTER V: REFERENCES

Abachin, E, Poyart, C, Pellegrini, E, Milohanic, E, Fiedler, F, Berche, P, Trieu-Cuot, P (2002) Formation of D-alanyl-lipoteichoic acid is required for adhesion and virulence of *Listeria monocytogenes*. Molecular microbiology 43: 1-14.

Abdullah, Z, Schlee, M, Roth, S, Mraheil, MA, Barchet, W, Bottcher, J, Hain, T, Geiger, S, Hayakawa, Y, Fritz, JH, Civril, F, Hopfner, KP, Kurts, C, Ruland, J, Hartmann, G, Chakraborty, T, Knolle, PA (2012) RIG-I detects infection with live *Listeria* by sensing secreted bacterial nucleic acids. The EMBO journal 31: 4153-4164.

Abumrad, NA, el-Maghrabi, MR, Amri, EZ, Lopez, E, Grimaldi, PA (1993) Cloning of a rat adipocyte membrane protein implicated in binding or transport of long-chain fatty acids that is induced during preadipocyte differentiation. Homology with human CD36. The Journal of biological chemistry 268: 17665-17668.

Adachi, H, Tsujimoto, M (2002) FEEL-1, a novel scavenger receptor with in vitro bacteria-binding and angiogenesis-modulating activities. The Journal of biological chemistry 277: 34264-34270.

Adachi, H, Tsujimoto, M (2010) Adaptor protein sorting nexin 17 interacts with the scavenger receptor FEEL-1/stabilin-1 and modulates its expression on the cell surface. Biochimica et biophysica acta 1803: 553-563.

Adachi, H, Tsujimoto, M, Arai, H, Inoue, K (1997) Expression cloning of a novel scavenger receptor from human endothelial cells. The Journal of biological chemistry 272: 31217-31220.

Aitman, TJ, Glazier, AM, Wallace, CA, Cooper, LD, Norsworthy, PJ, Wahid, FN, Al-Majali, KM, Trembling, PM, Mann, CJ, Shoulders, CC, Graf, D, St Lezin, E, Kurtz, TW, Kren, V, Pravenec, M, Ibrahimi, A, Abumrad, NA, Stanton, LW, Scott, J (1999) Identification of Cd36 (Fat) as an insulin-resistance gene causing defective fatty acid and glucose metabolism in hypertensive rats. Nature Genetics 21: 76-83.

Akira, S, Uematsu, S, Takeuchi, O (2006) Pathogen recognition and innate immunity. Cell 124: 783-801.

Al-Tawfiq, JA (2008) *Listeria monocytogenes* bacteremia in a twin pregnancy with differential outcome: fetus papyraceus and a full-term delivery. Journal of microbiology, immunology, and infection = Wei mian yu gan ran za zhi 41: 433-436.

Allerberger, F, Wagner, M (2010) Listeriosis: a resurgent foodborne infection. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases 16: 16-23.

Alvarez-Dominguez, C, Roberts, R, Stahl, PD (1997) Internalized *Listeria monocytogenes* modulates intracellular trafficking and delays maturation of the phagosome. J Cell Sci 110 ( Pt 6): 731-743.

Amiel, E, Nicholson-Dykstra, S, Walters, JJ, Higgs, H, Berwin, B (2007) Scavenger receptor-A functions in phagocytosis of *E. coli* by bone marrow dendritic cells. Experimental cell research 313: 1438-1448.

Anand, PK, Malireddi, RK, Lukens, JR, Vogel, P, Bertin, J, Lamkanfi, M, Kanneganti, TD (2012) NLRP6 negatively regulates innate immunity and host defence against bacterial pathogens. Nature 488: 389-393.

Anderson, KV, Bokla, L, Nusslein-Volhard, C (1985) Establishment of dorsal-ventral polarity in the Drosophila embryo: the induction of polarity by the Toll gene product. Cell 42: 791-798.

Archer, KA, Durack, J, Portnoy, DA (2014) STING-dependent type I IFN production inhibits cell-mediated immunity to *Listeria monocytogenes*. PLoS pathogens 10: e1003861.

Areschoug, T, Gordon, S (2008) Pattern recognition receptors and their role in innate immunity: focus on microbial protein ligands. Contributions to microbiology 15: 45-60.

Areschoug, T, Gordon, S (2009) Scavenger receptors: role in innate immunity and microbial pathogenesis. Cellular microbiology 11: 1160-1169.

Arredouani, M, Yang, Z, Ning, Y, Qin, G, Soininen, R, Tryggvason, K, Kobzik, L (2004) The scavenger receptor MARCO is required for lung defense against pneumococcal pneumonia and inhaled particles. The Journal of experimental medicine 200: 267-272.

Arredouani, MS, Yang, Z, Imrich, A, Ning, Y, Qin, G, Kobzik, L (2006) The macrophage scavenger receptor SR-AI/II and lung defense against pneumococci and particles. American journal of respiratory cell and molecular biology 35: 474-478.

Aruffo, A, Bowen, MA, Patel, DD, Haynes, BF, Starling, GC, Gebe, JA, Bajorath, J (1997) CD6-ligand interactions: a paradigm for SRCR domain function? Immunology today 18: 498-504.

Asano, K, Kakizaki, I, Nakane, A (2012) Interaction of *Listeria monocytogenes* autolysin amidase with glycosaminoglycans promotes listerial adhesion to mouse hepatocytes. Biochimie 94: 1291-1299.

Asano, K, Sashinami, H, Osanai, A, Asano, Y, Nakane, A (2011) Autolysin amidase of *Listeria monocytogenes* promotes efficient colonization of mouse hepatocytes and enhances host immune response. Int J Med Microbiol.

Asch, AS, Barnwell, J, Silverstein, RL, Nachman, RL (1987) Isolation of the thrombospondin membrane receptor. The Journal of clinical investigation 79: 1054-1061.

Ashley, JW, Shi, Z, Zhao, H, Li, X, Kesterson, RA, Feng, X (2011) Genetic ablation of CD68 results in mice with increased bone and dysfunctional osteoclasts. PloS one 6: e25838.

Aubry, C, Goulard, C, Nahori, MA, Cayet, N, Decalf, J, Sachse, M, Boneca, IG, Cossart, P, Dussurget, O (2011) OatA, a peptidoglycan O-acetyltransferase involved in *Listeria monocytogenes* immune escape, is critical for virulence. The Journal of infectious diseases 204: 731-740.

Auerbuch, V, Brockstedt, DG, Meyer-Morse, N, O'Riordan, M, Portnoy, DA (2004) Mice lacking the type I interferon receptor are resistant to *Listeria monocytogenes*. The Journal of experimental medicine 200: 527-533.

Baer, MT, Huang, N, Gibson, FC, 3rd (2009) Scavenger receptor A is expressed by macrophages in response to *Porphyromonas gingivalis*, and participates in TNF-alpha expression. Oral microbiology and immunology 24: 456-463.

Bak, SP, Walters, JJ, Takeya, M, Conejo-Garcia, JR, Berwin, BL (2007) Scavenger receptor-A-targeted leukocyte depletion inhibits peritoneal ovarian tumor progression. Cancer research 67: 4783-4789.

Baranova, IN, Kurlander, R, Bocharov, AV, Vishnyakova, TG, Chen, Z, Remaley, AT, Csako, G, Patterson, AP, Eggerman, TL (2008) Role of human CD36 in bacterial recognition, phagocytosis, and pathogen-induced JNK-mediated signaling. Journal of immunology 181: 7147-7156.

Baranova, IN, Vishnyakova, TG, Bocharov, AV, Leelahavanichkul, A, Kurlander, R, Chen, Z, Souza, AC, Yuen, PS, Star, RA, Csako, G, Patterson, AP, Eggerman, TL (2012) Class B scavenger receptor types I and II and CD36 mediate bacterial recognition and proinflammatory signaling induced by *Escherichia coli*, lipopolysaccharide, and cytosolic chaperonin 60. Journal of immunology 188: 1371-1380.

Barth, H, Schnober, EK, Neumann-Haefelin, C, Thumann, C, Zeisel, MB, Diepolder, HM, Hu, Z, Liang, TJ, Blum, HE, Thimme, R, Lambotin, M, Baumert, TF (2008) Scavenger receptor class B is required for hepatitis C virus uptake and cross-presentation by human dendritic cells. Journal of virology 82: 3466-3479.

Basset, C, Holton, J, O'Mahony, R, Roitt, I (2003) Innate immunity and pathogen-host interaction. Vaccine 21 Suppl 2: S12-23.

Baur, S, Rautenberg, M, Faulstich, M, Grau, T, Severin, Y, Unger, C, Hoffmann, WH, Rudel, T, Autenrieth, IB, Weidenmaier, C (2014) A nasal epithelial receptor for *Staphylococcus aureus* WTA governs adhesion to epithelial cells and modulates nasal colonization. PLoS pathogens 10: e1004089.

Beauvillain, C, Meloni, F, Sirard, JC, Blanchard, S, Jarry, U, Scotet, M, Magistrelli, G, Delneste, Y, Barnaba, V, Jeannin, P (2010) The scavenger receptors SRA-1 and SREC-I cooperate with TLR2 in the recognition of the hepatitis C virus non-structural protein 3 by dendritic cells. Journal of hepatology 52: 644-651.

Becker, M, Cotena, A, Gordon, S, Platt, N (2006) Expression of the class A macrophage scavenger receptor on specific subpopulations of murine dendritic cells limits their endotoxin response. European journal of immunology 36: 950-960.

Benard, EL, Roobol, SJ, Spaink, HP, Meijer, AH (2014) Phagocytosis of *mycobacteria* by zebrafish macrophages is dependent on the scavenger receptor Marco, a key control factor of pro-inflammatory signalling. Developmental and comparative immunology 47: 223-233.

Bertsch, D, Rau, J, Eugster, MR, Haug, MC, Lawson, PA, Lacroix, C, Meile, L (2013) *Listeria fleischmannii* sp. nov., isolated from cheese. International journal of systematic and evolutionary microbiology 63: 526-532.

Bessa Pereira, C, Bockova, M, Santos, RF, Santos, AM, Martins de Araujo, M, Oliveira, L, Homola, J, Carmo, AM (2016) The Scavenger Receptor SSc5D Physically Interacts with Bacteria through the SRCR-Containing N-Terminal Domain. Frontiers in immunology 7: 416.

Bierne, H, Mazmanian, SK, Trost, M, Pucciarelli, MG, Liu, G, Dehoux, P, Jansch, L, Garcia-del Portillo, F, Schneewind, O, Cossart, P (2002) Inactivation of the srtA gene in *Listeria monocytogenes* inhibits anchoring of surface proteins and affects virulence. Molecular microbiology 43: 869-881.

Birmingham, CL, Higgins, DE, Brumell, JH (2008a) Avoiding death by autophagy. Autophagy 4: 368-371.

Birmingham, CL, Canadien, V, Kaniuk, NA, Steinberg, BE, Higgins, DE, Brumell, JH (2008b) Listeriolysin O allows *Listeria monocytogenes* replication in macrophage vacuoles. Nature 451: 350-U313.

Bleriot, C, Dupuis, T, Jouvion, G, Eberl, G, Disson, O, Lecuit, M (2015) Liver-resident macrophage necroptosis orchestrates type 1 microbicidal inflammation and type-2-mediated tissue repair during bacterial infection. Immunity 42: 145-158.

Bodet, C, Chandad, F, Grenier, D (2007) [Pathogenic potential of *Porphyromonas gingivalis, Treponema denticola* and *Tannerella forsythia*, the red bacterial complex associated with periodontitis]. Pathologie-biologie 55: 154-162.

Boneca, IG, Dussurget, O, Cabanes, D, Nahori, MA, Sousa, S, Lecuit, M, Psylinakis, E, Bouriotis, V, Hugot, JP, Giovannini, M, Coyle, A, Bertin, J, Namane, A, Rousselle, JC, Cayet, N, Prevost, MC, Balloy, V, Chignard, M, Philpott, DJ, Cossart, P, Girardin, SE (2007) A critical role for peptidoglycan N-deacetylation in *Listeria* evasion from the host innate immune system. Proceedings of the National Academy of Sciences of the United States of America 104: 997-1002.

Booth, JL, Umstead, TM, Hu, S, Dybvig, KF, Cooper, TK, Wilson, RP, Chroneos, ZC (2014) Housing conditions modulate the severity of *Mycoplasma pulmonis* infection in mice deficient in class A scavenger receptor. Comparative medicine 64: 424-439.

Bowdish, DM, Sakamoto, K, Kim, MJ, Kroos, M, Mukhopadhyay, S, Leifer, CA, Tryggvason, K, Gordon, S, Russell, DG (2009) MARCO, TLR2, and CD14 are required for macrophage cytokine responses to mycobacterial trehalose dimycolate and *Mycobacterium tuberculosis*. PLoS pathogens 5: e1000474.

Braun, BJ, Slowik, A, Leib, SL, Lucius, R, Varoga, D, Wruck, CJ, Jansen, S, Podschun, R, Pufe, T, Brandenburg, LO (2011) The formyl peptide receptor like-1 and scavenger receptor MARCO are involved in glial cell activation in bacterial meningitis. Journal of neuroinflammation 8: 11.

Braun, L, Dramsi, S, Dehoux, P, Bierne, H, Lindahl, G, Cossart, P (1997) InlB: an invasion protein of *Listeria monocytogenes* with a novel type of surface association. Molecular microbiology 25: 285-294.

Bridges, CB, Harper, SA, Fukuda, K, Uyeki, TM, Cox, NJ, Singleton, JA (2003) Prevention and control of *influenza*. Recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Recommendations and reports : Morbidity and mortality weekly report Recommendations and reports 52: 1-34; quiz CE31-34.

Brinkmann, V, Reichard, U, Goosmann, C, Fauler, B, Uhlemann, Y, Weiss, DS, Weinrauch, Y, Zychlinsky, A (2004) Neutrophil extracellular traps kill bacteria. Science 303: 1532-1535.

Brodsky, IE, Medzhitov, R (2009) Targeting of immune signalling networks by bacterial pathogens. Nature cell biology 11: 521-526.

Brown, GD (2006) Dectin-1: a signalling non-TLR pattern-recognition receptor. Nature reviews Immunology 6: 33-43.

Brown, RL, Clarke, TB (2017) The regulation of host defences to infection by the microbiota. Immunology 150: 1-6.

Buchrieser, C (2007) Biodiversity of the species *Listeria monocytogenes* and the genus Listeria. Microbes Infect 9: 1147-1155.

Buchrieser, C, Rusniok, C, Garrido, P, Hain, T, Scortti, M, Lampidis, R, Karst, U, Chakraborty, T, Cossart, P, Kreft, J, Vazquez-Boland, JA, Goebel, W, Glaser, P (2011) Complete Genome Sequence of the Animal Pathogen *Listeria ivanovii*, Which Provides Insights into Host Specificities and Evolution of the Genus Listeria. Journal of Bacteriology 193: 6787-6788.

Burkholder, KM, Bhunia, AK (2010) *Listeria monocytogenes* uses Listeria adhesion protein (LAP) to promote bacterial transepithelial translocation and induces expression of LAP receptor Hsp60. Infection and immunity 78: 5062-5073.

Cabanes, D, Dussurget, O, Dehoux, P, Cossart, P (2004) Auto, a surface associated autolysin of *Listeria monocytogenes* required for entry into eukaryotic cells and virulence. Molecular microbiology 51: 1601-1614.

Cabanes, D, Dehoux, P, Dussurget, O, Frangeul, L, Cossart, P (2002) Surface proteins and the pathogenic potential of *Listeria monocytogenes*. Trends Microbiol 10: 238-245.

Cabanes, D, Sousa, S, Cebria, A, Lecuit, M, Garcia-del Portillo, F, Cossart, P (2005) Gp96 is a receptor for a novel *Listeria monocytogenes* virulence factor, Vip, a surface protein. Embo J 24: 2827-2838.

Calame, DG, Mueller-Ortiz, SL, Wetsel, RA (2016) Innate and adaptive immunologic functions of complement in the host response to *Listeria monocytogenes* infection. Immunobiology 221: 1407-1417.

Camejo, A, Carvalho, F, Reis, O, Leitao, E, Sousa, S, Cabanes, D (2011) The arsenal of virulence factors deployed by *Listeria monocytogenes* to promote its cell infection cycle. Virulence 2: 379-394.

Camejo, A, Buchrieser, C, Couve, E, Carvalho, F, Reis, O, Ferreira, P, Sousa, S, Cossart, P, Cabanes, D (2009) In vivo transcriptional profiling of *Listeria monocytogenes* and mutagenesis identify new virulence factors involved in infection. PLoS Pathog 5: e1000449.

Campbell, LA, Lee, AW, Rosenfeld, ME, Kuo, CC (2013) *Chlamydia pneumoniae* induces expression of pro-atherogenic factors through activation of the lectin-like oxidized LDL receptor-1. Pathogens and disease 69: 1-6.

Canton, J, Neculai, D, Grinstein, S (2013) Scavenger receptors in homeostasis and immunity. Nature reviews Immunology 13: 621-634.

Carlsson, F, Sandin, C, Lindahl, G (2005) Human fibrinogen bound to *Streptococcus pyogenes* M protein inhibits complement deposition via the classical pathway. Molecular microbiology 56: 28-39.

Carr, KD, Sieve, AN, Indramohan, M, Break, TJ, Lee, S, Berg, RE (2011) Specific depletion reveals a novel role for neutrophil-mediated protection in the liver during *Listeria monocytogenes* infection. European journal of immunology 41: 2666-2676.

Carrero, JA, Unanue, ER (2012) Mechanisms and immunological effects of apoptosis caused by *Listeria monocytogenes*. Advances in immunology 113: 157-174.

Carrero, JA, Calderon, B, Unanue, ER (2006) Lymphocytes are detrimental during the early innate immune response against *Listeria monocytogenes*. The Journal of experimental medicine 203: 933-940.

Castleman, MJ, Febbraio, M, Hall, PR (2015) CD36 Is Essential for Regulation of the Host Innate Response to *Staphylococcus aureus* alpha-Toxin-Mediated Dermonecrosis. Journal of immunology 195: 2294-2302.

Cha, SJ, Park, K, Srinivasan, P, Schindler, CW, van Rooijen, N, Stins, M, Jacobs-Lorena, M (2015) CD68 acts as a major gateway for malaria sporozoite liver infection. The Journal of experimental medicine 212: 1391-1403.

Chaemchuen, S, Rungpragayphan, S, Poovorawan, Y, Patarakul, K (2011) Identification of candidate host proteins that interact with LipL32, the major outer membrane protein of pathogenic *Leptospira*, by random phage display peptide library. Veterinary microbiology 153: 178-185.

Chatterjee, SS, Hossain, H, Otten, S, Kuenne, C, Kuchmina, K, Machata, S, Domann, E, Chakraborty, T, Hain, T (2006) Intracellular gene expression profile of *Listeria monocytogenes*. Infection and immunity 74: 1323-1338.

Chaturongakul, S, Raengpradub, S, Wiedmann, M, Boor, KJ (2008) Modulation of stress and virulence in *Listeria monocytogenes*. Trends in microbiology 16: 388-396.

Chen, Y, Wermeling, F, Sundqvist, J, Jonsson, AB, Tryggvason, K, Pikkarainen, T, Karlsson, MC (2010) A regulatory role for macrophage class A scavenger receptors in TLR4-mediated LPS responses. European journal of immunology 40: 1451-1460.

Chico-Calero, I, Suarez, M, Gonzalez-Zorn, B, Scortti, M, Slaghuis, J, Goebel, W, Vazquez-Boland, JA (2002) Hpt, a bacterial homolog of the microsomal glucose- 6-phosphate translocase, mediates rapid intracellular proliferation in *Listeria*. Proc Natl Acad Sci U S A 99: 431-436.

Chirathaworn, C, Pongpanich, A, Poovorawan, Y (2004) Herpes simplex virus 1 induced LOX-1 expression in an endothelial cell line, ECV 304. Viral immunology 17: 308-314.

Chu, Y, Li, J, Wu, X, Hua, Z, Wu, Z (2013) Identification of human immunodeficiency virus type 1 (HIV-1) gp120-binding sites on scavenger receptor cysteine rich 1 (SRCR1) domain of gp340. Journal of biomedical science 20: 44.

Chung, H, Brazil, MI, Irizarry, MC, Hyman, BT, Maxfield, FR (2001) Uptake of fibrillar beta-amyloid by microglia isolated from MSR-A (type I and type II) knockout mice. Neuroreport 12: 1151-1154.

Conlan, JW, North, RJ (1991) Neutrophil-Mediated Dissolution of Infected Host-Cells as a Defense Strategy against a Facultative Intracellular Bacterium. Journal of Experimental Medicine 174: 741-744.

Conlan, JW, North, RJ (1994) Neutrophils Are Essential for Early Anti-*Listeria* Defense in the Liver, but Not in the Spleen or Peritoneal-Cavity, as Revealed by a Granulocyte-Depleting Monoclonal-Antibody. Journal of Experimental Medicine 179: 259-268.

Coort, SL, Willems, J, Coumans, WA, van der Vusse, GJ, Bonen, A, Glatz, JF, Luiken, JJ (2002) Sulfo-N-succinimidyl esters of long chain fatty acids specifically inhibit fatty acid translocase (FAT/CD36)-mediated cellular fatty acid uptake. Molecular and cellular biochemistry 239: 213-219.

Cossart, P (2001) Met, the HGF-SF receptor: another receptor for *Listeria monocytogenes*. Trends Microbiol 9: 105-107.

Cossart, P (2007) Listeriology (1926-2007): the rise of a model pathogen. Microbes Infect 9: 1143-1146.

Cossart, P (2011) Illuminating the landscape of host-pathogen interactions with the bacterium *Listeria monocytogenes*. Proceedings of the National Academy of Sciences of the United States of America 108: 19484-19491.

Cossart, P, Toledo-Arana, A (2008) *Listeria monocytogenes*, a unique model in infection biology: an overview. Microbes Infect 10: 1041-1050.

Cossart, P, Pizarro-Cerda, J, Lecuit, M (2003) Invasion of mammalian cells by *Listeria monocytogenes*: functional mimicry to subvert cellular functions. Trends Cell Biol 13: 23-31.

Cousens, LP, Wing, EJ (2000) Innate defenses in the Liver during *Listeria* infection. Immunological Reviews 174: 150-159.

Czuczman, MA, Fattouh, R, van Rijn, JM, Canadien, V, Osborne, S, Muise, AM, Kuchroo, VK, Higgins, DE, Brumell, JH (2014) *Listeria monocytogenes* exploits efferocytosis to promote cell-to-cell spread. Nature 509: 230-234.

da Silva, RP, Gordon, S (1999) Phagocytosis stimulates alternative glycosylation of macrosialin (mouse CD68), a macrophage-specific endosomal protein. The Biochemical journal 338 ( Pt 3): 687-694.

Dalet, K, Gouin, E, Cenatiempo, Y, Cossart, P, Hechard, Y (1999) Characterisation of a new operon encoding a Zur-like protein and an associated ABC zinc permease in *Listeria monocytogenes*. FEMS microbiology letters 174: 111-116.

David, DJV, Cossart, P (2017) Recent advances in understanding *Listeria monocytogenes* infection: the importance of subcellular and physiological context. F1000Research 6.

de las Heras, A, Cain, RJ, Bielecka, MK, Vazquez-Boland, JA (2011) Regulation of *Listeria* virulence: PrfA master and commander. Curr Opin Microbiol 14: 118-127.

de Noordhout, CM, Devleesschauwer, B, Angulo, FJ, Verbeke, G, Haagsma, J, Kirk, M, Havelaar, A, Speybroeck, N (2014) The global burden of listeriosis: a systematic review and meta-analysis. The Lancet Infectious diseases 14: 1073-1082.

Den Bakker, HC, Warchocki, S, Wright, EM, Allred, AF, Ahlstrom, C, Manuel, CS, Stasiewicz, MJ, Burrell, A, Roof, S, Strawn, LK, Fortes, E, Nightingale, KK, Kephart, D, Wiedmann, M (2014) *Listeria floridensis sp. nov., Listeria aquatica sp. nov., Listeria cornellensis sp. nov., Listeria riparia sp. nov.* and *Listeria grandensis sp. nov.*, from agricultural and natural environments. International journal of systematic and evolutionary microbiology 64: 1882-1889.

Denny, J, McLauchlin, J (2008) Human *Listeria monocytogenes* infections in Europe--an opportunity for improved European surveillance. Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin 13.

Dewamitta, SR, Nomura, T, Kawamura, I, Hara, H, Tsuchiya, K, Kurenuma, T, Shen, Y, Daim, S, Yamamoto, T, Qu, H, Sakai, S, Xu, Y, Mitsuyama, M (2010) Listeriolysin O-dependent bacterial entry into the cytoplasm is required for calpain activation and interleukin-1 alpha secretion in macrophages infected with *Listeria monocytogenes*. Infection and immunity 78: 1884-1894.

DeWitte-Orr, SJ, Collins, SE, Bauer, CM, Bowdish, DM, Mossman, KL (2010) An accessory to the 'Trinity': SR-As are essential pathogen sensors of extracellular dsRNA, mediating entry and leading to subsequent type I IFN responses. PLoS pathogens 6: e1000829.

Dhar, G, Faull, KF, Schneewind, O (2000) Anchor structure of cell wall surface proteins in *Listeria monocytogenes*. Biochemistry 39: 3725-3733.

Dolowschiak, T, Chassin, C, Ben Mkaddem, S, Fuchs, TM, Weiss, S, Vandewalle, A, Hornef, MW (2010) Potentiation of epithelial innate host responses by intercellular communication. PLoS pathogens 6: e1001194.

Domann, E, Wehland, J, Rohde, M, Pistor, S, Hartl, M, Goebel, W, Leimeister-Wachter, M, Wuenscher, M, Chakraborty, T (1992) A novel bacterial virulence gene in *Listeria monocytogenes* required for host cell microfilament interaction with homology to the proline-rich region of vinculin. The EMBO journal 11: 1981-1990.

Dons, L, Eriksson, E, Jin, Y, Rottenberg, ME, Kristensson, K, Larsen, CN, Bresciani, J, Olsen, JE (2004) Role of flagellin and the two-component CheA/CheY system of *Listeria monocytogenes* in host cell invasion and virulence. Infection and immunity 72: 3237-3244.

Dorrington, MG, Roche, AM, Chauvin, SE, Tu, Z, Mossman, KL, Weiser, JN, Bowdish, DM (2013) MARCO is required for TLR2- and Nod2-mediated responses to *Streptococcus pneumoniae* and clearance of pneumococcal colonization in the murine nasopharynx. Journal of immunology 190: 250-258.

Dramsi, S, Cossart, P (2003) Listeriolysin O-mediated calcium influx potentiates entry of *Listeria monocytogenes* into the human Hep-2 epithelial cell line. Infection and immunity 71: 3614-3618.

Dramsi, S, Dehoux, P, Lebrun, M, Goossens, PL, Cossart, P (1997) Identification of four new members of the internalin multigene family of *Listeria monocytogenes* EGD. Infection and immunity 65: 1615-1625.

Dramsi, S, Biswas, I, Maguin, E, Braun, L, Mastroeni, P, Cossart, P (1995) Entry of *Listeria monocytogenes* into hepatocytes requires expression of inIB, a surface protein of the internalin multigene family. Molecular microbiology 16: 251-261.

Dramsi, S, Bourdichon, F, Cabanes, D, Lecuit, M, Fsihi, H, Cossart, P (2004) FbpA, a novel multifunctional *Listeria monocytogenes* virulence factor. Molecular microbiology 53: 639-649.

Dranoff, G (2004) Cytokines in cancer pathogenesis and cancer therapy. Nature Reviews Cancer 4: 11-22.

Drevets, DA, Campbell, PA (1991) Roles of complement and complement receptor type 3 in phagocytosis of *Listeria monocytogenes* by inflammatory mouse peritoneal macrophages. Infection and immunity 59: 2645-2652.

Drummond, RA, Brown, GD (2013) Signalling C-type lectins in antimicrobial immunity. PLoS pathogens 9: e1003417.

Dunne, DW, Resnick, D, Greenberg, J, Krieger, M, Joiner, KA (1994) The type I macrophage scavenger receptor binds to gram-positive bacteria and recognizes lipoteichoic acid. Proceedings of the National Academy of Sciences of the United States of America 91: 1863-1867.

Dussurget, O (2008) New insights into determinants of *Listeria monocytogenes* virulence. Int Rev Cell Mol Biol 270: 1-38.

Dussurget, O, Pizarro-Cerda, J, Cossart, P (2004) Molecular determinants of *Listeria monocytogenes* virulence. Annu Rev Microbiol 58: 587-610.

Dussurget, O, Dumas, E, Archambaud, C, Chafsey, I, Chambon, C, Hebraud, M, Cossart, P (2005) *Listeria monocytogenes* ferritin protects against multiple stresses and is required for virulence. FEMS microbiology letters 250: 253-261.

ECDC (2016) Listeriosis - Annual Epidemiological Report 2016.

Eckhardt, ER, Cai, L, Sun, B, Webb, NR, van der Westhuyzen, DR (2004) High density lipoprotein uptake by scavenger receptor SR-BII. The Journal of biological chemistry 279: 14372-14381.

Edelson, BT, Unanue, ER (2002) MyD88-dependent but Toll-like receptor 2-independent innate immunity to *Listeria*: No role for either in macrophage listericidal activity. Journal of immunology 169: 3869-3875.

EFSA (2015) The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2014. 4 p.

Eitel, J, Suttorp, N, Opitz, B (2010) Innate immune recognition and inflammasome activation in *listeria monocytogenes* infection. Frontiers in microbiology 1: 149.

El Khoury, JB, Moore, KJ, Means, TK, Leung, J, Terada, K, Toft, M, Freeman, MW, Luster, AD (2003) CD36 mediates the innate host response to beta-amyloid. The Journal of experimental medicine 197: 1657-1666.

Elomaa, O, Kangas, M, Sahlberg, C, Tuukkanen, J, Sormunen, R, Liakka, A, Thesleff, I, Kraal, G, Tryggvason, K (1995) Cloning of a novel bacteria-binding receptor structurally related to scavenger receptors and expressed in a subset of macrophages. Cell 80: 603-609.

Endo, G, Silver, S (1995) CadC, the transcriptional regulatory protein of the cadmium resistance system of *Staphylococcus aureus* plasmid pI258. Journal of bacteriology 177: 4437-4441.

Etzerodt, A, Maniecki, MB, Moller, K, Moller, HJ, Moestrup, SK (2010) Tumor necrosis factor alpha-converting enzyme (TACE/ADAM17) mediates ectodomain shedding of the scavenger receptor CD163. Journal of leukocyte biology 88: 1201-1205.

Fabriek, BO, van Bruggen, R, Deng, DM, Ligtenberg, AJ, Nazmi, K, Schornagel, K, Vloet, RP, Dijkstra, CD, van den Berg, TK (2009) The macrophage scavenger receptor CD163 functions as an innate immune sensor for bacteria. Blood 113: 887-892.

Fan, H, Qiao, L, Kang, KD, Fan, J, Wei, W, Luo, G (2017) Attachment and Post-Attachment Receptors Important for Hepatitis C Virus Infection and Cell-to-Cell Transmission. Journal of virology.

Fattouh, R, Kwon, H, Czuczman, MA, Copeland, JW, Pelletier, L, Quinlan, ME, Muise, AM, Higgins, DE, Brumell, JH (2015) The diaphanous-related formins promote protrusion formation and cell-to-cell spread of *Listeria monocytogenes*. The Journal of infectious diseases 211: 1185-1195.

Feng, H, Zhang, D, Palliser, D, Zhu, P, Cai, S, Schlesinger, A, Maliszewski, L, Lieberman, J (2005) *Listeria*-infected myeloid dendritic cells produce IFN-beta, priming T cell activation. Journal of immunology 175: 421-432.

Flor, HH (1942) Inheritance of pathogenicity in Melampsora lini. Phytopathology 32: 653-669.

Francis, MS, Thomas, CJ (1997) Mutants in the CtpA copper transporting P-type ATPase reduce virulence of *Listeria monocytogenes*. Microbial pathogenesis 22: 67-78.

Francis, MS, Thomas, CJ (1997) The *Listeria monocytogenes* gene ctpA encodes a putative P-type ATPase involved in copper transport. Molecular & general genetics : MGG 253: 484-491.

Fraser, IP, Koziel, H, Ezekowitz, RAB (1998) The serum mannose-binding protein and the macrophage mannose receptor are pattern recognition molecules that link innate and adaptive immunity. Seminars in Immunology 10: 363-372.

Freitag, NE, Port, GC, Miner, MD (2009) *Listeria monocytogenes* - from saprophyte to intracellular pathogen. Nat Rev Microbiol 7: 623-628.

Gaillard, JL, Berche, P, Sansonetti, P (1986) Transposon mutagenesis as a tool to study the role of hemolysin in the virulence of *Listeria monocytogenes*. Infection and immunity 52: 50-55.

Gaillard, JL, Berche, P, Mounier, J, Richard, S, Sansonetti, P (1987) In vitro model of penetration and intracellular growth of *Listeria monocytogenes* in the human enterocyte-like cell line Caco-2. Infection and immunity 55: 2822-2829.

Gaillard, JL, Berche, P, Frehel, C, Gouin, E, Cossart, P (1991) Entry of *L. monocytogenes* into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from gram-positive cocci. Cell 65: 1127-1141.

Gandhi, M, Chikindas, ML (2007) *Listeria*: A foodborne pathogen that knows how to survive. Int J Food Microbiol 113: 1-15.

Gao, X, Zhao, G, Li, C, Lin, J, Jiang, N, Wang, Q, Hu, L, Xu, Q, Peng, X, He, K, Zhu, G (2016) LOX-1 and TLR4 affect each other and regulate the generation of ROS in *A. fumigatus* keratitis. International immunopharmacology 40: 392-399.

Gebe, JA, Llewellyn, M, Hoggatt, H, Aruffo, A (2000) Molecular cloning, genomic organization and cell-binding characteristics of mouse Spalpha. Immunology 99: 78-86.

Gebe, JA, Kiener, PA, Ring, HZ, Li, X, Francke, U, Aruffo, A (1997) Molecular cloning, mapping to human chromosome 1 q21-q23, and cell binding characteristics of Spalpha, a new member of the scavenger receptor cysteine-rich (SRCR) family of proteins. The Journal of biological chemistry 272: 6151-6158.

Geijtenbeek, TBH, Torensma, R, van Vliet, SJ, van Duijnhoven, GCF, Adema, GJ, van Kooyk, Y, Figdor, CG (2000) Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. Cell 100: 575-585.

Ghosh, S, Gregory, D, Smith, A, Kobzik, L (2011) MARCO regulates early inflammatory responses against *influenza*: a useful macrophage function with adverse outcome. American journal of respiratory cell and molecular biology 45: 1036-1044.

Glaser, P, Frangeul, L, Buchrieser, C, Rusniok, C, Amend, A, Baquero, F, Berche, P, Bloecker, H, Brandt, P, Chakraborty, T, Charbit, A, Chetouani, F, Couve, E, de Daruvar, A, Dehoux, P, Domann, E, Dominguez-Bernal, G, Duchaud, E, Durant, L, Dussurget, O, Entian, KD, Fsihi, H, Garcia-del Portillo, F, Garrido, P, Gautier, L, Goebel, W, Gomez-Lopez, N, Hain, T, Hauf, J, Jackson, D, Jones, LM, Kaerst, U, Kreft, J, Kuhn, M, Kunst, F, Kurapkat, G, Madueno, E, Maitournam, A, Vicente, JM, Ng, E, Nedjari, H, Nordsiek, G, Novella, S, de Pablos, B, Perez-Diaz, JC, Purcell, R, Remmel, B, Rose, M, Schlueter, T, Simoes, N, Tierrez, A, Vazquez-Boland, JA, Voss, H, Wehland, J, Cossart, P (2001) Comparative genomics of *Listeria* species. Science 294: 849-852.

Goerdt, S, Walsh, LJ, Murphy, GF, Pober, JS (1991) Identification of a novel high molecular weight protein preferentially expressed by sinusoidal endothelial cells in normal human tissues. The Journal of cell biology 113: 1425-1437.

Gomes, IN, Palma, LC, Campos, GO, Lima, JG, TF, DEA, JP, DEM, Ferreira, CA, Santos, RR, Buck, GA, Manque, PA, Ozaki, LS, Probst, CM, LA, DEF, Krieger, MA, Veras, PS (2009) The scavenger receptor MARCO is involved in *Leishmania major* infection by CBA/J macrophages. Parasite immunology 31: 188-198.

Gong, J, Zhu, B, Murshid, A, Adachi, H, Song, B, Lee, A, Liu, C, Calderwood, SK (2009) T cell activation by heat shock protein 70 vaccine requires TLR signaling and scavenger receptor expressed by endothelial cells-1. Journal of immunology 183: 3092-3098.

Gordon, S (2002) Pattern recognition receptors: Doubling up for the innate immune response. Cell 111: 927-930.

Goulet, V, Hedberg, C, Le Monnier, A, de Valk, H (2008) Increasing incidence of listeriosis in France and other European countries. Emerging infectious diseases 14: 734-740.

Gowdy, KM, Madenspacher, JH, Azzam, KM, Gabor, KA, Janardhan, KS, Aloor, JJ, Fessler, MB (2015) Key role for scavenger receptor B-I in the integrative physiology of host defense during bacterial pneumonia. Mucosal immunology 8: 559-571.

Gowen, BB, Borg, TK, Ghaffar, A, Mayer, EP (2001) The collagenous domain of class A scavenger receptors is involved in macrophage adhesion to collagens. Journal of leukocyte biology 69: 575-582.

Granucci, F, Petralia, F, Urbano, M, Citterio, S, Di Tota, F, Santambrogio, L, Ricciardi-Castagnoli, P (2003) The scavenger receptor MARCO mediates cytoskeleton rearrangements in dendritic cells and microglia. Blood 102: 2940-2947.

Graves, LM, Helsel, LO, Steigerwalt, AG, Morey, RE, Daneshvar, MI, Roof, SE, Orsi, RH, Fortes, ED, Milillo, SR, den Bakker, HC, Wiedmann, M, Swaminathan, B, Sauders, BD (2010) *Listeria marthii sp. nov*., isolated from the natural environment, Finger Lakes National Forest. International journal of systematic and evolutionary microbiology 60: 1280-1288.

Greiffenberg, L, Sokolovic, Z, Schnittler, HJ, Spory, A, Bockmann, R, Goebel, W, Kuhn, M (1997) *Listeria monocytogenes*-infected human umbilical vein endothelial cells: internalin-independent invasion, intracellular growth, movement, and host cell responses. FEMS microbiology letters 157: 163-170.

Gronlund, J, Vitved, L, Lausen, M, Skjodt, K, Holmskov, U (2000) Cloning of a novel scavenger receptor cysteine-rich type I transmembrane molecule (M160) expressed by human macrophages. Journal of immunology 165: 6406-6415.

Guo, H, Callaway, JB, Ting, JP (2015) Inflammasomes: mechanism of action, role in disease, and therapeutics. Nature medicine 21: 677-687.

Guo, L, Chen, M, Song, ZQ, Daugherty, A, Li, XA (2011) C323 of SR-BI is required for SR-BI-mediated HDL binding and cholesteryl ester uptake. Journal of lipid research 52: 2272-2278.

Gutwein, P, Abdel-Bakky, MS, Schramme, A, Doberstein, K, Kampfer-Kolb, N, Amann, K, Hauser, IA, Obermuller, N, Bartel, C, Abdel-Aziz, AA, El Sayed el, SM, Pfeilschifter, J (2009) CXCL16 is expressed in podocytes and acts as a scavenger receptor for oxidized low-density lipoprotein. The American journal of pathology 174: 2061-2072.

Hain, T, Hossain, H, Chatterjee, SS, Machata, S, Volk, U, Wagner, S, Brors, B, Haas, S, Kuenne, CT, Billion, A, Otten, S, Pane-Farre, J, Engelmann, S, Chakraborty, T (2008) Temporal transcriptomic analysis of the *Listeria monocytogenes* EGD-e sigmaB regulon. BMC Microbiol 8: 20.

Hain, T, Steinweg, C, Kuenne, CT, Billion, A, Ghai, R, Chatterjee, SS, Domann, E, Karst, U, Goesmann, A, Bekel, T, Bartels, D, Kaiser, O, Meyer, F, Puehler, A, Weisshaar, B, Wehland, J, Liang, CG, Dandekar, T, Lampidis, R, Kreft, J, Goebel, W, Chakraborty, T (2006) Whole-genome sequence of Listeria welshimeri reveals common steps in genome reduction with *Listeria innocua* as compared to *Listeria monocytogenes*. Journal of Bacteriology 188: 7405-7415.

Haisma, HJ, Boesjes, M, Beerens, AM, van der Strate, BW, Curiel, DT, Pluddemann, A, Gordon, S, Bellu, AR (2009) Scavenger receptor A: a new route for adenovirus 5. Molecular pharmaceutics 6: 366-374.

Hamon, MA, Cossart, P (2008) Histone modifications and chromatin remodeling during bacterial infections. Cell host & microbe 4: 100-109.

Hamon, MA, Batsche, E, Regnault, B, Tham, TN, Seveau, S, Muchardt, C, Cossart, P (2007) Histone modifications induced by a family of bacterial toxins. Proceedings of the National Academy of Sciences of the United States of America 104: 13467-13472.

Hampton, RY, Raetz, CR (1991) Macrophage catabolism of lipid A is regulated by endotoxin stimulation. The Journal of biological chemistry 266: 19499-19509.

Hansson, GK, Hermansson, A (2011) The immune system in atherosclerosis. Nature immunology 12: 204-212.

Harty, JT, Bevan, MJ (1995) Specific immunity to *Listeria monocytogenes* in the absence of IFN gamma. Immunity 3: 109-117.

Havell, EA (1987) Production of tumor necrosis factor during murine listeriosis. Journal of immunology 139: 4225-4231.

Hawkes, M, Li, X, Crockett, M, Diassiti, A, Finney, C, Min-Oo, G, Liles, WC, Liu, J, Kain, KC (2010) CD36 deficiency attenuates experimental mycobacterial infection. BMC infectious diseases 10: 299.

Henneke, P, Dramsi, S, Mancuso, G, Chraibi, K, Pellegrini, E, Theilacker, C, Hubner, J, Santos-Sierra, S, Teti, G, Golenbock, DT, Poyart, C, Trieu-Cuot, P (2008) Lipoproteins are critical TLR2 activating toxins in *group B streptococcal* sepsis. Journal of immunology 180: 6149-6158.

Heneka, MT, O'Banion, MK (2007) Inflammatory processes in Alzheimer's disease. Journal of neuroimmunology 184: 69-91.

Hennessy, EJ, Parker, AE, O'Neill, LA (2010) Targeting Toll-like receptors: emerging therapeutics? Nature reviews Drug discovery 9: 293-307.

Hoebe, K, Janssen, E, Beutler, B (2004) The interface between innate and adaptive immunity. Nature immunology 5: 971-974.

Hollifield, M, Bou Ghanem, E, de Villiers, WJ, Garvy, BA (2007) Scavenger receptor A dampens induction of inflammation in response to the fungal pathogen *Pneumocystis carinii*. Infection and immunity 75: 3999-4005.

Holmskov, U, Lawson, P, Teisner, B, Tornoe, I, Willis, AC, Morgan, C, Koch, C, Reid, KBM (1997) Isolation and characterization of a new member of the scavenger receptor superfamily, glycoprotein-340 (gp-340), as a lung surfactant protein-D binding molecule. Journal of Biological Chemistry 272: 13743-13749.

Hopkins, PA, Sriskandan, S (2005) Mammalian Toll-like receptors: to immunity and beyond. Clinical and experimental immunology 140: 395-407.

Hornung, V, Latz, E (2010) Intracellular DNA recognition. Nature Reviews Immunology 10: 123-130.

Hornung, V, Hartmann, R, Ablasser, A, Hopfner, KP (2014) OAS proteins and cGAS: unifying concepts in sensing and responding to cytosolic nucleic acids. Nature Reviews Immunology 14: 521-528.

Hsieh, FL, Turner, L, Bolla, JR, Robinson, CV, Lavstsen, T, Higgins, MK (2016) The structural basis for CD36 binding by the malaria parasite. Nature communications 7: 12837.

Huang, S, Hendriks, W, Althage, A, Hemmi, S, Bluethmann, H, Kamijo, R, Vilcek, J, Zinkernagel, RM, Aguet, M (1993) Immune response in mice that lack the interferon-gamma receptor. Science 259: 1742-1745.

Hughes, DA, Fraser, IP, Gordon, S (1995) Murine macrophage scavenger receptor: in vivo expression and function as receptor for macrophage adhesion in lymphoid and non-lymphoid organs. European journal of immunology 25: 466-473.

Humann, J, Bjordahl, R, Andreasen, K, Lenz, LL (2007) Expression of the p60 autolysin enhances NK cell activation and is required for *listeria monocytogenes* expansion in IFN-gamma-responsive mice. Journal of immunology 178: 2407-2414.

Ibrahim, ZA, Armour, CL, Phipps, S, Sukkar, MB (2013) RAGE and TLRs: relatives, friends or neighbours? Molecular immunology 56: 739-744.

Imaizumi, T, Sashinami, H, Mori, F, Matsumiya, T, Yoshida, H, Nakane, A, Wakabayashi, K, Oyama, C, Satoh, K (2006) *Listeria monocytogenes* induces the expression of retinoic acid-inducible gene-I. Microbiology and immunology 50: 811-815.

Irjala, H, Elima, K, Johansson, EL, Merinen, M, Kontula, K, Alanen, K, Grenman, R, Salmi, M, Jalkanen, S (2003) The same endothelial receptor controls lymphocyte traffic both in vascular and lymphatic vessels. European journal of immunology 33: 815-824.

Isberg, RR, Falkow, S (1985) A single genetic locus encoded by Yersinia pseudotuberculosis permits invasion of cultured animal cells by *Escherichia coli* K-12. Nature 317: 262-264.

Ishiguro, T, Naito, M, Yamamoto, T, Hasegawa, G, Gejyo, F, Mitsuyama, M, Suzuki, H, Kodama, T (2001) Role of macrophage scavenger receptors in response to *Listeria monocytogenes* infection in mice. The American journal of pathology 158: 179-188.

Ishii, J, Adachi, H, Aoki, J, Koizumi, H, Tomita, S, Suzuki, T, Tsujimoto, M, Inoue, K, Arai, H (2002) SREC-II, a new member of the scavenger receptor type F family, trans-interacts with SREG-I through its extracellular domain. Journal of Biological Chemistry 277: 39696-39702.

Ishikawa, Y, Kimura-Matsumoto, M, Murakami, M, Yamamoto, K, Akasaka, Y, Uzuki, M, Yuri, Y, Inomata, N, Yokoo, T, Ishii, T (2009) Distribution of smooth muscle cells and macrophages expressing scavenger receptor BI/II in atherosclerosis. Journal of atherosclerosis and thrombosis 16: 829-839.

Ito, S, Naito, M, Kobayashi, Y, Takatsuka, H, Jiang, S, Usuda, H, Umezu, H, Hasegawa, G, Arakawa, M, Shultz, LD, Elomaa, O, Tryggvason, K (1999) Roles of a macrophage receptor with collagenous structure (MARCO) in host defense and heterogeneity of splenic marginal zone macrophages. Archives of histology and cytology 62: 83-95.

Jaillon, S, Galdiero, MR, Del Prete, D, Cassatella, MA, Garlanda, C, Mantovani, A (2013) Neutrophils in innate and adaptive immunity. Seminars in immunopathology 35: 377-394.

Janeway, C, Medzhitov, R (2002) Innate immune recognition. Annual review of immunology : 197-216.

Janeway, CJ (1989) Approaching the Asymptote? Evolution and Revolution in Immunology. Cold Spring Harb Symp Quant Biol.

Jang, S, Ohtani, K, Fukuoh, A, Yoshizaki, T, Fukuda, M, Motomura, W, Mori, K, Fukuzawa, J, Kitamoto, N, Yoshida, I, Suzuki, Y, Wakamiya, N (2009) Scavenger receptor collectin placenta 1 (CL-P1) predominantly mediates zymosan phagocytosis by human vascular endothelial cells. The Journal of biological chemistry 284: 3956-3965.

Janot, L, Secher, T, Torres, D, Maillet, I, Pfeilschifter, J, Quesniaux, VFJ, Landmann, R, Ryffel, B, Erard, F (2008) CD14 works with toll-like receptor 2 to contribute to recognition and control of *Listeria monocytogenes* infection. Journal of Infectious Diseases 198: 115-124.

Jeannin, P, Bottazzi, B, Sironi, M, Doni, A, Rusnati, M, Presta, M, Maina, V, Magistrelli, G, Haeuw, JF, Hoeffel, G, Thieblemont, N, Corvaia, N, Garlanda, C, Delneste, Y, Mantovani, A (2005) Complexity and complementarity of outer membrane protein A recognition by cellular and humoral innate immunity receptors. Immunity 22: 551-560.

Jiang, Y, Oliver, P, Davies, KE, Platt, N (2006) Identification and characterization of murine SCARA5, a novel class A scavenger receptor that is expressed by populations of epithelial cells. The Journal of biological chemistry 281: 11834-11845.

Johansson, J, Mandin, P, Renzoni, A, Chiaruttini, C, Springer, M, Cossart, P (2002) An RNA thermosensor controls expression of virulence genes in *Listeria monocytogenes*. Cell 110: 551-561.

Junttila, JR, Niemela, SI, Hirn, J (1988) Minimum growth temperatures of *Listeria monocytogenes* and non-haemolytic *Listeria*. The Journal of applied bacteriology 65: 321-327.

Kanneganti, TD, Lamkanfi, M, Nunez, G (2007) Intracellular NOD-like receptors in host Defense and disease. Immunity 27: 549-559.

Karikoski, M, Marttila-Ichihara, F, Elima, K, Rantakari, P, Hollmen, M, Kelkka, T, Gerke, H, Huovinen, V, Irjala, H, Holmdahl, R, Salmi, M, Jalkanen, S (2014) Clever-1/stabilin-1 controls cancer growth and metastasis. Clinical cancer research : an official journal of the American Association for Cancer Research 20: 6452-6464.

Karikoski, M, Irjala, H, Maksimow, M, Miiluniemi, M, Granfors, K, Hernesniemi, S, Elima, K, Moldenhauer, G, Schledzewski, K, Kzhyshkowska, J, Goerdt, S, Salmi, M, Jalkanen, S (2009) Clever-1/Stabilin-1 regulates lymphocyte migration within lymphatics and leukocyte entrance to sites of inflammation. European journal of immunology 39: 3477-3487.

Kataoka, H, Kume, N, Miyamoto, S, Minami, M, Morimoto, M, Hayashida, K, Hashimoto, N, Kita, T (2001) Oxidized LDL modulates Bax/Bcl-2 through the lectinlike Ox-LDL receptor-1 in vascular smooth muscle cells. Arteriosclerosis Thrombosis and Vascular Biology 21: 955-960.

Kato, H, Sato, S, Yoneyama, M, Yamamoto, M, Uematsu, S, Matsui, K, Tsujimura, T, Takeda, K, Fujita, T, Takeuchi, O, Akira, S (2005) Cell type-specific involvement of RIG-I in antiviral response. Immunity 23: 19-28.

Kaufmann, SH (2008) Immunology's foundation: the 100-year anniversary of the Nobel Prize to Paul Ehrlich and Elie Metchnikoff. Nature immunology 9: 705-712.

Kayal, S, Lilienbaum, A, Poyart, C, Memet, S, Israel, A, Berche, P (1999) Listeriolysin O-dependent activation of endothelial cells during infection with *Listeria monocytogenes*: activation of NF-kappa B and upregulation of adhesion molecules and chemokines. Molecular microbiology 31: 1709-1722.

Kazmierczak, MJ, Mithoe, SC, Boor, KJ, Wiedmann, M (2003) *Listeria monocytogenes* sigma B regulates stress response and virulence functions. J Bacteriol 185: 5722-5734.

Kennedy, DJ, Kashyap, SR (2011) Pathogenic role of scavenger receptor CD36 in the metabolic syndrome and diabetes. Metabolic syndrome and related disorders 9: 239-245.

Khelef, N, Lecuit, M, Bierne, H, Cossart, P (2006) Species specificity of the *Listeria monocytogenes* InlB protein. Cellular microbiology 8: 457-470.

Kim, S, Watarai, M, Suzuki, H, Makino, S, Kodama, T, Shirahata, T (2004) Lipid raft microdomains mediate class A scavenger receptor-dependent infection of *Brucella abortus*. Microbial pathogenesis 37: 11-19.

Kim, S, Bauernfeind, F, Ablasser, A, Hartmann, G, Fitzgerald, KA, Latz, E, Hornung, V (2010) *Listeria monocytogenes* is sensed by the NLRP3 and AIM2 inflammasome. European journal of immunology 40: 1545-1551.

Kirchner, M, Higgins, DE (2008) Inhibition of ROCK activity allows InlF-mediated invasion and increased virulence of *Listeria monocytogenes*. Molecular microbiology 68: 749-767.

Kneidl, J, Loffler, B, Erat, MC, Kalinka, J, Peters, G, Roth, J, Barczyk, K (2012) Soluble CD163 promotes recognition, phagocytosis and killing of *Staphylococcus aureus* via binding of specific fibronectin peptides. Cellular microbiology 14: 914-936.

Kocks, C, Gouin, E, Tabouret, M, Berche, P, Ohayon, H, Cossart, P (1992) *L. monocytogenes*-induced actin assembly requires the actA gene product, a surface protein. Cell 68: 521-531.

Kodama, T, Freeman, M, Rohrer, L, Zabrecky, J, Matsudaira, P, Krieger, M (1990) Type I macrophage scavenger receptor contains alpha-helical and collagen-like coiled coils. Nature 343: 531-535.

Korhonen, JT, Puolakkainen, M, Haveri, A, Tammiruusu, A, Sarvas, M, Lahesmaa, R (2012) *Chlamydia pneumoniae* entry into epithelial cells by clathrin-independent endocytosis. Microbial pathogenesis 52: 157-164.

Krawczyk-Balska, A, Markiewicz, Z (2016) The intrinsic cephalosporin resistome of *Listeria monocytogenes* in the context of stress response, gene regulation, pathogenesis and therapeutics. Journal of applied microbiology 120: 251-265.

Kristiansen, M, Graversen, JH, Jacobsen, C, Sonne, O, Hoffman, HJ, Law, SK, Moestrup, SK (2001) Identification of the haemoglobin scavenger receptor. Nature 409: 198-201.

Kruger, P, Saffarzadeh, M, Weber, AN, Rieber, N, Radsak, M, von Bernuth, H, Benarafa, C, Roos, D, Skokowa, J, Hartl, D (2015) Neutrophils: Between host defence, immune modulation, and tissue injury. PLoS pathogens 11: e1004651.

Kumar, H, Kawai, T, Akira, S (2011) Pathogen recognition by the innate immune system. International reviews of immunology 30: 16-34.

Kumar, S, Ingle, H, Prasad, DV, Kumar, H (2013) Recognition of bacterial infection by innate immune sensors. Critical reviews in microbiology 39: 229-246.

Kume, N, Kita, T (2001) Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) in atherogenesis. Trends in Cardiovascular Medicine 11: 22-25.

Kuroki, Y, Voelker, DR (1994) Pulmonary surfactant proteins. The Journal of biological chemistry 269: 25943-25946.

Kuronuma, K, Sano, H, Kato, K, Kudo, K, Hyakushima, N, Yokota, S, Takahashi, H, Fujii, N, Suzuki, H, Kodama, T, Abe, S, Kuroki, Y (2004) Pulmonary surfactant protein A augments the phagocytosis of *Streptococcus pneumoniae* by alveolar macrophages through a casein kinase 2-dependent increase of cell surface localization of scavenger receptor A. The Journal of biological chemistry 279: 21421-21430.

Kurtz, J (2004) Memory in the innate and adaptive immune systems. Microbes and infection 6: 1410-1417.

Kvarnhammar, AM, Cardell, LO (2012) Pattern-recognition receptors in human eosinophils. Immunology 136: 11-20.

Kzhyshkowska, J (2010) Multifunctional receptor stabilin-1 in homeostasis and disease. TheScientificWorldJournal 10: 2039-2053.

Kzhyshkowska, J, Workman, G, Cardo-Vila, M, Arap, W, Pasqualini, R, Gratchev, A, Krusell, L, Goerdt, S, Sage, EH (2006) Novel function of alternatively activated macrophages: stabilin-1-mediated clearance of SPARC. Journal of immunology 176: 5825-5832.

Kzhyshkowska, J, Gratchev, A, Martens, JH, Pervushina, O, Mamidi, S, Johansson, S, Schledzewski, K, Hansen, B, He, X, Tang, J, Nakayama, K, Goerdt, S (2004) Stabilin-1 localizes to endosomes and the trans-Golgi network in human macrophages and interacts with GGA adaptors. Journal of leukocyte biology 76: 1151-1161.

Kzhyshkowska, J, Gratchev, A, Schmuttermaier, C, Brundiers, H, Krusell, L, Mamidi, S, Zhang, J, Workman, G, Sage, EH, Anderle, C, Sedlmayr, P, Goerdt, S (2008)

Alternatively activated macrophages regulate extracellular levels of the hormone placental lactogen via receptor-mediated uptake and transcytosis. Journal of immunology 180: 3028-3037.

Lam, GY, Fattouh, R, Muise, AM, Grinstein, S, Higgins, DE, Brumell, JH (2011) Listeriolysin O suppresses phospholipase C-mediated activation of the microbicidal NADPH oxidase to promote *Listeria monocytogenes* infection. Cell host & microbe 10: 627-634.

Lamkanfi, M, Dixit, VM (2012) Inflammasomes and Their Roles in Health and Disease. Annual Review of Cell and Developmental Biology, Vol 28 28: 137-161.

Landschulz, KT, Pathak, RK, Rigotti, A, Krieger, M, Hobbs, HH (1996) Regulation of scavenger receptor, class B, type I, a high density lipoprotein receptor, in liver and steroidogenic tissues of the rat. The Journal of clinical investigation 98: 984-995.

Lang Halter, E, Neuhaus, K, Scherer, S (2013) *Listeria weihenstephanensis sp. nov.*, isolated from the water plant Lemna trisulca taken from a freshwater pond. International journal of systematic and evolutionary microbiology 63: 641-647.

Larsen, H, Seeliger, H (1966) A mannitol fermenting listeria, *listeria grayi sp*. n. Proceedings of the Third International Symposium on Listeriosis 35.

Law, SK, Micklem, KJ, Shaw, JM, Zhang, XP, Dong, Y, Willis, AC, Mason, DY (1993) A new macrophage differentiation antigen which is a member of the scavenger receptor superfamily. European journal of immunology 23: 2320-2325.

Leber, JH, Crimmins, GT, Raghavan, S, Meyer-Morse, NP, Cox, JS, Portnoy, DA (2008) Distinct TLR- and NLR-mediated transcriptional responses to an intracellular pathogen. PLoS pathogens 4: e6.

Lebreton, A, Stavru, F, Brisse, S, Cossart, P (2016) 1926-2016: 90 Years of listeriology. Microbes and infection 18: 711-723.

Lebrun, M, Audurier, A, Cossart, P (1994) Plasmid-borne cadmium resistance genes in *Listeria monocytogenes* are present on Tn5422, a novel transposon closely related to Tn917. Journal of bacteriology 176: 3049-3061.

Lebrun, M, Audurier, A, Cossart, P (1994) Plasmid-borne cadmium resistance genes in *Listeria monocytogenes* are similar to cadA and cadC of Staphylococcus aureus and are induced by cadmium. Journal of bacteriology 176: 3040-3048.

Leclercq, A, Clermont, D, Bizet, C, Grimont, PA, Le Fleche-Mateos, A, Roche, SM, Buchrieser, C, Cadet-Daniel, V, Le Monnier, A, Lecuit, M, Allerberger, F (2010) *Listeria rocourtiae sp. nov*. International journal of systematic and evolutionary microbiology 60: 2210-2214.

Lecuit, M (2005) Understanding how *Listeria monocytogenes* targets and crosses host barriers. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases 11: 430-436.

Lecuit, M, Hurme, R, Pizarro-Cerda, J, Ohayon, H, Geiger, B, Cossart, P (2000) A role for alpha-and beta-catenins in bacterial uptake. Proceedings of the National Academy of Sciences of the United States of America 97: 10008-10013.

Lecuit, M, Nelson, DM, Smith, SD, Khun, H, Huerre, M, Vacher-Lavenu, MC, Gordon, JI, Cossart, P (2004) Targeting and crossing of the human maternofetal barrier by *Listeria monocytogenes*: role of internalin interaction with trophoblast E-cadherin. Proceedings of the National Academy of Sciences of the United States of America 101: 6152-6157.

Ledala, N, Sengupta, M, Muthaiyan, A, Wilkinson, BJ, Jayaswal, RK (2010) Transcriptomic response of *Listeria monocytogenes* to iron limitation and Fur mutation. Applied and environmental microbiology 76: 406-416.

Lee, SJ, Park, SY, Jung, MY, Bae, SM, Kim, IS (2011) Mechanism for phosphatidylserine-dependent erythrophagocytosis in mouse liver. Blood 117: 5215-5223.

Leito, JT, Ligtenberg, AJ, van Houdt, M, van den Berg, TK, Wouters, D (2011) The bacteria binding glycoprotein salivary agglutinin (SAG/gp340) activates complement via the lectin pathway. Molecular immunology 49: 185-190.

Lemaitre, B, Nicolas, E, Michaut, L, Reichhart, JM, Hoffmann, JA (1996) The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell 86: 973-983.

Li, C, Zhao, G, Che, C, Lin, J, Li, N, Hu, L, Jiang, N, Liu, Y (2015) The Role of LOX-1 in Innate Immunity to *Aspergillus fumigatus* in Corneal Epithelial Cells. Investigative ophthalmology & visual science 56: 3593-3603.

Li, JY, Paragas, N, Ned, RM, Qiu, A, Viltard, M, Leete, T, Drexler, IR, Chen, X, Sanna-Cherchi, S, Mohammed, F, Williams, D, Lin, CS, Schmidt-Ott, KM, Andrews, NC, Barasch, J (2009) Scara5 is a ferritin receptor mediating non-transferrin iron delivery. Developmental cell 16: 35-46.

Liang, DY, Liu, F, Chen, JX, He, XL, Zhou, YL, Ge, BX, Luo, LJ (2016) *Porphyromonas gingivalis* infected macrophages upregulate CD36 expression via ERK/NF-kappaB pathway. Cellular signalling 28: 1292-1303.

Liani, R, Halvorsen, B, Sestili, S, Handberg, A, Santilli, F, Vazzana, N, Formoso, G, Aukrust, P, Davi, G (2012) Plasma levels of soluble CD36, platelet activation, inflammation, and oxidative stress are increased in type 2 diabetic patients. Free Radical Biology and Medicine 52: 1318-1324.

Limmon, GV, Arredouani, M, McCann, KL, Corn Minor, RA, Kobzik, L, Imani, F (2008) Scavenger receptor class-A is a novel cell surface receptor for double-stranded RNA. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 22: 159-167.

Lingnau, A, Domann, E, Hudel, M, Bock, M, Nichterlein, T, Wehland, J, Chakraborty, T (1995) Expression of the *Listeria monocytogenes* EGD inlA and inlB genes, whose products mediate bacterial entry into tissue culture cell lines, by PrfA-dependent and -independent mechanisms. Infection and immunity 63: 3896-3903.

Lloyd-Jones, D, Adams, RJ, Brown, TM, Carnethon, M, Dai, S, De Simone, G, Ferguson, TB, Ford, E, Furie, K, Gillespie, C, Go, A, Greenlund, K, Haase, N, Hailpern, S, Ho, M, Howard, V, Kissela, B, Kittner, S, Lackland, D, Lisabeth, L, Marelli, A, McDermott, MM, Meigs, J, Mozaffarian, D, Mussolino, M, Nichol, G, Roger, VL, Rosamond, W, Sacco, R, Sorlie, P, Stafford, R, Thom, T, Wasserthiel-Smoller, S, Wong, ND, Wylie-Rosett, J, Stroke, AHASC (2010) Executive Summary: Heart Disease and Stroke Statistics-2010 Update A Report From the American Heart Association. Circulation 121: 948-954.

Loh, E, Dussurget, O, Gripenland, J, Vaitkevicius, K, Tiensuu, T, Mandin, P, Repoila, F, Buchrieser, C, Cossart, P, Johansson, J (2009) A trans-acting riboswitch controls expression of the virulence regulator PrfA in *Listeria monocytogenes*. Cell 139: 770-779.

Lomonaco, S, Nucera, D, Filipello, V (2015) The evolution and epidemiology of *Listeria monocytogenes* in Europe and the United States. Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases 35: 172-183.

Low, JS, Tao, Q, Ng, KM, Goh, HK, Shu, XS, Woo, WL, Ambinder, RF, Srivastava, G, Shamay, M, Chan, AT, Popescu, NC, Hsieh, WS (2011) A novel isoform of the 8p22 tumor suppressor gene DLC1 suppresses tumor growth and is frequently silenced in multiple common tumors. Oncogene 30: 1923-1935.

Machata, S, Tchatalbachev, S, Mohamed, W, Jansch, L, Hain, T, Chakraborty, T (2008) Lipoproteins of *Listeria monocytogenes* are critical for virulence and TLR2-mediated immune activation. Journal of immunology 181: 2028-2035.

Mackaness, GB (1962) Cellular resistance to infection. The Journal of experimental medicine 116: 381-406.

MacLeod, DT, Nakatsuji, T, Yamasaki, K, Kobzik, L, Gallo, RL (2013) HSV-1 exploits the innate immune scavenger receptor MARCO to enhance epithelial adsorption and infection. Nature communications 4: 1963.

MacMicking, JD, Nathan, C, Hom, G, Chartrain, N, Fletcher, DS, Trumbauer, M, Stevens, K, Xie, QW, Sokol, K, Hutchinson, N, et al. (1995) Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. Cell 81: 641-650.

Madjunkov, M, Chaudhry, S, Ito, S (2017) Listeriosis during pregnancy. Archives of gynecology and obstetrics 296: 143-152.

Magalhaes, R, Almeida, G, Ferreira, V, Santos, I, Silva, J, Mendes, MM, Pita, J, Mariano, G, Mancio, I, Sousa, MM, Farber, J, Pagotto, F, Teixeira, P (2015) Cheese-related listeriosis outbreak, Portugal, March 2009 to February 2012. Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin 20.

Makitie, T, Summanen, P, Tarkkanen, A, Kivela, T (2001) Tumor-infiltrating macrophages (CD68(+) cells) and prognosis in malignant uveal melanoma. Investigative ophthalmology & visual science 42: 1414-1421.

Maler, MD, Nielsen, PJ, Stichling, N, Cohen, I, Ruzsics, Z, Wood, C, Engelhard, P, Suomalainen, M, Gyory, I, Huber, M, Muller-Quernheim, J, Schamel, WWA, Gordon, S, Jakob, T, Martin, SF, Jahnen-Dechent, W, Greber, UF, Freudenberg, MA, Fejer, G (2017) Key Role of the Scavenger Receptor MARCO in Mediating Adenovirus Infection and Subsequent Innate Responses of Macrophages. mBio 8.

Mandin, P, Fsihi, H, Dussurget, O, Vergassola, M, Milohanic, E, Toledo-Arana, A, Lasa, I, Johansson, J, Cossart, P (2005) VirR, a response regulator critical for L*isteria monocytogenes* virulence. Molecular microbiology 57: 1367-1380.

Martins, M, Custodio, R, Camejo, A, Almeida, MT, Cabanes, D, Sousa, S (2012) *Listeria monocytogenes* triggers the cell surface expression of Gp96 protein and interacts with its N terminus to support cellular infection. The Journal of biological chemistry 287: 43083-43093.

Matloubian, M, David, A, Engel, S, Ryan, JE, Cyster, JG (2000) A transmembrane CXC chemokine is a ligand for HIV-coreceptor Bonzo. Nature immunology 1: 298-304.

Matsumura, S, Wang, B, Kawashima, N, Braunstein, S, Badura, M, Cameron, TO, Babb, JS, Schneider, RJ, Formenti, SC, Dustin, ML, Demaria, S (2008) Radiation-induced CXCL16 release by breast cancer cells attracts effector T cells. Journal of immunology 181: 3099-3107.

Matzinger, P (1994) Tolerance, danger, and the extended family. Annual review of immunology 12: 991-1045.

Matzinger, P (1998) An innate sense of danger. Seminars in Immunology 10: 399-415.

Matzinger, P (2002) The danger model: a renewed sense of self. Science 296: 301-305.

McCourt, PA, Smedsrod, BH, Melkko, J, Johansson, S (1999) Characterization of a hyaluronan receptor on rat sinusoidal liver endothelial cells and its functional relationship to scavenger receptors. Hepatology 30: 1276-1286.

McGreal, EP, Miller, JL, Gordon, S (2005) Ligand recognition by antigen-presenting cell C-type lectin receptors. Current opinion in immunology 17: 18-24.

McGuire, VA, Arthur, JS (2015) Subverting Toll-Like Receptor Signaling by Bacterial Pathogens. Frontiers in immunology 6: 607.

McMahon, HT, Boucrot, E (2011) Molecular mechanism and physiological functions of clathrin-mediated endocytosis. Nature reviews Molecular cell biology 12: 517-533.

Means, TK, Mylonakis, E, Tampakakis, E, Colvin, RA, Seung, E, Puckett, L, Tai, MF, Stewart, CR, Pukkila-Worley, R, Hickman, SE, Moore, KJ, Calderwood, SB, Hacohen, N, Luster, AD, El Khoury, J (2009) Evolutionarily conserved recognition and innate immunity to fungal pathogens by the scavenger receptors SCARF1 and CD36. The Journal of experimental medicine 206: 637-653.

Medzhitov, R (2010) Innate immunity: quo vadis? Nature immunology 11: 551-553.

Medzhitov, R, Janeway, C (2000a) Innate immune recognition: mechanisms and pathways. Immunological Reviews 173: 89-97.

Medzhitov, R, Janeway, C (2000b) The Toll receptor family and microbial recognition. Trends in Microbiology 8: 452-456.

Mengaud, J, Ohayon, H, Gounon, P, Mege, RM, Cossart, P (1996) E-cadherin is the receptor for internalin, a surface protein required for entry of *L. monocytogenes* into epithelial cells. Cell 84: 923-932.

Mesquita, FS, Brito, C, Mazon Moya, MJ, Pinheiro, JC, Mostowy, S, Cabanes, D, Sousa, S (2017) Endoplasmic reticulum chaperone Gp96 controls actomyosin dynamics and protects against pore-forming toxins. EMBO reports 18: 303-318.

Metchnikoff, E (1984) Classics in infectious diseases. Concerning the relationship between phagocytes and anthrax bacilli. By Elie Metchnikoff. 1884. Reviews of Infectious Diseases 6: 761-770.

Meyer-Morse, N, Robbins, JR, Rae, CS, Mochegova, SN, Swanson, MS, Zhao, Z, Virgin, HW, Portnoy, D (2010) Listeriolysin O is necessary and sufficient to induce autophagy during *Listeria monocytogenes* infection. PloS one 5: e8610.

Milohanic, E, Pron, B, Berche, P, Gaillard, JL (2000) Identification of new loci involved in adhesion of *Listeria monocytogenes* to eukaryotic cells. European Listeria Genome Consortium. Microbiology 146 ( Pt 3): 731-739.

Milohanic, E, Jonquieres, R, Cossart, P, Berche, P, Gaillard, JL (2001) The autolysin Ami contributes to the adhesion of *Listeria monocytogenes* to eukaryotic cells via its cell wall anchor. Molecular microbiology 39: 1212-1224.

Milohanic, E, Glaser, P, Coppee, JY, Frangeul, L, Vega, Y, Vazquez-Boland, JA, Kunst, F, Cossart, P, Buchrieser, C (2003) Transcriptome analysis of *Listeria monocytogenes* identifies three groups of genes differently regulated by PrfA. Molecular microbiology 47: 1613-1625.

Mogensen, TH (2009) Pathogen recognition and inflammatory signaling in innate immune defenses. Clinical microbiology reviews 22: 240-273, Table of Contents.

Moretti, J, Roy, S, Bozec, D, Martinez, J, Chapman, JR, Ueberheide, B, Lamming, DW, Chen, ZJ, Horng, T, Yeretssian, G, Green, DR, Blander, JM (2017) STING Senses Microbial Viability to Orchestrate Stress-Mediated Autophagy of the Endoplasmic Reticulum. Cell 171: 809-823 e813.

Moser, M, Leo, O (2010) Key concepts in immunology. Vaccine 28 Suppl 3: C2-13.

Mukhopadhyay, S, Gordon, S (2004) The role of scavenger receptors in pathogen recognition and innate immunity. Immunobiology 209: 39-49.

Mukhopadhyay, S, Chen, Y, Sankala, M, Peiser, L, Pikkarainen, T, Kraal, G, Tryggvason, K, Gordon, S (2006) MARCO, an innate activation marker of macrophages, is a class A scavenger receptor for *Neisseria meningitidis*. European journal of immunology 36: 940-949.

Mukouhara, T, Arimoto, T, Cho, K, Yamamoto, M, Igarashi, T (2011) Surface lipoprotein PpiA of *Streptococcus mutans* suppresses scavenger receptor MARCO-dependent phagocytosis by macrophages. Infection and immunity 79: 4933-4940.

Murphy, JE, Tedbury, PR, Homer-Vanniasinkam, S, Walker, JH, Ponnambalam, S (2005) Biochemistry and cell biology of mammalian scavenger receptors. Atherosclerosis 182: 1-15.

Murray, RE, W, MBR, S (1926) A disease of rabbits characterized by a large mononuclear leucocytosis, caused by a hitherto undescribed bacillus bacterium monocytogenes. Pathol Bacteriol 29: 407-439.

Murshid, A, Gong, J, Calderwood, SK (2012) The role of heat shock proteins in antigen cross presentation. Frontiers in immunology 3: 63.

Nakamura, K, Funakoshi, H, Miyamoto, K, Tokunaga, F, Nakamura, T (2001) Molecular cloning and functional characterization of a human scavenger receptor with C-type lectin (SRCL), a novel member of a scavenger receptor family. Biochemical and biophysical research communications 280: 1028-1035.

Nakayama, H, Kurokawa, K, Lee, BL (2012) Lipoproteins in bacteria: structures and biosynthetic pathways. The FEBS journal 279: 4247-4268.

Nesargikar, PN, Spiller, B, Chavez, R (2012) The complement system: history, pathways, cascade and inhibitors. European journal of microbiology & immunology 2: 103-111.

Neyen, C, Pluddemann, A, Mukhopadhyay, S, Maniati, E, Bossard, M, Gordon, S, Hagemann, T (2013) Macrophage scavenger receptor a promotes tumor progression in murine models of ovarian and pancreatic cancer. Journal of immunology 190: 3798-3805.

Nieman, RE, Lorber, B (1980) Listeriosis in Adults - a Changing Pattern - Report of 8 Cases and Review of the Literature, 1968-1978. Reviews of Infectious Diseases 2: 207-227.

Nikitas, G, Deschamps, C, Disson, O, Niault, T, Cossart, P, Lecuit, M (2011) Transcytosis of *Listeria monocytogenes* across the intestinal barrier upon specific targeting of goblet cell accessible E-cadherin. The Journal of experimental medicine 208: 2263-2277.

Nish, S, Medzhitov, R (2011) Host defense pathways: role of redundancy and compensation in infectious disease phenotypes. Immunity 34: 629-636.

Nucifora, G, Chu, L, Misra, TK, Silver, S (1989) Cadmium resistance from *Staphylococcus aureus* plasmid pI258 cadA gene results from a cadmium-efflux ATPase. Proceedings of the National Academy of Sciences of the United States of America 86: 3544-3548.

Nyfelt, A (1929) Etiologie de la mononucléose infectieuse. Comptes Rendus de la Societé de Biologie 101: 590-592.

O'Brien, DK, Melville, SB (2000) The anaerobic pathogen *Clostridium perfringens* can escape the phagosome of macrophages under aerobic conditions. Cellular microbiology 2: 505-519.

O'Brien, DK, Melville, SB (2003) Multiple effects on *Clostridium perfringens* binding, uptake and trafficking to lysosomes by inhibitors of macrophage phagocytosis receptors. Microbiology 149: 1377-1386.

Ohtaki, Y, Ishii, G, Nagai, K, Ashimine, S, Kuwata, T, Hishida, T, Nishimura, M, Yoshida, J, Takeyoshi, I, Ochiai, A (2010) Stromal macrophage expressing CD204 is associated with tumor aggressiveness in lung adenocarcinoma. Journal of thoracic oncology : official publication of the International Association for the Study of Lung Cancer 5: 1507-1515.

Ohtani, K, Suzuki, Y, Eda, S, Kawai, T, Kase, T, Keshi, H, Sakai, Y, Fukuoh, A, Sakamoto, T, Itabe, H, Suzutani, T, Ogasawara, M, Yoshida, I, Wakamiya, N (2001) The membrane-type collectin CL-P1 is a scavenger receptor on vascular endothelial cells. The Journal of biological chemistry 276: 44222-44228.

Ollinger, J, Wiedmann, M, Boor, KJ (2008) SigmaB- and PrfA-dependent transcription of genes previously classified as putative constituents of the *Listeria monocytogenes* PrfA regulon. Foodborne pathogens and disease 5: 281-293.

Olonisakin, TF, Li, H, Xiong, Z, Kochman, EJ, Yu, M, Qu, Y, Hulver, M, Kolls, JK, St Croix, C, Doi, Y, Nguyen, MH, Shanks, RM, Mallampalli, RK, Kagan, VE, Ray, A, Silverstein,

RL, Ray, P, Lee, JS (2016) CD36 Provides Host Protection Against *Klebsiella pneumoniae* Intrapulmonary Infection by Enhancing Lipopolysaccharide Responsiveness and Macrophage Phagocytosis. The Journal of infectious diseases 214: 1865-1875.

Orndorff, PE, Hamrick, TS, Smoak, IW, Havell, EA (2006) Host and bacterial factors in listeriosis pathogenesis. Veterinary microbiology 114: 1-15.

Orsi, RH, Wiedmann, M (2016) Characteristics and distribution of *Listeria spp.*, including *Listeria* species newly described since 2009. Applied Microbiology and Biotechnology 100: 5273-5287.

Ortega, FE, Rengarajan, M, Chavez, N, Radhakrishnan, P, Gloerich, M, Bianchini, J, Siemers, K, Luckett, WS, Lauer, P, Nelson, WJ, Theriot, JA (2017) Adhesion to the host cell surface is sufficient to mediate *Listeria monocytogenes* entry into epithelial cells. Molecular biology of the cell 28: 2945-2957.

Osanai, A, Li, SJ, Asano, K, Sashinami, H, Hu, DL, Nakane, A (2013) Fibronectin-binding protein, FbpA, is the adhesin responsible for pathogenesis of *Listeria monocytogenes* infection. Microbiology and immunology 57: 253-262.

Osborne, SE, Brumell, JH (2017) Listeriolysin O: from bazooka to Swiss army knife. Philosophical transactions of the Royal Society of London Series B, Biological sciences 372.

Osman, D, Cavet, JS (2010) Bacterial metal-sensing proteins exemplified by ArsR-SmtB family repressors. Natural product reports 27: 668-680.

Ozoren, N, Masumoto, J, Franchi, L, Kanneganti, TD, Body-Malapel, M, Erturk, I, Jagirdar, R, Zhu, L, Inohara, N, Bertin, J, Coyle, A, Grant, EP, Nunez, G (2006) Distinct roles of TLR2 and the adaptor ASC in IL-1beta/IL-18 secretion in response to *Listeria monocytogenes*. Journal of immunology 176: 4337-4342.

Padilla, O, Calvo, J, Vila, JM, Arman, M, Gimferrer, I, Places, L, Arias, MT, Pujana, MA, Vives, J, Lozano, F (2000) Genomic organization of the human CD5 gene. Immunogenetics 51: 993-1001.

Pamer, EG (2004) Immune responses to *Listeria monocytogenes*. Nat Rev Immunol 4: 812-823.

Pandiripally, VK, Westbrook, DG, Sunki, GR, Bhunia, AK (1999) Surface protein p104 is involved in adhesion of *Listeria monocytogenes* to human intestinal cell line, Caco-2. J Med Microbiol 48: 117-124.

Parida, SK, Domann, E, Rohde, M, Muller, S, Darji, A, Hain, T, Wehland, J, Chakraborty, T (1998) Internalin B is essential for adhesion and mediates the invasion of *Listeria monocytogenes* into human endothelial cells. Molecular microbiology 28: 81-93.

Park, D, Tosello-Trampont, AC, Elliott, MR, Lu, M, Haney, LB, Ma, Z, Klibanov, AL, Mandell, JW, Ravichandran, KS (2007) BAI1 is an engulfment receptor for apoptotic cells upstream of the ELMO/Dock180/Rac module. Nature 450: 430-434.

Park, SY, Jung, MY, Lee, SJ, Kang, KB, Gratchev, A, Riabov, V, Kzhyshkowska, J, Kim, IS (2009) Stabilin-1 mediates phosphatidylserine-dependent clearance of cell corpses in alternatively activated macrophages. Journal of cell science 122: 3365-3373.

Pearson, A, Lux, A, Krieger, M (1995) Expression cloning of dSR-CI, a class C macrophage-specific scavenger receptor from Drosophila melanogaster. Proceedings of the National Academy of Sciences of the United States of America 92: 4056-4060.

Peiser, L, de Winther, MPJ, Makepeace, K, Hollinshead, M, Coull, P, Plested, J, Kodama, T, Moxon, ER, Gordon, S (2002) The class A macrophage scavenger receptor is a major pattern recognition receptor for *Neisseria meningitidis* which is independent of lipopolysaccharide and not required for secretory responses. Infection and immunity 70: 5346-5354.

Peiser, L, Makepeace, K, Pluddemann, A, Savino, S, Wright, JC, Pizza, M, Rappuoli, R, Moxon, ER, Gordon, S (2006) Identification of *Neisseria meningitidis* nonlipopolysaccharide ligands for class A macrophage scavenger receptor by using a novel assay. Infection and immunity 74: 5191-5199.

Percy, MG, Karinou, E, Webb, AJ, Grundling, A (2016) Identification of a Lipoteichoic Acid Glycosyltransferase Enzyme Reveals that GW-Domain-Containing Proteins Can Be Retained in the Cell Wall of *Listeria monocytogenes* in the Absence of Lipoteichoic Acid or Its Modifications. Journal of bacteriology 198: 2029-2042.

Pfeiler, S, Khandagale, AB, Magenau, A, Nichols, M, Heijnen, HF, Rinninger, F, Ziegler, T, Seveau, S, Schubert, S, Zahler, S, Verschoor, A, Latz, E, Massberg, S, Gaus, K, Engelmann, B (2016) Distinct surveillance pathway for immunopathology during acute infection via autophagy and SR-BI. Scientific reports 6: 34440.

Pierini, LM (2006) Uptake of serum-opsonized *Francisella tularensis* by macrophages can be mediated by class A scavenger receptors. Cellular microbiology 8: 1361-1370.

Pillich, H, Puri, M, Chakraborty, T (2017) ActA of *Listeria monocytogenes* and Its Manifold Activities as an Important Listerial Virulence Factor. Current topics in microbiology and immunology 399: 113-132.

Pirie, AH (1927) Rodent ulcer. Canadian Medical Association Journal 17: 1326-1328.

Pirie, JH (1940) The Genus Listerella Pirie. Science 91: 383.

Pizarro-Cerda, J, Cossart, P (2006) Bacterial adhesion and entry into host cells. Cell 124: 715-727.

Pizarro-Cerda, J, Kuhbacher, A, Cossart, P (2012) Entry of *Listeria monocytogenes* in mammalian epithelial cells: an updated view. Cold Spring Harbor perspectives in medicine 2.

Plato, A, Willment, JA, Brown, GD (2013) C-Type Lectin-Like Receptors of the Dectin-1 Cluster: Ligands and Signaling Pathways. International reviews of immunology 32: 134-156.

Platt, N, Gordon, S (2001) Is the class A macrophage scavenger receptor (SR-A) multifunctional? - The mouse's tale. Journal of Clinical Investigation 108: 649-654.

Pluddemann, A, Mukhopadhyay, S, Gordon, S (2006) The interaction of macrophage receptors with bacterial ligands. Expert reviews in molecular medicine 8: 1-25.

Pluddemann, A, Neyen, C, Gordon, S (2007) Macrophage scavenger receptors and host-derived ligands. Methods 43: 207-217.

Pluddemann, A, Mukhopadhyay, S, Gordon, S (2011) Innate immunity to intracellular pathogens: macrophage receptors and responses to microbial entry. Immunological reviews 240: 11-24.

Pluddemann, A, Hoe, JC, Makepeace, K, Moxon, ER, Gordon, S (2009) The macrophage scavenger receptor A is host-protective in experimental meningococcal septicaemia. PLoS pathogens 5: e1000297.

Politz, O, Gratchev, A, McCourt, PA, Schledzewski, K, Guillot, P, Johansson, S, Svineng, G, Franke, P, Kannicht, C, Kzhyshkowska, J, Longati, P, Velten, FW, Goerdt, S (2002) Stabilin-1 and -2 constitute a novel family of fasciclin-like hyaluronan receptor homologues. The Biochemical journal 362: 155-164.

Port, GC, Freitag, NE (2007) Identification of novel *Listeria monocytogenes* secreted virulence factors following mutational activation of the central virulence regulator, PrfA. Infection and immunity 75: 5886-5897.

Portnoy, DA, Jacks, PS, Hinrichs, DJ (1988) Role of hemolysin for the intracellular growth of *Listeria monocytogenes*. The Journal of experimental medicine 167: 1459-1471.

Prabhudas, M, Bowdish, D, Drickamer, K, Febbraio, M, Herz, J, Kobzik, L, Krieger, M, Loike, J, Means, TK, Moestrup, SK, Post, S, Sawamura, T, Silverstein, S, Wang, XY, El Khoury, J (2014) Standardizing scavenger receptor nomenclature. Journal of immunology 192: 1997-2006.

Prasadarao, NV, Srivastava, PK, Rudrabhatla, RS, Kim, KS, Huang, SH, Sukumaran, SK (2003) Cloning and expression of the *Escherichia coli* K1 outer membrane protein A receptor, a gp96 homologue. Infection and immunity 71: 1680-1688.

Prins, JM, Lauw, FN, Derkx, BH, Speelman, P, Kuijper, EJ, Dankert, J, van Deventer, SJ (1998) Endotoxin release and cytokine production in acute and chronic meningococcaemia. Clinical and experimental immunology 114: 215-219.

Qian, H, Johansson, S, McCourt, P, Smedsrod, B, Ekblom, M, Johansson, S (2009) Stabilins are expressed in bone marrow sinusoidal endothelial cells and mediate scavenging and cell adhesive functions. Biochemical and biophysical research communications 390: 883-886.

Qiu, Y, Dayrit, JK, Davis, MJ, Carolan, JF, Osterholzer, JJ, Curtis, JL, Olszewski, MA (2013) Scavenger receptor A modulates the immune response to pulmonary *Cryptococcus neoformans* infection. Journal of immunology 191: 238-248.

Rabinovich, L, Sigal, N, Borovok, I, Nir-Paz, R, Herskovits, AA (2012) Prophage excision activates *Listeria* competence genes that promote phagosomal escape and virulence. Cell 150: 792-802.

Radoshevich, L, Cossart, P (2017) *Listeria monocytogenes*: towards a complete picture of its physiology and pathogenesis. Nature reviews Microbiology.

Rae, CS, Geissler, A, Adamson, PC, Portnoy, DA (2011) Mutations of the *Listeria monocytogenes* peptidoglycan N-deacetylase and O-acetylase result in enhanced lysozyme sensitivity, bacteriolysis, and hyperinduction of innate immune pathways. Infection and immunity 79: 3596-3606.

Rajabian, T, Gavicherla, B, Heisig, M, Muller-Altrock, S, Goebel, W, Gray-Owen, SD, Ireton, K (2009) The bacterial virulence factor InlC perturbs apical cell junctions and promotes cell-to-cell spread of *Listeria*. Nature cell biology 11: 1212-1218.

Ramasamy, R, Yan, SF, Schmidt, AM (2009) RAGE: therapeutic target and biomarker of the inflammatory response--the evidence mounts. Journal of leukocyte biology 86: 505-512.

Ramet, M, Pearson, A, Manfruelli, P, Li, X, Koziel, H, Gobel, V, Chung, E, Krieger, M, Ezekowitz, RA (2001) Drosophila scavenger receptor CI is a pattern recognition receptor for bacteria. Immunity 15: 1027-1038.

Ramprasad, MP, Terpstra, V, Kondratenko, N, Quehenberger, O, Steinberg, D (1996) Cell surface expression of mouse macrosialin and human CD68 and their role as macrophage receptors for oxidized low density lipoprotein. Proceedings of the National Academy of Sciences of the United States of America 93: 14833-14838.

Rantakari, P, Patten, DA, Valtonen, J, Karikoski, M, Gerke, H, Dawes, H, Laurila, J, Ohlmeier, S, Elima, K, Hubscher, SG, Weston, CJ, Jalkanen, S, Adams, DH, Salmi, M, Shetty, S (2016) Stabilin-1 expression defines a subset of macrophages that mediate tissue homeostasis and prevent fibrosis in chronic liver injury. Proceedings of the National Academy of Sciences of the United States of America 113: 9298-9303.

Ray, K, Marteyn, B, Sansonetti, PJ, Tang, CM (2009) Life on the inside: the intracellular lifestyle of cytosolic bacteria. Nat Rev Microbiol 7: 333-340.

Rea, RB, Gahan, CG, Hill, C (2004) Disruption of putative regulatory loci in *Listeria monocytogenes* demonstrates a significant role for Fur and PerR in virulence. Infection and immunity 72: 717-727.

Rechner, C, Kuhlewein, C, Muller, A, Schild, H, Rudel, T (2007) Host glycoprotein Gp96 and scavenger receptor SREC interact with PorB of disseminating *Neisseria gonorrhoeae* in an epithelial invasion pathway. Cell host & microbe 2: 393-403.

Regan, T, MacSharry, J, Brint, E (2014) Tracing innate immune defences along the path of *Listeria monocytogenes* infection. Immunology and Cell Biology 92: 563-569.

Regan, T, Nally, K, Carmody, R, Houston, A, Shanahan, F, Macsharry, J, Brint, E (2013) Identification of TLR10 as a key mediator of the inflammatory response to *Listeria monocytogenes* in intestinal epithelial cells and macrophages. Journal of immunology 191: 6084-6092.

Reis, O, Sousa, S, Camejo, A, Villiers, V, Gouin, E, Cossart, P, Cabanes, D (2010) LapB, a novel *Listeria monocytogenes* LPXTG surface adhesin, required for entry into eukaryotic cells and virulence. J Infect Dis 202: 551-562.

Reniere, ML, Whiteley, AT, Hamilton, KL, John, SM, Lauer, P, Brennan, RG, Portnoy, DA (2015) Glutathione activates virulence gene expression of an intracellular pathogen. Nature 517: 170-173.

Riabov, V, Yin, S, Song, B, Avdic, A, Schledzewski, K, Ovsiy, I, Gratchev, A, Llopis Verdiell, M, Sticht, C, Schmuttermaier, C, Schonhaber, H, Weiss, C, Fields, AP, Simon-Keller, K, Pfister, F, Berlit, S, Marx, A, Arnold, B, Goerdt, S, Kzhyshkowska, J (2016) Stabilin-1 is expressed in human breast cancer and supports tumor growth in mammary adenocarcinoma mouse model. Oncotarget 7: 31097-31110.

Ricklin, D, Hajishengallis, G, Yang, K, Lambris, JD (2010) Complement: a key system for immune surveillance and homeostasis. Nature immunology 11: 785-797.

Rocourt, J, Grimont, PAD (1983) *Listeria-Welshimeri Sp-Nov* and *Listeria-Seeligeri Sp-Nov*. International Journal of Systematic Bacteriology 33: 866-869.

Rogers, HW, Unanue, ER (1993) Neutrophils Are Involved in Acute, Nonspecific Resistance to *Listeria*-Monocytogenes in Mice. Infection and immunity 61: 5090-5096.

Rolhion, N, Cossart, P (2017) How the study of *Listeria monocytogenes* has led to new concepts in biology. Future microbiology 12: 621-638.

Saier, MH, Jr., Paulsen, IT, Sliwinski, MK, Pao, SS, Skurray, RA, Nikaido, H (1998) Evolutionary origins of multidrug and drug-specific efflux pumps in bacteria. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 12: 265-274.

Saijo, S, Ikeda, S, Yamabe, K, Kakuta, S, Ishigame, H, Akitsu, A, Fujikado, N, Kusaka, T, Kubo, S, Chung, SH, Komatsu, R, Miura, N, Adachi, Y, Ohno, N, Shibuya, K,

Yamamoto, N, Kawakami, K, Yamasaki, S, Saito, T, Akira, S, Iwakura, Y (2010) Dectin-2 recognition of alpha-mannans and induction of Th17 cell differentiation is essential for host defense against *Candida albicans*. Immunity 32: 681-691.

Salmi, M, Koskinen, K, Henttinen, T, Elima, K, Jalkanen, S (2004) CLEVER-1 mediates lymphocyte transmigration through vascular and lymphatic endothelium. Blood 104: 3849-3857.

Samba-Louaka, A, Pereira, JM, Nahori, MA, Villiers, V, Deriano, L, Hamon, MA, Cossart, P (2014) *Listeria monocytogenes* dampens the DNA damage response. PLoS pathogens 10: e1004470.

Sander, LE, Davis, MJ, Boekschoten, MV, Amsen, D, Dascher, CC, Ryffel, B, Swanson, JA, Muller, M, Blander, JM (2011) Detection of prokaryotic mRNA signifies microbial viability and promotes immunity. Nature 474: 385-389.

Sankala, M, Brannstrom, A, Schulthess, T, Bergmann, U, Morgunova, E, Engel, J, Tryggvason, K, Pikkarainen, T (2002) Characterization of recombinant soluble macrophage scavenger receptor MARCO. The Journal of biological chemistry 277: 33378-33385.

Santiago-Garcia, J, Kodama, T, Pitas, RE (2003) The class A scavenger receptor binds to proteoglycans and mediates adhesion of macrophages to the extracellular matrix. The Journal of biological chemistry 278: 6942-6946.

Sarrias, MR, Rosello, S, Sanchez-Barbero, F, Sierra, JM, Vila, J, Yelamos, J, Vives, J, Casals, C, Lozano, F (2005) A role for human Sp alpha as a pattern recognition receptor. The Journal of biological chemistry 280: 35391-35398.

Sashinami, H, Hu, DL, Li, SJ, Mitsui, T, Hakamada, K, Ishiguro, Y, Fukuda, S, Nakane, A (2010) Virulence factor p60 of *Listeria monocytogenes* modulates innate immunity by inducing tumor necrosis factor alpha. FEMS immunology and medical microbiology 59: 100-107.

Sauer, JD, Witte, CE, Zemansky, J, Hanson, B, Lauer, P, Portnoy, DA (2010) *Listeria monocytogenes* Triggers AIM2-Mediated Pyroptosis upon Infrequent Bacteriolysis in the Macrophage Cytosol. Cell host & microbe 7: 412-419.

Sauer, JD, Sotelo-Troha, K, von Moltke, J, Monroe, KM, Rae, CS, Brubaker, SW, Hyodo, M, Hayakawa, Y, Woodward, JJ, Portnoy, DA, Vance, RE (2011) The N-ethyl-N-nitrosourea-induced Goldenticket mouse mutant reveals an essential function of Sting in the in vivo interferon response to *Listeria monocytogenes* and cyclic dinucleotides. Infection and Immunity 79: 688-694

Sawamura, T, Kume, N, Aoyama, T, Moriwaki, H, Hoshikawa, H, Aiba, Y, Tanaka, T, Miwa, S, Katsura, Y, Kita, T, Masaki, T (1997) An endothelial receptor for oxidized low-density lipoprotein. Nature 386: 73-77.

Scarselli, E, Ansuini, H, Cerino, R, Roccasecca, RM, Acali, S, Filocamo, G, Traboni, C, Nicosia, A, Cortese, R, Vitelli, A (2002) The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. Embo Journal 21: 5017-5025.

Schafer, G, Guler, R, Murray, G, Brombacher, F, Brown, GD (2009) The role of scavenger receptor B1 in infection with *Mycobacterium tuberculosis* in a murine model. PloS one 4: e8448.

Schifferli, JA, Ng, YC, Peters, DK (1986) The role of complement and its receptor in the elimination of immune complexes. The New England journal of medicine 315: 488-495.

Schlech, WF, 3rd, Lavigne, PM, Bortolussi, RA, Allen, AC, Haldane, EV, Wort, AJ, Hightower, AW, Johnson, SE, King, SH, Nicholls, ES, Broome, CV (1983) Epidemic listeriosis--evidence for transmission by food. The New England journal of medicine 308: 203-206.

Schmeck, B, Beermann, W, van Laak, V, Zahlten, J, Opitz, B, Witzenrath, M, Hocke, AC, Chakraborty, T, Kracht, M, Rosseau, S, Suttorp, N, Hippenstiel, S (2005) Intracellular bacteria differentially regulated endothelial cytokine release by MAPK-dependent histone modification. Journal of immunology 175: 2843-2850.

Schmid, MW, Ng, EY, Lampidis, R, Emmerth, M, Walcher, M, Kreft, J, Goebel, W, Wagner, M, Schleifer, KH (2005) Evolutionary history of the genus *Listeria* and its virulence genes. Syst Appl Microbiol 28: 1-18.

Schwarz, AK, Grove, J, Hu, K, Mee, CJ, Balfe, P, McKeating, JA (2009) Hepatoma cell density promotes claudin-1 and scavenger receptor BI expression and hepatitis C virus internalization. Journal of virology 83: 12407-12414.

Schwartz, KT, Carleton, JD, Quillin, SJ, Rollins, SD, Portnoy, DA, Leber, JH (2012) Hyperinduction of host beta interferon by a *Listeria monocytogenes* strain naturally overexpressing the multidrug efflux pump MdrT. Infection and immunity 80: 1537-1545.

Scortti, M, Monzo, HJ, Lacharme-Lora, L, Lewis, DA, Vazquez-Boland, JA (2007) The PrfA virulence regulon. Microbes Infect 9: 1196-1207.

Seeliger, H, Hohne, K (1979) Serotyping of *listeria monocytogenes* and related species. Methods in microbiology 13: 31-49.

Seeliger, HP (1981) [Nonpathogenic listeriae: *L. innocua sp.* n. (Seeliger et Schoofs, 1977) (author's transl)]. Zentralblatt fur Bakteriologie, Mikrobiologie und Hygiene 1 Abt Originale A, Medizinische Mikrobiologie, Infektionskrankheiten und Parasitologie = International journal of microbiology and hygiene A, Medical microbiology, infectious 249: 487-493.

Seeliger, HPR, Jones, D (1986) *Listeria*. In Bergey's Manual of Systematic Bacteriology. Edited by P H A Sneath, N S Nair, N E Sharpe & J G Holt Baltimore: Williams and Wilkins 2: 1235–1245.

Seeliger, HPR, Langer, B (1989) Serological analysis of the genus *Listeria* - Its values and Limitations. International journal of food microbiology 8 (3): 245-248.

Seeliger, HPR, Rocourt, J, Schrettenbrunner, A, Grimont, PAD, Jones, D (1984) Listeria-Ivanovii Sp-Nov. International Journal of Systematic Bacteriology 34: 336-337.

Selman, L, Skjodt, K, Nielsen, O, Floridon, C, Holmskov, U, Hansen, S (2008) Expression and tissue localization of collectin placenta 1 (CL-P1, SRCL) in human tissues. Molecular immunology 45: 3278-3288.

Seveau, S, Pizarro-Cerda, J, Cossart, P (2007) Molecular mechanisms exploited by *Listeria monocytogenes* during host cell invasion. Microbes Infect 9: 1167-1175.

Sever-Chroneos, Z, Tvinnereim, A, Hunter, RL, Chroneos, ZC (2011) Prolonged survival of scavenger receptor class A-deficient mice from pulmonary *Mycobacterium tuberculosis* infection. Tuberculosis 91 Suppl 1: S69-74.

Shahamat, M, Seaman, A, Woodbine, M (1980) Survival of *Listeria monocytogenes* in high salt concentrations. Zentralblatt fur Bakteriologie 1 Abt Originale A: Medizinische Mikrobiologie, Infektionskrankheiten und Parasitologie 246: 506-511.

Sharif, O, Matt, U, Saluzzo, S, Lakovits, K, Haslinger, I, Furtner, T, Doninger, B, Knapp, S (2013) The scavenger receptor CD36 downmodulates the early inflammatory response while enhancing bacterial phagocytosis during pneumococcal pneumonia. Journal of immunology 190: 5640-5648.

Shatursky, O, Heuck, AP, Shepard, LA, Rossjohn, J, Parker, MW, Johnson, AE, Tweten, RK (1999) The mechanism of membrane insertion for a cholesterol-dependent cytolysin: a novel paradigm for pore-forming toxins. Cell 99: 293-299.

Shen, Y, Naujokas, M, Park, M, Ireton, K (2000) InlB-dependent internalization of *Listeria* is mediated by the Met receptor tyrosine kinase. Cell 103: 501-510.

Shimaoka, T, Kume, N, Minami, M, Hayashida, K, Kataoka, H, Kita, T, Yonehara, S (2000) Molecular cloning of a novel scavenger receptor for oxidized low density lipoprotein, SR-PSOX, on macrophages. The Journal of biological chemistry 275: 40663-40666.

Shimaoka, T, Kume, N, Minami, M, Hayashida, K, Sawamura, T, Kita, T, Yonehara, S (2001) LOX-1 supports adhesion of Gram-positive and Gram-negative bacteria. Journal of immunology 166: 5108-5114.

Shimaoka, T, Nakayama, T, Fukumoto, N, Kume, N, Takahashi, S, Yamaguchi, J, Minami, M, Hayashida, K, Kita, T, Ohsumi, J, Yoshie, O, Yonehara, S (2004) Cell surface-anchored SR-PSOX/CXC chemokine ligand 16 mediates firm adhesion of CXC chemokine receptor 6-expressing cells. Journal of leukocyte biology 75: 267-274.

Silver, S, Phung, LT (1996) Bacterial heavy metal resistance: new surprises. Annual review of microbiology 50: 753-789.

Silverstein, RL, Li, W, Park, YM, Rahaman, SO (2010) Mechanisms of cell signaling by the scavenger receptor CD36: implications in atherosclerosis and thrombosis. Transactions of the American Clinical and Climatological Association 121: 206-220.

Smith, GA, Marquis, H, Jones, S, Johnston, NC, Portnoy, DA, Goldfine, H (1995) The two distinct phospholipases C of *Listeria monocytogenes* have overlapping roles in escape from a vacuole and cell-to-cell spread. Infection and immunity 63: 4231-4237.

Snyder, DT, Hedges, JF, Jutila, MA (2017) Getting "Inside" Type I IFNs: Type I IFNs in Intracellular Bacterial Infections. Journal of immunology research 2017: 9361802.

Soldevila, G, Raman, C, Lozano, F (2011) The immunomodulatory properties of the CD5 lymphocyte receptor in health and disease. Current opinion in immunology 23: 310-318.

Song, L, Lee, C, Schindler, C (2011) Deletion of the murine scavenger receptor CD68. Journal of lipid research 52: 1542-1550.

Stavru, F, Bouillaud, F, Sartori, A, Ricquier, D, Cossart, P (2011) *Listeria monocytogenes* transiently alters mitochondrial dynamics during infection. Proceedings of the National Academy of Sciences of the United States of America 108: 3612-3617.

Steinbrecher, UP, Fisher, M, Witztum, JL, Curtiss, LK (1984) Immunogenicity of homologous low density lipoprotein after methylation, ethylation, acetylation, or carbamylation: generation of antibodies specific for derivatized lysine. Journal of lipid research 25: 1109-1116.

Steinman, RM, Cohn, ZA (1973) Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. The Journal of experimental medicine 137: 1142-1162.

Steinman, RM, Cohn, ZA (1974) Identification of a novel cell type in peripheral lymphoid organs of mice. II. Functional properties in vitro. The Journal of experimental medicine 139: 380-397.

Steinweg, C, Kuenne, CT, Billion, A, Mraheil, MA, Domann, E, Ghai, R, Barbuddhe, SB, Karst, U, Goesmann, A, Puhler, A, Weisshaar, B, Wehland, J, Lampidis, R, Kreft, J, Goebel, W, Chakraborty, T, Hain, T (2010) Complete Genome Sequence of *Listeria seeligeri*, a Nonpathogenic Member of the Genus Listeria. Journal of Bacteriology 192: 1473-1474.

Stewart, CR, Stuart, LM, Wilkinson, K, van Gils, JM, Deng, J, Halle, A, Rayner, KJ, Boyer, L, Zhong, R, Frazier, WA, Lacy-Hulbert, A, El Khoury, J, Golenbock, DT, Moore, KJ (2010) CD36 ligands promote sterile inflammation through assembly of a Toll-like receptor 4 and 6 heterodimer. Nature immunology 11: 155-161.

Suzuki, H, Kurihara, Y, Takeya, M, Kamada, N, Kataoka, M, Jishage, K, Ueda, O, Sakaguchi, H, Higashi, T, Suzuki, T, Takashima, Y, Kawabe, Y, Cynshi, O, Wada, Y, Honda, M, Kurihara, H, Aburatani, H, Doi, T, Matsumoto, A, Azuma, S, Noda, T, Toyoda, Y, Itakura, H, Yazaki, Y, Kodama, T, et al. (1997) A role for macrophage scavenger receptors in atherosclerosis and susceptibility to infection. Nature 386: 292-296.

Swaminathan, B, Gerner-Smidt, P (2007) The epidemiology of human listeriosis. Microbes Infect 9: 1236-1243.

Swanson, JA (2008) Shaping cups into phagosomes and macropinosomes. Nature Reviews Molecular Cell Biology 9: 639-649.

Tabuchi, N, Akasaki, K, Sasaki, T, Kanda, N, Tsuji, H (1997) Identification and characterization of a major lysosomal membrane glycoprotein, LGP85/LIMP II in mouse liver. Journal of biochemistry 122: 756-763.

Takahasi, K, Yoneyama, M, Nishihori, T, Hirai, R, Kumeta, H, Narita, R, Gale, M, Inagaki, F, Fujita, T (2008) Nonself RNA-sensing mechanism of RIG-I helicase and activation of antiviral immune responses. Molecular Cell 29: 428-440.

Tarakhovsky, A, Kanner, SB, Hombach, J, Ledbetter, JA, Muller, W, Killeen, N, Rajewsky, K (1995) A role for CD5 in TCR-mediated signal transduction and thymocyte selection. Science 269: 535-537.

Thedieck, K, Hain, T, Mohamed, W, Tindall, BJ, Nimtz, M, Chakraborty, T, Wehland, J, Jansch, L (2006) The MprF protein is required for lysinylation of phospholipids in listerial membranes and confers resistance to cationic antimicrobial peptides (CAMPs) on *Listeria monocytogenes*. Molecular microbiology 62: 1325-1339.

Thelen, T, Hao, Y, Medeiros, AI, Curtis, JL, Serezani, CH, Kobzik, L, Harris, LH, Aronoff, DM (2010) The class A scavenger receptor, macrophage receptor with collagenous structure, is the major phagocytic receptor for *Clostridium sordellii* expressed by human decidual macrophages. Journal of immunology 185: 4328-4335.

Theriault, JR, Adachi, H, Calderwood, SK (2006) Role of scavenger receptors in the binding and internalization of heat shock protein 70. Journal of immunology 177: 8604-8611.

Thomas, CA, Li, Y, Kodama, T, Suzuki, H, Silverstein, SC, El Khoury, J (2000) Protection from lethal gram-positive infection by macrophage scavenger receptor-dependent phagocytosis. The Journal of experimental medicine 191: 147-156.

Torres, D, Barrier, M, Bihl, F, Quesniaux, VJ, Maillet, I, Akira, S, Ryffel, B, Erard, F (2004) Toll-like receptor 2 is required for optimal control of *Listeria monocytogenes* infection. Infection and immunity 72: 2131-2139.

Trinchieri, G, Sher, A (2007) Cooperation of Toll-like receptor signals in innate immune defence. Nature reviews Immunology 7: 179-190.

Tripp, CS, Wolf, SF, Unanue, ER (1993) Interleukin 12 and tumor necrosis factor alpha are costimulators of interferon gamma production by natural killer cells in severe combined immunodeficiency mice with listeriosis, and interleukin 10 is a physiologic antagonist. Proceedings of the National Academy of Sciences of the United States of America 90: 3725-3729.

Truong, D, Copeland, JW, Brumell, JH (2014) Bacterial subversion of host cytoskeletal machinery: hijacking formins and the Arp2/3 complex. BioEssays : news and reviews in molecular, cellular and developmental biology 36: 687-696.

Truong-Bolduc, QC, Khan, NS, Vyas, JM, Hooper, DC (2017) Tet38 Efflux Pump Affects Staphylococcus aureus Internalization by Epithelial Cells through Interaction with CD36 and Contributes to Bacterial Escape from Acidic and Nonacidic Phagolysosomes. Infection and immunity 85.

Tsuchiya, K, Kawamura, I, Takahashi, A, Nomura, T, Kohda, C, Mitsuyama, M (2005) Listeriolysin O-induced membrane permeation mediates persistent interleukin-6 production in Caco-2 cells during *Listeria monocytogenes* infection in vitro. Infection and immunity 73: 3869-3877.

Tweten, RK, Parker, MW, Johnson, AE (2001) The cholesterol-dependent cytolysins. Current topics in microbiology and immunology 257: 15-33.

Ulrichs, T, Kaufmann, SHE (2006) New insights into the function of granulomas in human tuberculosis. Journal of Pathology 208: 261-269.

Vadia, S, Seveau, S (2014) Fluxes of Ca2+ and K+ are required for the listeriolysin O-dependent internalization pathway of *Listeria monocytogenes*. Infection and immunity 82: 1084-1091.

van der Laan, LJ, Dopp, EA, Haworth, R, Pikkarainen, T, Kangas, M, Elomaa, O, Dijkstra, CD, Gordon, S, Tryggvason, K, Kraal, G (1999) Regulation and functional involvement of macrophage scavenger receptor MARCO in clearance of bacteria in vivo. Journal of immunology 162: 939-947.

van der Veen, S, Abee, T (2011) Contribution of *Listeria monocytogenes* RecA to acid and bile survival and invasion of human intestinal Caco-2 cells. International journal of medical microbiology : IJMM 301: 334-340.

van Dijk, R, Montenegro-Miranda, PS, Riviere, C, Schilderink, R, ten Bloemendaal, L, van Gorp, J, Duijst, S, de Waart, DR, Beuers, U, Haisma, HJ, Bosma, PJ (2013) Polyinosinic acid blocks adeno-associated virus macrophage endocytosis in vitro and enhances adeno-associated virus liver-directed gene therapy in vivo. Human gene therapy 24: 807-813.

van Kessel, KP, Antonissen, AC, van Dijk, H, Rademaker, PM, Willers, JM (1981) Interactions of killed *Listeria monocytogenes* with the mouse complement system. Infection and immunity 34: 16-19.

Vance, RE, Isberg, RR, Portnoy, DA (2009) Patterns of pathogenesis: discrimination of pathogenic and nonpathogenic microbes by the innate immune system. Cell host & microbe 6: 10-21.

Varol, C, Mildner, A, Jung, S (2015) Macrophages: development and tissue specialization. Annual review of immunology 33: 643-675.

Vazquez-Boland, JA, Kuhn, M, Berche, P, Chakraborty, T, Dominguez-Bernal, G, Goebel, W, Gonzalez-Zorn, B, Wehland, J, Kreft, J (2001) *Listeria* pathogenesis and molecular virulence determinants. Clinical microbiology reviews 14: 584-640.

Villwock, A, Schmitt, C, Schielke, S, Frosch, M, Kurzai, O (2008) Recognition via the class A scavenger receptor modulates cytokine secretion by human dendritic cells after contact with *Neisseria meningitidis*. Microbes and infection 10: 1158-1165.

Vishnyakova, TG, Kurlander, R, Bocharov, AV, Baranova, IN, Chen, ZG, Abu-Asab, MS, Tsokos, M, Malide, D, Basso, F, Remaley, A, Csako, G, Eggerman, TL, Patterson, AP (2006) CLA-1 and its splicing variant CLA-2 mediate bacterial adhesion and cytosolic bacterial invasion in mammalian cells. Proceedings of the National Academy of Sciences of the United States of America 103: 16888-16893.

Wagsater, D, Olofsson, PS, Norgren, L, Stenberg, B, Sirsjo, A (2004) The chemokine and scavenger receptor CXCL16/SR-PSOX is expressed in human vascular smooth muscle cells and is induced by interferon gamma. Biochemical and biophysical research communications 325: 1187-1193.

Wang, R, Chandawarkar, RY (2010) Phagocytosis of fungal agents and yeast via macrophage cell surface scavenger receptors. The Journal of surgical research 164: e273-279.

Watanabe, K, Shin, EK, Hashino, M, Tachibana, M, Watarai, M (2010) Toll-like receptor 2 and class B scavenger receptor type I are required for bacterial uptake by trophoblast giant cells. Molecular immunology 47: 1989-1996.

Welch, MD, Rosenblatt, J, Skoble, J, Portnoy, DA, Mitchison, TJ (1998) Interaction of human Arp2/3 complex and the *Listeria monocytogenes* ActA protein in actin filament nucleation. Science 281: 105-108.

Weller, D, Andrus, A, Wiedmann, M, den Bakker, HC (2015) *Listeria booriae sp. nov.* and *Listeria newyorkensis sp. nov.*, from food processing environments in the USA. International journal of systematic and evolutionary microbiology 65: 286-292.

Wheelock, EF, Sibley, WA (1965) Circulating Virus, Interferon and Antibody after Vaccination with the 17-D Strain of Yellow-Fever Virus. The New England journal of medicine 273: 194-198.

Whelan, FJ, Meehan, CJ, Golding, GB, McConkey, BJ, Bowdish, DM (2012) The evolution of the class A scavenger receptors. BMC evolutionary biology 12: 227.

White, NJ (2017) Malaria parasite clearance. Malaria journal 16: 88.

Wilkinson, K, El Khoury, J (2012) Microglial scavenger receptors and their roles in the pathogenesis of Alzheimer's disease. International journal of Alzheimer's disease 2012: 489456.

Williams, MA, Schmidt, RL, Lenz, LL (2012) Early events regulating immunity and pathogenesis during *Listeria monocytogenes* infection. Trends in immunology 33: 488-495.

Witter, AR, Okunnu, BM, Berg, RE (2016) The Essential Role of Neutrophils during Infection with the Intracellular Bacterial Pathogen *Listeria monocytogenes*. Journal of immunology 197: 1557-1565.

Workman, G, Sage, EH (2011) Identification of a Sequence in the Matricellular Protein SPARC That Interacts With the Scavenger Receptor Stabilin-1. Journal of Cellular Biochemistry 112: 1003-1008.

Wu, J, Fernandes-Alnemri, T, Alnemri, ES (2010) Involvement of the AIM2, NLRC4, and NLRP3 inflammasomes in caspase-1 activation by *Listeria monocytogenes*. Journal of clinical immunology 30: 693-702.

Wu, Z, Sawamura, T, Kurdowska, AK, Ji, HL, Idell, S, Fu, J (2011) LOX-1 deletion improves neutrophil responses, enhances bacterial clearance, and reduces lung injury in a murine polymicrobial sepsis model. Infection and immunity 79: 2865-2870.

Wuenscher, MD, Kohler, S, Bubert, A, Gerike, U, Goebel, W (1993) The iap gene of *Listeria monocytogenes* is essential for cell viability, and its gene product, p60, has bacteriolytic activity. Journal of bacteriology 175: 3491-3501.

Xayarath, B, Alonzo, F, 3rd, Freitag, NE (2015) Identification of a peptide-pheromone that enhances *Listeria monocytogenes* escape from host cell vacuoles. PLoS pathogens 11: e1004707.

Xayarath, B, Marquis, H, Port, GC, Freitag, NE (2009) *Listeria monocytogenes* CtaP is a multifunctional cysteine transport-associated protein required for bacterial pathogenesis. Molecular microbiology 74: 956-973.

Xu, J, Flaczyk, A, Neal, LM, Fa, Z, Eastman, AJ, Malachowski, AN, Cheng, D, Moore, BB, Curtis, JL, Osterholzer, JJ, Olszewski, MA (2017) Scavenger Receptor MARCO Orchestrates Early Defenses and Contributes to Fungal Containment during Cryptococcal Infection. Journal of immunology 198: 3548-3557.

Xu, Z, Xu, L, Li, W, Jin, X, Song, X, Chen, X, Zhu, J, Zhou, S, Li, Y, Zhang, W, Dong, X, Yang, X, Liu, F, Bai, H, Chen, Q, Su, C (2017) Innate scavenger receptor-A regulates adaptive T helper cell responses to pathogen infection. Nature communications 8: 16035.

Yalaoui, S, Huby, T, Franetich, JF, Gego, A, Rametti, A, Moreau, M, Collet, X, Siau, A, van Gemert, GJ, Sauerwein, RW, Luty, AJ, Vaillant, JC, Hannoun, L, Chapman, J, Mazier, D, Froissard, P (2008) Scavenger receptor BI boosts hepatocyte permissiveness to *Plasmodium* infection. Cell host & microbe 4: 283-292.

Yates, M, Kolmakova, A, Zhao, YL, Rodriguez, A (2011) Clinical impact of scavenger receptor class B type I gene polymorphisms on human female fertility. Human Reproduction 26: 1910-1916.

Yeung, OW, Lo, CM, Ling, CC, Qi, X, Geng, W, Li, CX, Ng, KT, Forbes, SJ, Guan, XY, Poon, RT, Fan, ST, Man, K (2015) Alternatively activated (M2) macrophages promote tumour growth and invasiveness in hepatocellular carcinoma. Journal of hepatology 62: 607-616.

Yoneyama, M, Kikuchi, M, Natsukawa, T, Shinobu, N, Imaizumi, T, Miyagishi, M, Taira, K, Akira, S, Fujita, T (2004) The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nature immunology 5: 730-737.

Yoneyama, M, Kikuchi, M, Matsumoto, K, Imaizumi, T, Miyagishi, M, Taira, K, Foy, E, Loo, YM, Gale, M, Jr., Akira, S, Yonehara, S, Kato, A, Fujita, T (2005) Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. Journal of immunology 175: 2851-2858.

Yoshida, T, Koide, N, Mori, I, Ito, H, Yokochi, T (2006) *Chlamydia pneumoniae* infection enhances lectin-like oxidized low-density lipoprotein receptor (LOX-1) expression on human endothelial cells. FEMS microbiology letters 260: 17-22.

Yoshikawa, K, Mitsunaga, S, Kinoshita, T, Konishi, M, Takahashi, S, Gotohda, N, Kato, Y, Aizawa, M, Ochiai, A (2012) Impact of tumor-associated macrophages on invasive ductal carcinoma of the pancreas head. Cancer science 103: 2012-2020.

Yoshikawa, Y, Ogawa, M, Hain, T, Yoshida, M, Fukumatsu, M, Kim, M, Mimuro, H, Nakagawa, I, Yanagawa, T, Ishii, T, Kakizuka, A, Sztul, E, Chakraborty, T, Sasakawa, C (2009) *Listeria monocytogenes* ActA-mediated escape from autophagic recognition. Nat Cell Biol 11: 1233-1240.

Yoshimoto, R, Fujita, Y, Kakino, A, Iwamoto, S, Takaya, T, Sawamura, T (2011) The discovery of LOX-1, its ligands and clinical significance. Cardiovascular drugs and therapy 25: 379-391.

Yu, G, Tseng, GC, Yu, YP, Gavel, T, Nelson, J, Wells, A, Michalopoulos, G, Kokkinakis, D, Luo, JH (2006) CSR1 suppresses tumor growth and metastasis of prostate cancer. The American journal of pathology 168: 597-607.

Yu, X, Guo, C, Fisher, PB, Subjeck, JR, Wang, XY (2015) Scavenger Receptors: Emerging Roles in Cancer Biology and Immunology. Advances in cancer research 128: 309-364.

Zani, IA, Stephen, SL, Mughal, NA, Russell, D, Homer-Vanniasinkam, S, Wheatcroft, SB, Ponnambalam, S (2015) Scavenger receptor structure and function in health and disease. Cells 4: 178-201.

Zenewicz, LA, Shen, H (2007) Innate and adaptive immune responses to *Listeria monocytogenes*: a short overview. Microbes Infect 9: 1208-1215.

Zevini, A, Olagnier, D, Hiscott, J (2017) Crosstalk between Cytoplasmic RIG-I and STING Sensing Pathways. Trends in immunology 38: 194-205.

Zhu, FG, Reich, CF, Pisetsky, DS (2001) The role of the macrophage scavenger receptor in immune stimulation by bacterial DNA and synthetic oligonucleotides. Immunology 103: 226-234.

Zhu, H, Lee, C, Zhang, D, Wu, W, Wang, L, Fang, X, Xu, X, Song, D, Xie, J, Ren, S, Gu, J (2013) Surface-associated GroEL facilitates the adhesion of *Escherichia coli* to macrophages through lectin-like oxidized low-density lipoprotein receptor-1. Microbes and infection 15: 172-180.

Zhu, Q, Gooneratne, R, Hussain, MA (2017) *Listeria monocytogenes* in Fresh Produce: Outbreaks, Prevalence and Contamination Levels. Foods 6.

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***Curriculum Vitae***

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## Personal Data

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## Education

**2013-2017**    PhD in biomedical sciences - Instituto de Biologia Molecular e Celular (IBMC), I3S
                   Scientific area: Health Sciences
                   Thesis title: Unraveling the role of Scavenger Receptors in *Listeria* infection
                   Supervisor: Dr. Didier Cabanes
                   Co-supervisor: Dr. Rui Appelberg

**2009-2011**    Master in Microbiology (17 values out of 20), *Universidade Católica Portuguesa – Escola Superior de Biotecnologia*
                   Scientific area: Health Sciences
                   Thesis title: Identification and characterization of new *Listeria monocytogenes* virulence factors
                   Supervisor: Dr. Didier Cabanes
                   Co-supervisor: Dra. Sandra Sousa

**2006-2009**    Degree in Microbiology (15 values out of 20), *Universidade Católica Portuguesa – Escola Superior de Biotecnologia*
                   Scientific area: Biological Sciences
                   Thesis title: The effect of wine consumption on *Lactobacillus acidophilus*
                   Supervisor: Professor José António Couto
                   Co-supervisor: Professor Tim Hogg

## Key capabilities

- Wide-ranging expertise in cell biology, molecular biology and animal experimentation.
- PhD in biomedical sciences (Host-pathogen interactions).
- Effective communication skills (speaking, writing, listening).
- Adaptability, self-motivation and team working capacity.

## Professional skills

**Research and Technical**
- <u>Cellular Biology</u>: Routine culture cell lines, cell transfection and transduction, flow cytometry, immunofluorescence, electron microscopy, immunohistochemistry.
- <u>Molecular Biology</u>: Cloning and mutagenesis, protein-RNA-DNA extraction, qRT-PCR, ChIP.
- <u>Protein Biochemistry</u>: Western Blot, immunoprecipitation, biotinylation, protein purification, ELISA.
- <u>Animal Experimentation</u>: Mice infection (oral, intravenous and intraperitoneal), organs and cells removal, perfusion, bone marrow-derived macrophages.

**Interpersonal skills**

- Strong communication skills, regular contributor to scientific conferences and project meetings giving talks and presenting posters.
- Experience writing scientific and technical reports.
- Capacity to set goals, design and plan experiments, identify problems and seek alternative solutions.
- Experience as a scientific adviser – supervision of Master students.
- Management of research budget during PhD.
- Willingness to cooperate and collaborate, willpower to learn and do always better.

**Computer skills**

- Good knowledge of Microsoft Office Word, Excel, PowerPoint
- Good knowledge of GraphPad, EndNote, Illustrator, Image J

## Participation in R&D projects

- Infect-ERA/0001/2013, IBMC, FCT (2014-2017), "Subversive pro- and anti-inflammation trade-offs promote infection by *Listeria monocytogenes*", team member.

- PTDC/SAU-MIC/111581/2009, IBMC, FCT (2011-2015), "Characterization of two new *Listeria monocytogenes virulence* genes up-regulated during in vivo infection: role of cadmium efflux system and teichoic acid decoration in Listeria pathogenicity", team member.

- ERA-PTG/0003/2010, IBMC, FCT (2009-2014), "LISTRESS. Analysis of the cellular mechanisms underlying the early response of the host to stress induced by *Listeria* infection: Teichoic acid modifications, surface protein anchoring and virulence", team member.

- PTDC/BIA-BCM/111215/2009, IBMC, FCT (2009-2014), "Unraveling the interplay between *Listeria monocytogenes* infection and the host cell cycle", team member.

## Published works

- Pombinho R, Camejo A, Vieira A, Reis O, Carvalho F, Almeida MT, Pinheiro JC, Sousa S, Cabanes D (2017) *Listeria monocytogenes* CadC Regulates Cadmium Efflux and Fine-tunes Lipoprotein Localization to Escape the Host Immune Response and Promote Infection. J Infect Dis, doi: 10.1093/infdis/jix118

- Carvalho F, Atilano ML, Pombinho R, Covas G, Gallo RL, Filipe SR, Sousa S, Cabanes D (2015) L-Rhamnosylation of *Listeria monocytogenes* Wall Teichoic Acids Promotes Resistance to Antimicrobial Peptides by Delaying Interaction with the Membrane. PLoS Pathogen, doi:10.1371/journal.ppat.1004919

- Leitão E, Costa AC, Brito C, Costa L, Pombinho R, Cabanes D, Sousa S (2014) *Listeria monocytogenes* induces host DNA damage and delays the host cell cycle to promote infection. Cell Cycle, doi:10.4161/cc.27780

- Henriques A, Carvalho F, Pombinho R, Reis O, Sousa S, Cabanes D (2012) PCR-based screening of targeted mutants for the fast and simultaneous identification of bacterial virulence factors. BioTechniques, doi:10.2144/000113906

## Poster communications

- Pombinho R., Pinheiro J., Jalkanen S., Sousa S., Cabanes D. (2016) Unraveling the role of Scavenger Receptors in *Listeria* infection. 100 years of Phagocytes 19-22 September 2016. Giardini Naxos, Sicily, Italy.

- Pombinho R., Sousa S., Cabanes D. (2014) Unraveling the role of Scavenger Receptors in *Listeria* infection. I3S Scientific Retreat 30-31 October 2014. Póvoa de Varzim, Portugal.

- Pombinho, R., Camejo, A., Vieira, A., Reis O., Carvalho F., Almeida MT., Archambaud C., Cossart P., Sousa S., Cabanes D. (2013) *Listeria monocytogenes* CadC regulates cadmium efflux and virulence genes. EMBO-FEBS Lecture Course: Host-Microbes Interactions 30 August–7 September 2013 Spetses, Greece.

- Pombinho, R., Henriques, A., Carvalho, F., Reis, O., Vieira, A., Pinheiro, J., Sousa, S., Cabanes, D. (2012) Lmo0463, a new *Listeria monocytogenes* virulence factor identified by Targeted Signature Mutagenesis and PCR Screening. I3S Scientific Retreat 10-11 May 2012. Póvoa de Varzim, Portugal.

- Pombinho, R., Ferreira, P., Camejo, A., Carvalho, F., Sousa, S., Cabanes, D. (2011) Characterization of the role of the multifunctional glyceraldehyde-3-phosphate dehydrogenase in *Listeria monocytogenes* virulence. Microbiotec 1-3 December 2011. Braga, Portugal.

- Pombinho, R., Camejo, A., Reis, O., Sousa, S., Cabanes, D. (2011) Identification and characterization of novel virulence factors implicated in *Listeria monocytogenes* virulence. I3S Scientific Retreat 5-6 May 2011. Póvoa de Varzim, Portugal.

## Other interests

- Volunteer at *Hospital S. João* in the field of neurology, pediatrics A and B (2007-2008).
- Involvement in CASO projects – Supermarket and warehouse (*Banco Alimentar Contra a Fome*).
- Football and other teamwork sports.
- Classical ballet since 1992 and modern dance since 2015.
- Surf since 2004 and wakeboard since 2015.