Characterisation of the hepatocyte response to long-term Listeria monocytogenes infection

Natalie Descoeudres

université PARIS-SACLAY

Characterisation of the hepatocyte response to long-term *Listeria monocytogenes* infection

Caractérisation de la réponse des hépatocytes à l'infection à long terme de Listeria monocytogenes

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Natalie DESCOEUDRES

Composition du Jury

Stéphanie BURY-MONÉ Professeure des Universités, Université Paris-Saclay	Présidente
Olivier DISSON Chargé de Recherche, INSERM, Institut Pasteur	Rapporteur & Examinateur
Shaynoor DRAMSI Directrice de Recherche, CNRS, Institut Pasteur,	Rapporteure & Examinatrice
Nadejda BERKOVA Directrice de Recherche, INRAE, Agrocampus Ouest Rennes	Examinatrice
Hélène BIERNE Directrice de Recherche, Institut Micalis, INRAE, Université Paris Saclay, AgroParisTech	Directrice de thèse

THESE DE DOCTORAT

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ActA	Actin-assembly-inducing protein
Akt	Protein kinase B
ALT	Alanine aminotransferase
APC	Antigen presenting cell
AP-1	Activator protein 1
APP	Acute phase protein
APR	Acute phase response
BAH	Bromo adjacent homology
BAHD1	BAH domain-containing 1
BMDM	Bone marrow derived macrophage
BMMCs	Bone marrow derived mast cell
BRD4	Bromodomain containing 4
C1q	Complement component 1q
C3	Complement component C3
C3aR1	Complement component 3a receptor 1
C5aR1	Complement C5a receptor 1 (CD88)
C5aR2	Complement C5a receptor 2
CC	Clonal complex
CCL	C-C motif chemokine ligand
CCR	C-C chemokine receptor type
CD	Cluster of differentiation
CD4 T	CD4+ helper T cell
CD8 T	CD8+ cytotoxic T cell
CDC	Cholesterol-dependent cytolysin
c-di-AMP	Cyclic di-adenosine monophosphate
CDYL	Chromodomain on Y-like
C/EBP	CCAAT/enhancer binding protein
CFU	Colony forming units
cgMLST	Core genome multilocus sequence typing
cGAMP	Cyclic guanosine monophosphate–adenosine monophosphate
cGAS	Cyclic GMP-AMP synthase
CH25H	Cholesterol 25-hydroxylase
c-Met	Hepatocyte growth factor receptor (HGFR)
CNS	Central nervous system
CP	Canonical Pathway (QIAGEN)
CR1	Complement C3b/C4b receptor 1 (CD35)
CR3	Complement receptor 3 (CD11b/CD18)
CR4	Complement receptor 4 (CD11c/CD18)
CREBBP	CREB-binding protein
CRIg	Complement receptor of the immunoglobulin superfamily
CRP	C reactive protein
CXCL	C-X-C motif chemokine ligand
CXCR	C-X-C motif chemokine receptor
DAMP	Damage associated molecular pattern
DAVID	Database for Annotation, Visualization and Integrated Discovery
DC	Dendritic cell
DEG	Differentially expressed gene
dsRNA	Double-stranded RNA
E-cad	E-cadherin
eSLAP	Epithelial spacious *Listeria*-containing phagosome
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
EU	European Union
FACS	Fluorescence-activated cell sorting
FKPM	Fragments per kilobase of transcript per million mapped reads
fMLP	N-formyl-methionyl-leucyl-phenylalanine
FC	Fold change
FOS	Fos proto-oncogene, AP-1 transcription factor subunit
FXR	Farnesoid X receptor
G9a	Euchromatic histone-lysine N-methyltransferase 2 (EHMT2)
GAF	IFN-γ activation factor
GAS	γ-activation sequence
GI	Gastrointestinal
GM-CSF	Granulocyte-macrophage colony-stimulating factor

GOBP	Gene Ontology Biological Process
H3	Histone H3
H4	Histone H4
HBMEC	Human brain microvascular endothelial cells
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HDAC	Histone deacetylase
HGF	Hepatocyte growth factor
HNF	Hepatic nuclear factor
HP	Haptoglobin
HP1	Heterochromatin protein 1
HSC	Hepatic stellate cell
Hsp60	Heat shock protein 60
ICAM-1	Intercellular adhesion molecule 1 (CD54)
IκB	Inhibitor of kappa B
IKK	IκB kinase
IFN	Interferon
IFN-I	Type I interferon: IFN-α, -β, -ε, -κ, -δ, -ζ, -ω
IFN-II	Type II interferon: IFN-γ
IFN-III	Type III interferon: IFN-λ1, -λ2, -λ3, -λ4
IFNAR	IFN-I receptor (IFNAR1/IFNAR2)
IFNLR	IFN-III receptor (IFNLR1/IL10R2)
IFNGR	IFN-γ receptor (IFNGR1/IFNGR2)
IG	Intragastric
IHL	Intrahepatic lymphocyte
IL	Interleukin
InlA	Internalin A
InlB	Internalin B
InlC	Internalin C
InlH	Internalin H
iNOS	Inducible nitric oxide synthase
IP	Intraperitoneal
IPA	Ingenuity Pathway Analysis (QIAGEN)
IRF	Interferon regulatory factor
ISG	Interferon stimulated gene
ISGF3	IFN-stimulated gene factor 3
ISRE	IFN-stimulated response element
IκB	Inhibitor of κB
IKK	IκB kinase
IV	Intravenous
JAK	Janus activated kinase
JNK	Jun N-terminal kinase
JUN	Jun proto-oncogene, AP-1 transcription factor subunit
KAP1	KRAB (Kruppel-associated box)-associated protein 1
KC	Kupffer cell
KD	Knock-down
KEGG	Kyoto Encyclopedia of Genes and Genomes
KMT	Histone lysine methyltransferase
KO	Knock-out
LAMP1	Lysosome-associated membrane protein 1
LAP	*Listeria* adhesion protein
LC3	Microtubule-associated proteins 1A/1B light chain 3B
LIPI-1	*Listeria* pathogenicity island I
LisCV	*Listeria*-containing vacuole
LLO	Listeriolysin O
Lm	*Listeria monocytogenes*
LntA	*Listeria* nuclear targeted protein A
LRR	Leucine rich repeat
LSEC	Liver sinusoidal endothelial cell
LXR	Liver X receptor
mAb	Monoclonal antibody
MAC	Membrane attack complex
Mb	Megabase pair
MAPK	Mitogen-activated protein kinase
MAVS	Mitochondrial antiviral signalling protein
MBD1	Methyl-CpG-binding domain protein 1
MBL	Mannose-binding lectin
M-CSF	Macrophage colony-stimulating factor
MHC	Major histocompatibility complex
MIER	Mesoderm induction early response protein
MLCK	Myosin light-chain kinase
MLEE	Multilocus enzyme electrophoresis
MLN	Mesenteric lymph nodes
MLST	Multilocus sequence typing
MOI	Multiplicity of infection

Mpl	Metalloprotease
MTA	Metastasis-associated protein
mTOR	Mammalian target of rapamycin
MYC	Myc proto-oncogene protein
MyD88	Myeloid differentiation factor 88
NF-κB	Nuclear factor kappa B
NI	Non-infected
NIK	NF-κB inducing kinase
NK	Natural killer
NKT	Natural killer T
NLR	NOD-like receptor
NOD	Nucleotide-binding oligomerization domain
NuRD	Nucleosome remodeling and deacetylase
ORF	Open reading frame
ORM1/2	Alpha-1-acid glycoprotein 1/2
OXPHOS	Oxidative phosphorylation
p38	Mitogen-activated protein kinase 14 (MAPK14)
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PC-PLC	Phosphatidyl-choline phospholipase C
PFGE	Pulsed-field gel electrophoresis
PFT	Pore-forming toxin
p.i.	Post-infection
PI3K	Phosphoinositide 3-kinase
PI-PLC	Phosphatidyl-inositol phospholipase C
PlcA	Phosphatidylinositol-specific phospholipase C
PlcB	Phospholipase C/sphingomyelinase
PMH	Primary mouse hepatocytes
PMN	Polymorphonuclear cell
PPAR	Peroxisome proliferator-activated receptor
PPP-RNA	5'triphosphate RNA
PrfA	Positive regulatory factor A
PRR	Pathogen recognition receptor
PTM	Post-translational modification
RE	Response element
RER	Rough endoplasmic reticulum
RIG-I	Retinoic acid-inducible gene I
RNA-seq	RNA-sequencing
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT-qPCR	Reverse transcription quantitative PCR
SAA	Serum amyloid A
SCID	Severe combined immunodeficiency
SER	Smooth endoplasmic reticulum
SIRT2	NAD-dependent protein deacetylase sirtuin-2
SLAP	Spacious *Listeria*-containing phagosomes
SNP	Single-nucleotide polymorphism
SR	Scavenger receptor
SREBP2	Sterol regulatory element-binding protein 2
ST	Sequence type
STAT	Signal transducer and activator of transcription
STING	Stimulator of interferon genes
TAK1	TGF-β-activated kinase 1
TBK1	Serine/threonine-protein kinase
TEM	Transmission electron microscopy
TGF-β	Transforming growth factor beta
Tip-DC	TNF- and iNOS-producing DC
TIR	Toll/interleukin-1 receptor
TIRAP	TIR domain containing adaptor protein
TF	Transcription factor
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TRIF	TIR-domain-containing adapter-inducing interferon-β
TYK2	Tyrosine kinase 2
URA	Upstream Regulator Analysis (QIAGEN)
VBNC	Viable but nonculturable
wgMLST	Whole genome MLST
WASP	Wiskott-Aldrich syndrome protein
WAVE	WASP-family verprolin-homologous protein
WGS	Whole genome sequencing
WT	Wild type

INTRODUCTION

A. *Listeria monocytogenes* and listeriosis

Listeria monocytogenes (*Lm*) is a foodborne pathogen that causes listeriosis, one of the most dangerous foodborne bacterial zoonosis. Although rare, listeriosis has a high case fatality rate and is of particular concern for pregnant women. *Lm* is a Gram-positive bacterium that is extremely adaptable, capable of transiting from life as a soil-living saprophyte to that of an invasive pathogen, adept at survival and proliferation in the host cell. This adaptability increases *Lm*'s foodborne pathogen potential, as the bacterium can resist the high salt and acid and low oxygen concentrations and low temperatures used for food conservation. *Lm*'s adaptability has also facilitated this bacterium's use in the laboratory and has seen *Lm* emerge as an extremely versatile model pathogen over the last half of a century. The analysis of the *Lm* infectious process both *in vitro* and *in vivo* has led to numerous breakthroughs in our understanding of cell biology, host-pathogen interactions, and innate and cell mediated immunology.

1. Discovery

Lm was first described in 1926 by Murray, Webb and Swann following their meticulous investigation into the cause of the sudden and concomitant death of six laboratory rabbits. The isolated causative microorganism was named *Bacterium monocytogenes*: the "indefinite" *Bacterium* because it couldn't be attributed at the time to a known genus, and *monocytogenes* due to the characteristic production of large mononuclear leukocytosis in infected animals (Murray et al., 1926). A year later, Pirie et al., reported the isolation of the same species in the liver of wild gerbils (Mitchell et al., 1927). He initially named it *Listerella hepatolytica* – the specific epithet in honour of Lord Joseph Lister, the pioneer of antisepsis, and the generic because of the liver lesions observed in infected animals – before withdrawing *hepatolytica* in favour of *monocytogenes* to acknowledge Murray's discovery. Pirie subsequently proposed the generic name *Listeria* in 1940 due to *Listerella* having already been attributed to a mycetozoan (Pirie, 1940). It is likely that the first cases of human listeriosis were documented well before the first characterisations of the bacteria by Murray et al., with reports of "pseudotuberculosis" and "neonatal septicaemia" due to "argentophile rods" as early as 1891. The first confirmed report, however, was in 1929 by Nyfeldt et al. (Gray and Killinger, 1966; Seeliger, 1988). Human cases remained sporadic, however, and listeriosis was essentially regarded as a rare zoonosis, its epidemiology an unresolved mystery. Laboratory interest for the bacterium grew from the mid- to late 1950s with the pioneering work of Mackaness et al. in cellular immunity

using *Lm* as a prototypic intracellular parasite (Mackaness, 1962). The first International Symposium on Problems of *Listeria* and Listeriosis (ISOPOL) was held in 1957 and has since seen *Lm* become one of the most well-established models in the study of host-pathogen interactions (Cossart, 2011; Lebreton et al., 2016; Lecuit, 2020; Radoshevich and Cossart, 2017). It wasn't until the early 1980s, however, that *Lm*'s importance as a risk to public health and food safety was formally recognised (Schlech et al., 1983), and tremendous research efforts have since been employed in order to protect consumers from listeriosis.

2. Taxonomy, phylogeny and classification

The *Listeria* genus belongs to the Listeriaceae family (along with the *Brochothrix* genus), the Bacillales order (as do the genera *Bacillus* and *Staphylococcus* to whom *Listeria* is closely related), the Bacilli class and the Firmicutes phylum. The *Listeria* genus comprises 26 species, 20 of which have been discovered since 2009, including five in 2021 (Carlin et al., 2021). *Listeria* species are further subdivided into two clades: "*Listeria sensu stricto*" (clade I) and "*Listeria sensu lato*" (or clade II). *Lm* and eight other species (*L. ivanovii*, *L. seeligeri*, *L. welshimeri*, *L. innocua*, *L. marthii*, *L. farberi*, *L. immobilis*, and *L. cossartiae*) form the *Listeria sensu strictu* clade, a tight monophyletic group within the genus. Members of this clade have been found in faeces or the gastrointestinal (GI) tract of animals, as well as in food of animal origin, and two – *Lm* and *L. ivanovii* – are pathogens, the latter affecting predominantly ovine and bovine (Disson et al., 2021; Orsi and Wiedmann, 2016; Schardt et al., 2017). Members of the second clade, "*Listeria sensu lato*", are believed to be environmental bacteria unable to colonise mammalian hosts (Schardt et al., 2017).

3. *Lm* intraspecies biodiversity

3.1. Serotypes

Serotyping was the first method employed to investigate the biodiversity of *Lm*, elaborated by Paterson et al. (Paterson, 1940), and extended by Seeliger et al., among others (Seeliger and Langer, 1989). Adding an antiserum raised against one of 15 different somatic (O) or one of four flagellar (H) *Lm* surface antigens to a strain displaying the same antigen results in agglutination. Thirteen different *Lm* serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7) have been identified based on unique combinations of O and H antigens (Seeliger and Langer, 1989). Traditional immunological serotyping has since been replaced by genetic-based molecular serotyping (or genoserotyping), eliminating the need for antisera (Doumith et

al., 2004a; Nightingale et al., 2007). While genoserotyping remains a first step in routine strain analysis, it is of poor discriminatory power as the majority of food and clinical isolates belong to 1/2a, 1/2b, 1/2c or 4b serotypes (Datta and Burall, 2018; Liu, 2006).

3.2. Lineages

Other phenotypic (multilocus enzyme electrophoresis (MLEE) (Graves et al., 1994; Piffaretti et al., 1989)) and genetic subtyping methods (pulsed-field gel electrophoresis (PFGE) (Brosch et al., 1991; 1994), ribotyping (Graves et al., 1994; Wiedmann et al., 1997), partial virulence gene sequencing (Rasmussen et al., 1995; Roberts et al., 2006), DNA arrays (Doumith et al., 2004b), and multilocus genotyping (Ward et al., 2008)) allowed the clustering of *Lm* serotypes into four distinct evolutionary lineages (I, II, III, and IV). The two main lineages I and II, into which the majority of *Lm* isolates cluster, were first identified in 1989 by MLEE (Piffaretti et al., 1989), and include serotypes 1/2b, 3b, 7, 4b, 4d et 4e (lineage I), and 1/2a, 1/2c, 3a et 3c (lineage II). Although isolates from all four lineages have been associated with human listeriosis, lineage I and in particular serotype 4b occurs more frequently among clinical isolates, whereas lineage II isolates (chiefly 1/2a and 1/2c) are overrepresented among isolates from food and environmental sources (Orsi et al., 2011).

A third lineage was identified in 1995 (Rasmussen et al., 1995), and further subdivided into 3 subgroups: IIIA, IIIB, IIIC (Roberts et al., 2006); the evolutionary diverse subgroup IIIB later became lineage IV (Ward et al., 2008). Lineage III and IV isolates are rare and are predominantly isolated from animal sources (Lee et al., 2018; Orsi et al., 2011).

3.3. Sequence type, clonal complex, cgMLST type, and sublineage

Our understanding of *Lm* ecology and evolution advanced greatly with the development and world-wide application of multilocus sequence typing (MLST) (Stessl et al., 2014). MLST exploits the sequence variation in seven housekeeping genes to class *Lm* strains into sequence types (ST) based on allelic profiles (Ragon et al., 2008; Salcedo et al., 2003). Isolates with the same allelic profile are grouped in a single ST, and STs differing by only one gene are grouped into a clonal complex (CC) (Ragon et al., 2008). Serotypes were confirmed to be markers of *Lm* biodiversity, with each CC having a unique or dominant serotype. The absence of even one common allele among the three lineages confirmed the existence of four distinct evolutionary lineages (Ragon et al., 2008) (Figure 1).

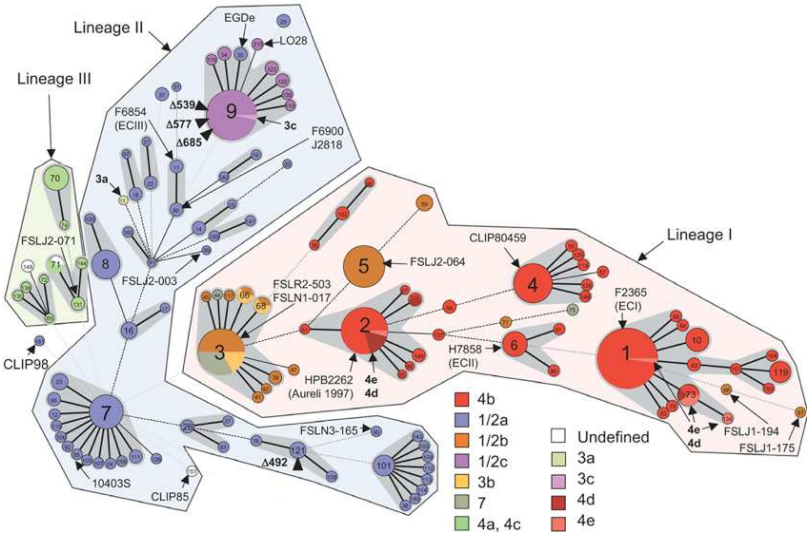

Figure 1 | Phylogenetic tree of *Lm* strains constructed using MLST data.
Minimum spanning tree analysis of 360 *Lm* strains based on MLST data. Each circle corresponds to a ST that is coloured according to the serotype of the individual isolates it contains. CCs are indicted by grey zones that regroup STs and are numbered according the dominant ST of the group (enlarged numbers). The three major lineages are represented by polygons. The lines between STs indicate inferred phylogenetic relationships and are represented as bold, plain, discontinuous and light discontinuous depending on the number of allelic mismatches between profiles (1, 2, 3 and 4 or more, respectively). As no common alleles exist between the three major lineages, they were arbitrarily linked through ST7. (Adapted from Ragon et al., 2008)

Several medium to large scale MLST analyses have highlighted the clonal nature of *Lm* (the majority of isolates cluster into a small number of CCs) and have identified the most prevalent STs and CCs and their ecological distribution (Cabal et al., 2019; Chenal-Francisque et al., 2011; Filipello et al., 2020; Henri et al., 2016; Jennison et al., 2017; Maury et al., 2019; 2016; Painset et al., 2019; Ragon et al., 2008). CC1, CC2, CC4, and CC6 are strongly associated with a clinical origin whereas CC121 and CC9 are overrepresented in environmental samples, confirming the uneven prevalence of *Lm* isolates of food versus clinical origin previously observed at the lineage and serotype levels (Orsi et al., 2011) (Figure 2).

With large scale sequencing increasingly accessible and affordable, routine sequencing of strains is now standard practice in several countries including the USA (Jackson et al., 2016), the UK (McLauchlin et al., 2020), Australia (Kwong et al., 2016a), France (Moura et al., 2017), Germany (Halbedel et al., 2020), Austria (Cabal et al., 2019), and Denmark (Jensen et al., 2016b). Several whole genome sequencing (WGS) approaches have been developed, based either on single nucleotide polymorphisms within the whole genome (Jagadeesan et al., 2019), or whole genome (wg) (Jackson et al., 2016; Schmid et al., 2014) or core gene (cg) (Chen et

al., 2016; Moura et al., 2016; Ruppitsch et al., 2015) allelic profiling similar to MLST in which the set of genes is increased from seven to 1701 (Ruppitsch et al., 2015), 1748 (Moura et al., 2016), 1827 (Chen et al., 2016), 2298 (Schmid et al., 2014), or 4804 (Jackson et al., 2016). cgMLST defines cgMLST types and sublineages by allelic distances of 7 and 150 genes respectively. cgMLST sublineages largely correspond to CCs, facilitating the matching of data from MLST and cgMLST analyses (Moura et al., 2016).

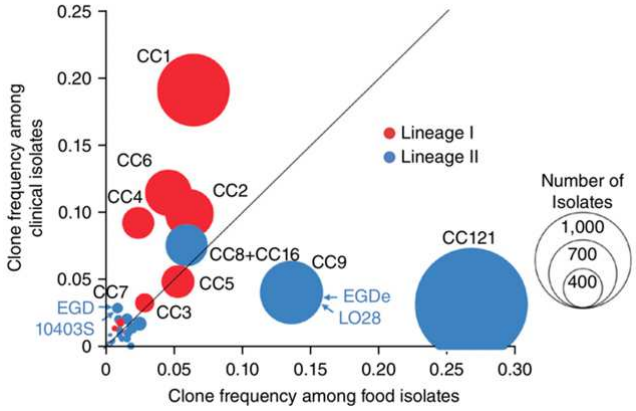

Figure 2 | Prevalence and distribution of MLST clones in food and clinical sources.
The frequencies of clones among food (*x* axis) and clinical (*y* axis) isolates are shown by a circle whose size is proportional to the number of isolates. (Source: Maury et al., 2016)

3.4. Strain discrimination for epidemiological analysis

For outbreak detection, surveillance and source-tracking, highly discriminative molecular subtyping methods are instrumental (Jensen et al., 2016a). PFGE has long been the current gold standard in *Lm* strain discrimination (Gerner-Smidt et al., 2006; Graves et al., 1994) and has been essential for outbreak investigation worldwide (Jackson et al., 2016; Ruppitsch et al., 2015). WGS-based strain discrimination methods, however, surpass all others in discriminatory power and have transformed real-time listeriosis surveillance, increasing substantially the number of clinical cases that could be linked to a specific food or to other seemingly unrelated clinical cases (Jackson et al., 2016; McLauchlin et al., 2020). As an example, WGS data and its immediate publication allowed an isolated case in Australia to be linked to a USA outbreak involving stone fruit; the imported fruit was subsequently recalled, preventing any further contaminations (Kwong et al., 2016b). This illustrates the utility of WGS-based typing methods but also the need for the standardisation of protocols, tools, and nomenclature, and the rapid public release of data, an objective yet to be achieved (Barretto et al., 2021; Kwong et al., 2016b).

4. General microbiology

Lm is a Gram-positive, rod-shaped (~0.5 × 1–2 µm), non-encapsulated, non-sporulating, facultative anaerobic bacterium (Thakur et al., 2018). With its 2–6 polar peritrichous flagella, it exhibits a characteristic tumbling motility at temperatures of up to 30°C, but motility – at least in laboratory strains – is repressed by thermosensitive transcriptional repression of flagellar export apparatus genes at 37°C (Lebreton and Cossart, 2017), as visible by transmission electron microscopy (TEM) (Figure 3). All *Listeria* spp. are catalase positive, indole and oxidase negative, and can hydrolyse aesculin, but not urea; these common biochemical characteristics have been used to determine genus (Liu, 2006). *Lm* is morphologically indistinguishable from the other *Listeria* species but can be distinguished through a set of biochemical tests that determine haemolytic activity (only *Lm*, *L. seeligeri*, and *L. ivanovii* are haemolytic), and the ability to produce acid from L-rhamnose, D-xylose, or α-methyl-D-mannoside (of the 3 haemolytic species, only *Lm* can ferment L-rhamnose and α-methyl-D-mannoside, but not D-xylose) (Allerberger, 2003).

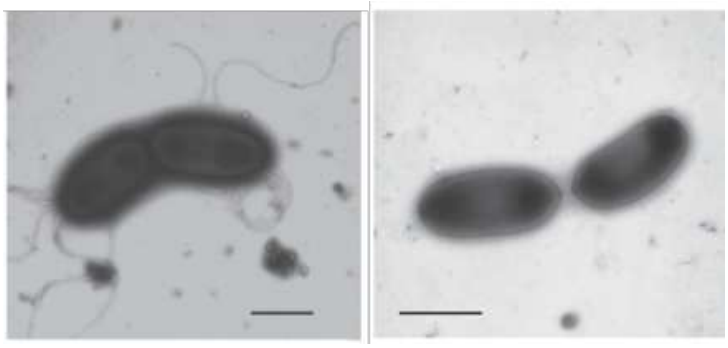

Figure 3 | TEM images of *Lm* in liquid culture at 20°C and 36°C.
Flagellated bacteria are observed at 20°C (left) but not at 36°C (right) (scale bar: 1 µm). (Source: Allerberger et al., 2015)

Lm can multiply at temperatures ranging from 0–45°C, with optimal growth at 30–37°C, at which the growth peak is reached in 16–18 h (Gray and Killinger, 1966; McLauchlin et al., 2014; Membré et al., 2005). *Lm* can also maintain growth at pH 4.6–9.2, as well as at high osmotic pressure (growth at up to 10% NaCl, survival at up to 20–30%), and in microaerobic conditions (Rees et al., 2017). This resistance to high salt and acid concentrations, low oxygen conditions, and the capacity to multiply at refrigeration temperatures used for food processing and preservation, as well as the ability to form antimicrobial-resistant biofilms, renders *Lm* a serious concern for food safety (Colagiorgi et al., 2017; Vázquez-Boland et al., 2001). The biological properties and robustness of *Lm* also contribute to its ubiquitous nature: *Lm* is widely

distributed throughout the environment and is found in soil, vegetation, water, sewage, and in the GI tract and faeces of humans and many species of animals, as well as in the food processing environment and in a large variety of foods. The natural habitat of *Lm* is thought to be decomposing plant matter, however, in which this species lives as a saprophyte (Abu Mraheil et al., 2013; Vázquez-Boland et al., 2001).

The genome organisation of *Listeria* spp. is highly conserved. *Lm* contains one circular chromosome of approximately three Mb, with an average G+C content of 38%, containing approximately 3000 protein-coding sequences, two-thirds of which are common to all species of the genus (Bakker et al., 2010; Buchrieser, 2007, Glaser et al., 2001).

5. Pathology

5.1. The epidemiology of human listeriosis

5.1.1. Transmission, clinical manifestations, outcomes, and treatment

Lm's importance as a risk to public health and food safety was recognized in the early 1980s when the investigation of a Canadian outbreak with a high case-fatality rate provided conclusive evidence of foodborne transmission (Schlech et al., 1983). Listeriosis is today considered to occur almost exclusively through the consumption of contaminated food, with the exception of vertical transmission from the mother to the unborn foetus and transmission during birth (Rees et al., 2017). *Lm* is responsible for a quarter of the burden of food-related deaths in the USA (Scallan et al., 2011) and in France (Van Cauteren et al., 2017) (Table 1). Rare cases of transmission from animals to humans or humans to humans have however been documented (Allerberger and Wagner, 2010; Scallan et al., 2011).

Listeriosis is used to describe both the mild non-invasive form of the disease, as well as the severe invasive form. Mild non-invasive listeriosis manifests as a typical febrile gastroenteritis and affects immuno-competent adults and children (Allerberger and Wagner, 2010; Swaminathan and Gerner-Smidt, 2007) or, rarely, as cutaneous listeriosis (Godshall et al., 2013). These non-invasive forms of listeriosis are generally self-limiting and resolve without antibiotic treatment (Dalton et al., 1997; Godshall et al., 2013; Swaminathan and Gerner-Smidt, 2007). Invasive listeriosis generally occurs when cell-mediated immunity is impaired, and manifests in three forms: bacteraemia, neurolisteriosis, and maternal-neonatal listeriosis, where *Lm* has crossed the intestinal barrier, or the intestinal and blood-brain barriers, or the intestinal

and placental barriers, respectively (Charlier et al., 2017; Madjunkov et al., 2017). Diagnosis is based on the isolation of *Lm* or the detection of *Lm* nucleic acid from a normally sterile site (blood, cerebrospinal fluid, or placental sample), or for pregnancy-associated cases also non-sterile sites such as vaginal swabs (European Food Safety Authority, European Centre for Disease Prevention and Control, 2021).

Foodborne pathogen	Foodborne transmission (% total cases)	Hospitalisations (% total)	Deaths (% total)
Bacillus cereus	100%	2,6	0,4
Campylobacter spp.	73-86%	31,4	16,0
Clostridium botulinum	100%	0,1	0,1
Clostridium perfringens	100%	4,6	0,8
STEC	59-87%	2,1	1,6
Listeria monocytogenes	100%	1,8	25,4
Salmonella spp.	91-95%	23,3	26,2
Shigella spp.	23-40%	0,4	0,1
Staphylococcus aureus	100%	2,8	0,5
Yersinia spp.	80-100%	1,1	3,5
Total bactéries		70,3	74,6
Total virus		23,8	12,1
Total parasites		6	13
Total		**100**	**100**

Table 1 | Estimated percent of foodborne disease related hospitalisations and deaths attributed to *Lm* compared to other pathogens.
Around 2% of foodborne disease related hospitalisations and 25% of deaths are attributed to *Lm* each year in France. (Adapted from Van Cauteren et al., 2017)

Maternal–neonatal listeriosis affects pregnant women, foetuses, or infants up to one month old and while the mother presents only a fever and/or flu-like symptoms – or no symptoms at all – the maternal infection often has severe consequences on the pregnancy outcome or on the new-born infant (Charlier et al., 2017; Elinav et al., 2014; Jeffs et al., 2020). Spontaneous abortion or stillbirth occur in around 25% of cases and of the remaining pregnancies, many are pre-term, and up to two-thirds of neonates are infected (Charlier et al., 2017; Elinav et al., 2014; Girard et al., 2014; Jeffs et al., 2020; McLauchlin, 1990). Neonatal listeriosis is most commonly of early onset (diagnosis in the first two days of life), and presents as pneumonia, sepsis, or meningitis with poor prognosis (Charlier et al., 2017; Girard et al., 2014; Mylonakis et al., 2002). Only 50% of maternal blood cultures of confirmed maternal-listeriosis cases are positive for *Lm* however (Charlier et al., 2017; Mylonakis et al., 2002), and diagnosis of early foetal loss may therefore be under-estimated due to the variable, non-specific (or lack of) symptoms experienced by the mother, and the absence of microbiological testing of aborted foetal tissue that would confirm infection (Lamont and Postlethwaite, 1986). Today, maternal-neonatal listeriosis represents 10–20% of all invasive listeriosis cases (Charlier et al., 2017; de

Noordhout et al., 2014), a sharp decrease from studies anterior to 1990 where 30–55% of listeriosis cases were maternal-neonatal (Cherubin et al., 1991; Gellin et al., 1991; McLauchlin, 1990).

Non-pregnancy related invasive listeriosis manifests as septicaemia with or without the involvement of the central nervous system (CNS) (meningitis most commonly but also meningoencephalitis, encephalitis, and rhombencephalitis) (Schlech, 2019). Endovascular infections (endocarditis or aneurysm/prosthetic graft infections) have also been reported, and although rare (representing 1% of non-maternal-neonatal listeriosis cases in France), mortality in *Lm*-associated endocarditis is twice as high as that reported for other pathogens (Shoai-Tehrani et al., 2019).

Bacteraemia is currently the most common clinical form of listeriosis, observed in around 60–80% of non-maternal-neonatal listeriosis cases. The remaining 20–40% of cases involve the CNS, with or without bacteraemia (Charlier et al., 2017; de Noordhout et al., 2014; Jensen et al., 2016a; Scobie et al., 2019). *Lm* is responsible for approximately 5% of bacterial meningitis and 1–4% of infectious encephalitis cases in adults, with, as for endovascular infections, poorer prognosis compared to other etiological forms of these diseases (Granerod et al., 2010; Mailles et al., 2011; 2009; Putz et al., 2013; Quist-Paulsen et al., 2013; Sunwoo et al., 2021; van de Beek et al., 2004). Prognosis for non-pregnancy related listeriosis is poor, with case fatality rates of around 20–40% reported for both bacteraemia and neurolisteriosis (Charlier et al., 2017; de Noordhout et al., 2014; Mook et al., 2012; Scobie et al., 2019). A high proportion (25–50%) of neurolisteriosis survivors suffer neurological sequelae (Büla et al., 1995; Charlier et al., 2017; Koopmans et al., 2013). The hospitalisation rate (estimated at 92% in the European Union (EU) (European Food Safety Authority, European Centre for Disease Prevention and Control, 2021) and 94% in the USA (Scallan et al., 2011)) and mortality rate of listeriosis are indeed the highest of any zoonotic pathogen in the western world, and prognosis has not improved over the last decades (Charlier et al., 2017; European Food Safety Authority, European Centre for Disease Prevention and Control, 2021) (Table 2).

Listeriosis presents an unusually long and highly variable incubation period that depends, at least in part, on the clinical form of the disease. While short incubation periods (around 24 h) are generally observed for non-invasive listeriosis – similar to those observed for other enteric bacteria such as *Salmonella* – incubation periods exceeding 10 days have been documented (Goulet et al., 2013; McIntyre et al., 2015). The asymptomatic incubation period for invasive

listeriosis can be much longer, with a median of around 10 days (range 1–70) and incubation periods superior to 28 days reported in around 15% of cases, the majority of which are maternal-neonatal (Angelo et al., 2016; Goulet et al., 2013).

Disease	Number of confirmed human cases	Hospitalisation				Deaths			
		Status available (%)	Number of reporting MS[b]	Reported hospitalised cases	Proportion hospitalised (%)	Outcome available (%)	Number of reporting MS[b]	Reported deaths	Case fatality (%)
Campylobacteriosis	220,682	29.1	16	20,432	31.8	78.0	17	47	0.03
Salmonellosis	87,923	44.5	15	16,628	42.5	71.8	17	140	0.22
STEC infections	7,775	37.3	18	1,100	37.9	61.0	20	10	0.21
Yersiniosis	6,961	27.4	15	648	33.9	57.0	14	2	0.05
Listeriosis	2,621	51.1	19	1,234	92.1	65.1	20	300	17.6
Tularaemia	1,280	22.8	12	149	51.0	21.6	13	1	0.36
Echinococcosis	739	33.3	14	109	44.3	31.4	14	2	0.86
Q fever	950	NA[c]	NA	NA	NA	67.3	13	4	0.63
West Nile virus infection[a]	443	83.7	9	347	93.5	99.3	11	52	11.8
Brucellosis	310	44.5	11	98	71.0	36.8	12	2	1.75
Trichinellosis	96	16.7	5	6	37.5	25.0	7	1	4.20
Rabies	4	NA[c]	NA	NA	NA	75.0	3	3	100.0

MS: Member State.
(a): Instead of confirmed human cases, the total number of human cases was included.
(b): Not all countries observed cases for all diseases.
(c): NA: Not applicable as the information is not collected for this disease.

Table 2 | Reported hospitalisations and case fatalities due to zoonoses in confirmed human cases in the EU, 2019. (Source: European Food Safety Authority, European Centre for Disease Prevention and Control, 2021)

Standard treatment for all forms of listeriosis is intravenous administration of β-lactam antibiotics (ampicillin or penicillin) – or meropenem or trimethoprim-sulfamethoxazole in the case of allergy – and an aminoglycoside such as gentamicin (Schlech, 2019). The recommended duration of treatment is at least two weeks for bacteraemia, and three weeks for meningitis (Donovan, 2015).

5.1.2. Prevalence

Reliable epidemiological data is generally only available for developed countries where listeriosis is a notifiable disease, and effective laboratory surveillance networks have been put in place, such as in the European Economic Area member states (European Food Safety Authority, European Centre for Disease Prevention and Control, 2021), the USA (Scallan et al., 2011), Australia (Kirk et al., 2014), and New Zealand (Jeffs et al., 2020). As diagnosis is based on the confirmed presence of *Lm* (as described in section A.5.1.1), only the severe, invasive form of the disease is notified (Official Journal of the EU, 06.07.2018, page 28). Non-invasive listeriosis is thus rarely diagnosed or reported, partly because *Lm* is not detected by routine stool culture, and partly because symptoms are generally non-specific and self-limiting, with the illness generally resolving spontaneously without antibiotic treatment within 48 h (Dalton et al., 1997; Donovan, 2015; Salamina et al., 1996). Invasive listeriosis has a low

incidence rate, with three to six cases per million population (de Noordhout et al., 2014; Fox et al., 2012; Jeffs et al., 2020; Kirk et al., 2014; Scallan et al., 2011); the most recent data from the European Food Safety Authority (EFSA) documented 4.6 confirmed cases per million in 2019 in member states (European Food Safety Authority, European Centre for Disease Prevention and Control, 2021). Pregnancy-associated listeriosis is today reported to affect 4–10 per 100 000 pregnant women (Charlier et al., 2020) and has significantly decreased since the 1980s in both the USA and the EU, independent of the incidence rate of non-maternal-neonatal listeriosis (Bertrand et al., 2016; Girard et al., 2014).

Although outbreaks often make headlines and involve a large number of cases, as the most recent large outbreak of 947 cases in South Africa highlighted (Thomas et al., 2020), listeriosis is mainly sporadic, with only 13.4% of invasive listeriosis cases in the EU in 2019 attributed to outbreaks (European Food Safety Authority, European Centre for Disease Prevention and Control, 2021). This proportion is likely to increase however as the global shift to WGS-based typing methods in foodborne-pathogen surveillance accelerates the identification of epidemically related clusters, as described in section A.3.4.

5.1.3. Risk factors

Risk factors for invasive listeriosis are multiple and combinatorial and include the risk of exposure to contaminated food and the level of contamination, the virulence of the *Lm* strain, as well as host susceptibility to infection (EFSA Panel on Biological Hazards (BIOHAZ) et al., 2018). Foods at risk include ready-to-eat food products, meat and fish products, and dairy products, especially soft and semi-soft cheeses (EFSA Panel on Biological Hazards (BIOHAZ) et al., 2018). Specific ethnic or socioeconomic populations have been shown to be more at risk of listeriosis because of increased consumption of certain foods (Elinav et al., 2014; Jackson et al., 2018; Pohl et al., 2019). The dose-response relationship of *Lm*, which represents an essential component of risk assessment, is still a pivotal question (Allerberger et al., 2015) and depends on both the strain and the susceptibility of the host (Pouillot et al., 2015). In an identified outbreak of 45 cases of exclusively non-invasive listeriosis in healthy individuals, the median dose of *Lm* may have been as high as 2.9×10^{11} organisms (Dalton et al., 1997). On the other hand, an infectious dose of approximately 10^3 cells was sufficient to cause invasive disease and death in highly susceptible hospitalised patients (Pouillot et al., 2016). Prediction models based on outbreak data estimate that both the susceptibility of the host and the virulence of the strain can each modify by at least 100-fold the probability of developing invasive

listeriosis, with this probability increasing approximately 10-fold for every log increase in the ingested dose (Farber et al., 1996; Pouillot et al., 2015; 2016).

Certain conditions have been identified as risk factors: pregnant women are up to 100 times more likely to develop listeriosis than healthy adults with no comorbidities because of impaired cell-mediated immunity during pregnancy and *Lm*'s placental tropism (Charlier et al., 2020) (Table 3). For non-pregnancy-related invasive listeriosis: advanced age, non-haematological cancer, diabetes, and heart disease concern the most individuals but the highest risk-associated comorbidities identified are haematological malignancies in general and chronic lymphocytic leukaemia in particular (relative risk of 1488), and liver cancer (relative risk of 748), both of which also had a higher risk of mortality (Goulet et al., 2012). The use of gastric acid-suppressing drugs such as protein pump inhibitors, histamine-2 receptor antagonists and antacids has also been associated with an increased risk of listeriosis (Fisher and Fisher, 2017; Mook et al., 2012). Less than 10% of invasive listeriosis cases are thus observed in non-pregnant healthy adults under 65 years of age (Table 3).

Population subgroup	Number of individuals per subgroup	Percent of total population	Number of cases per subgroup	Percent of total cases	Relative risk (CI 95%)
Less than 65 years old, no known underlying condition (i.e., "healthy adult")	48 909 403	76.71%	189	9.65%	Reference group
More than 65 years old, no known underlying condition	7 038 068	11.04%	377	19.24%	13.9 (8.6, 23.1)
Pregnancy	774 000	1.21%	347	17.71%	116 (71, 194.4)
Non-haematological cancer	2 065 000	3.24%	437	22.31%	54.8 (34.2, 90.3)
Haematological cancer	160 000	0.25%	231	11.79%	373.6 (217.3, 648.9)
Renal or liver failure (dialysis, cirrhosis)	284 000	0.45%	164	8.37%	149.4 (82, 270.1)
Solid organ transplant	25 300	0.04%	16	0.82%	163.7 (26.3, 551.5)
Inflammatory diseases (rheumatoid arthritis, ulcerative colitis, giant cell arthritis, Crohn's disease)	300 674	0.47%	68	3.47%	58.5 (25.2, 123.4)
HIV/AIDS	120 000	0.19%	22	1.12%	47.4 (10.5, 140.4)
Diabetes (type I or type II)	2 681 000	4.20%	79	4.03%	7.6 (3.5, 15.6)
Heart diseases	1 400 000	2.20%	29	1.48%	5.4 (1.5, 14.4)
Total population	63 757 445	100%	1 959	100%	

Table 3 | Incidence of listeriosis and relative risk of incidence per subgroup of the French population with underlying conditions. (Adapted from Goulet et al., 2012; Pouillot et al., 2015)

CCs have been termed as "infection-associated", "food-associated" or "intermediate" depending on the relative proportion of isolates from clinical cases, food or both (Maury et al., 2016; 2019) and there is ample *in vivo* and *in vitro* evidence that "infection-associated" clones are more virulent (Ghosh et al., 2019; Maury et al., 2016; 2019; Stratakos et al., 2020; Vázquez-Boland et al., 2020). However, the more susceptible the host, the more likely even a hypovirulent, food-associated clone of lineage II will cause invasive disease (Figure 4).

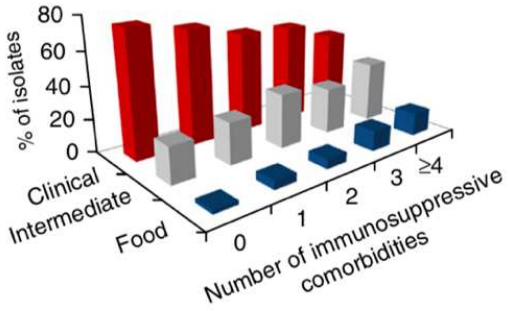

Figure 4 | The infectious potential of MLST clones.
The histograms show the distribution of food-associated (food; CC9 and CC121; blue), infection-associated (clinical; CC1, CC2, CC4 and CC6; red) and intermediate (intermediate; CC8+CC16, CC5, CC3, CC37, CC155 and CC18; grey) clones in patient groups with different numbers of immunosuppressive comorbidities. (Source: Maury et al., 2016)

5.1.4. Asymptomatic carriage

As *Lm* is frequently isolated from the environment, from food, and from the faeces of healthy adults and ruminants, and yet invasive listeriosis is so rare, the question of the existence of asymptomatic carriage of *Lm* is naturally raised (Painter and Slutsker, 2007). The unusually long asymptomatic incubation period observed in listeriosis compared to other foodborne pathogens further suggests the existence of a silent, subclinical phase during *Lm* host colonisation (Vázquez-Boland et al., 2001). This silent phase could either persist long-term to be qualified as asymptomatic carriage or develop into invasive listeriosis within a highly variable timeframe (as described in section A.5.1.1). Asymptomatic persistence could also occur following invasive disease, as suggested in the rare documented cases of recurrent listeriosis. Recurrent listeriosis in children and non-pregnant adults in which strains isolated from the paired episodes were indistinguishable by genetic subtyping strongly suggests incomplete eradication of the original infection, chronic infection or long-term carriage, and subsequent recrudescence of infection (Ciceri et al., 2017; Levett et al., 1993; McLauchlin et al., 1986; 1991; Sauders et al., 2001). Cases were separated by intervals as short as three weeks, but also as long as 2 years. Recurrence of listeriosis within three months was reported in 1% of cases in a recent national study (Charlier et al., 2017). A case of delayed bacteraemia was observed following the administration to a cancer patient of a highly attenuated *Lm* strain used as a tumour immunotherapy vaccine vector. PFGE and wgMLST confirmed the isolated strain as identical to the *Lm* vaccine vector that had been administered 31 months prior to the bacteraemia (Fares et al., 2018). The colonisation of prosthetic material could be implicated in some cases of recurrent listeriosis but cannot explain the majority (Ciceri et al., 2017). The

possibility of asymptomatic carriage is of particular concern in women, as recurrent foetal loss due to *Lm* has been suggested but unfortunately never explored or rigorously documented (Gray and Killinger, 1966; Rocourt et al., 2000). Humans are exposed regularly to *Lm*: it is estimated that adults ingest 10^7 *Lm* organisms approximately once a year, and around 10^5 *Lm* at least four times per year, with smaller doses ingested much more frequently (Hitchins, 1996; Notermans et al., 1998). Large scale studies have isolated *Lm* from the faeces of around 1–5% of healthy adults (Grif et al., 2001; Iida et al., 1998; MacGowan et al., 1991; Mascola et al., 1992; Müller, 1990); an individual was found to excrete *Lm* 5–9 times per year for up to four days, averaging two episodes of faecal carriage per person per year (Grif et al., 2003). It is unknown, however, whether *Lm* disseminates to the liver or spleen during this asymptomatic intestinal colonization. The gall bladder and bone marrow could be sites for *Lm* asymptomatic persistence, as suggested by experiments in mice (Hardy et al., 2009; 2004). Oral inoculation of guinea pigs with a nonlethal dose of *Lm* resulted in asymptomatic colonisation of the GI tract and the liver suggesting that this could also occur in humans, with further dissemination occurring only in the case of a compromised immune system (Melton-Witt et al., 2012).

5.2. Animal listeriosis from a food safety and public health perspective

Although direct transmission of listeriosis from animals to humans is very rare, human listeriosis is considered a zoonotic disease because of the indirect transmission through food, milk, or water contaminated by wild or domestic animal carriers (Chlebicz and Śliżewska, 2018). *Lm* has been reported to colonise the GI tract of or cause invasive disease in more than 40 species of animals, both domestic and wild (including monkeys, cattle, sheep, goats, buffalos, horses, deer, black bears, wild boars, domestic pigs, camelids, dogs, cats, rodents, lagomorphs, domestic fowl, wild birds, fish, and crustaceans) (Allerberger et al., 2015; Fredriksson-Ahomaa et al., 2020; Gray and Killinger, 1966; Leclercq, 2021; Parsons et al., 2020; Yoshida et al., 2000), although disease occurs primarily in farm ruminants (Clune et al., 2020; Esteban et al., 2009; Oevermann et al., 2010). Listeriosis manifests in ruminants mainly as uterine infections (resulting in abortion or neonatal infection) and CNS infections typically characterised by rhombencephalitis, and less commonly as gastroenteritis, bacteraemia and mastitis (Dreyer et al., 2016). *Lm* faecal shedding has been shown to be frequent in asymptomatic ruminants, swine, and poultry (Castro et al., 2018; Esteban et al., 2009; Hurtado et al., 2017; Nightingale et al., 2004), and in particular in cattle where the 4b serotype was predominantly isolated (Esteban et al., 2009). Source attribution analysis revealed that 50% of human listeriosis cases could be imputed to dairy products, followed by poultry and pork

(Filipello et al., 2020). Animal listeriosis is thus of concern not only because of the important economic burden on livestock production due to morbidity and mortality in sheep, cattle and goats, but also from a food safety and public health perspective as an important reservoir of *Lm* and as a frequent source of food contamination – both direct and indirect through cross contamination or the use of manure. As has been observed for human listeriosis (as described in section A.3.3), the prevalence of CCs varied strongly between ruminant clinical and environmental sources (Dreyer et al., 2016). Ruminants were shown to be exposed to a large diversity of *Lm* strains in their immediate environment, yet lineage I and in particular ST1 (CC1) and to a lesser extent ST4 (CC4) clones were predominantly isolated from clinical infections in cattle (Dreyer et al., 2016; Papić et al., 2019; Rocha et al., 2013), both of which are predominant CCs in human listeriosis (Maury et al., 2016). CC1 clones in particular are overrepresented in human CNS infections (Maury et al., 2019), have a strong association with dairy products (Filipello et al., 2020; Maury 2019, Jennison 2017), and have been linked to several high case fatality rate human listeriosis outbreaks involving either unpasteurised dairy products (Büla et al., 1995; Linnan et al., 1988), or the use of contaminated ovine manure (Cantinelli et al., 2013; Schlech et al., 1983). This illustrates the need to identify the sources of *Lm* especially in primary meat and milk production, in fresh produce from manure-amended soils, as well as from cross-contaminations possible along the food chain.

6. The *Lm* infection process

6.1. The *in vivo* infection process

Upon ingestion of contaminated food, *Lm* encounters and can cross the intestinal barrier to disseminate via the lymph and blood to target tissues such as the liver and spleen. *Lm* can also cross the blood-brain barrier and fetoplacental barriers to disseminate to the brain (neurolisteriosis) or placental tissue and foetus (maternal-neonatal listeriosis) (Radoshevich and Cossart, 2017). Our knowledge of the *in vivo Lm* infection process derives mainly from the murine model of listeriosis, which generally involves the intravenous (IV), or less commonly, the intragastric (IG) or intraperitoneal (IP) inoculation of mice. Guinea pigs or gerbils infected by the IG route have also been used, especially in the study of maternal-neonatal listeriosis (Bakardjiev et al., 2005; 2006; Disson et al., 2008; Lecuit et al., 2001; Melton-Witt et al., 2012). Contrary to the mouse, both these rodents express a form of E-cadherin (E-cad) that interacts with the *Lm* surface protein internalin A (InlA) to mediate cellular entry (as described below and in section A.6.3.1) and are natural hosts for *Lm* (Disson

and Lecuit, 2013; Hoelzer et al., 2012). In this work, unless otherwise specified, *in vivo* results refer to those obtained using the IV murine model of listeriosis.

Once ingested, *Lm* passes though the GI tract, encountering the low pH of the stomach and duodenum, as well as membrane-disrupting bile acids, bacteriocin-producing commensal bacteria, and antimicrobial peptides, against all of which *Lm* has developed highly efficient survival mechanisms enabling it to penetrate the intestinal mucus (Gahan and Hill, 2014; Matereke and Okoh, 2020; Maudet et al., 2021). Nonetheless, the vast majority of the initial ingested inoculum is killed in the stomach or shed in the faeces within hours and explains, in part, why an inoculum at least 1000 times higher is required in the IG compared to the IV murine model of infection (Pitts and D'Orazio, 2018); only one bacterium in a million is estimated to invade the intestinal villi (Bou Ghanem et al., 2012; Melton-Witt et al., 2012). Once invaded, however, extensive intracellular replication and cell-to-cell spread take place within enterocytes (Melton-Witt et al., 2012).

Three different mechanisms by which *Lm* breaches the intestinal barrier have been identified (Figure 5). Receptor-specific entry occurs through the interaction between the *Lm* surface protein InlA and E-cad, the major transmembrane protein required for adherens junction formation in epithelial cells (Lecuit et al., 2001; Nikitas et al., 2011). As adherens junctions are situated beneath tight junctions that maintain taut cell-to-cell contact between enterocytes, forming a hermetic barrier, E-cad is not luminally accessible (Nikitas et al., 2011). E-cad is however accessible on goblet cells (specialized mucus secreting cells), around extruding enterocytes at the tip and lateral sides of villi, in villus epithelial folds, and in zones of senescent epithelial cell extrusion (Gessain et al., 2015; Nikitas et al., 2011; Pentecost et al., 2006). Through the interaction of InlA with E-cad, *Lm* hijacks the Rab11-mediated E-cad recycling machinery, and is rapidly transcytosed within its internalization vacuole across the intestinal epithelium by dynamin-mediated endocytosis and released at the basolateral pole into the lamina propria (Kim et al., 2021; Nikitas et al., 2011).

Lm can also initiate the cellular redistribution of tight junction proteins claudin-1 and occludin, and E-cad, to allow bacterial paracellular translocation (Drolia et al., 2018). The *Listeria* adhesion protein (LAP) increases *Lm* adhesion to enterocytes through its interaction with the cellular surface receptor heat shock protein 60 (Hsp60) (Jagadeesan et al., 2010), which activates canonical nuclear factor κB (NF-κB) signalling and pro-inflammatory cytokine production, inducing myosin light-chain kinase (MLCK)-mediated opening of the epithelial

barrier (Drolia et al., 2018). This dysregulation of cellular junctions also increases the accessibility of E-cad, further facilitating *Lm* invasion.

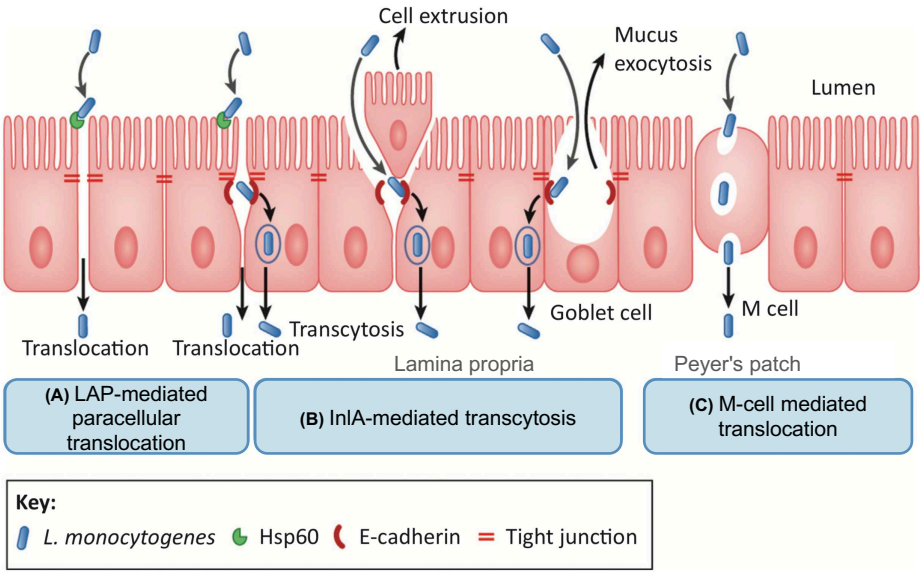

Figure 5 | Schematic representation of the different mechanisms employed by *Lm* to cross the intestinal epithelial barrier.
(**A**) LAP-mediated *Lm* translocation involves the interaction of LAP with epithelial Hsp60 for redistribution of tight junction proteins (claudin-1 and occludin) and E-cad and subsequent epithelial barrier opening. (**B**) InlA/E-cad-mediated *Lm* transcytosis, which occurs during epithelial cell extrusion or goblet cell mucus exocytosis, providing InlA access to E-cad at the adherens junction. (**C**) M cell mediated *Lm* translocation occurs in Peyer's patches. (Adapted from Drolia and Bhunia, 2019)

Finally, *Lm* can cross the intestinal barrier in a non-specific manner, through the phagocytic activity of M cells to reach the underlying Peyer's patch (Bou Ghanem et al., 2012; Chiba et al., 2011; Jensen et al., 1998; Marco et al., 1997). *Lm* invasion of Peyer's patches has been shown to trigger the global intestinal host response to infection, which includes the modification of the intestinal epithelium by increasing enterocyte proliferation while decreasing the maturation of goblet cells and thus reducing E-cad accessibility (Disson et al., 2018).

Once in the lamina propria or the Peyer's patch, *Lm* is phagocytosed by dendritic cells (DCs) or macrophages and trafficked via the lymphatic system to mesenteric lymph nodes (MLNs) and eventually the spleen, or via the hepatic portal vein to the liver (Melton-Witt et al., 2012) and gall bladder (Hardy et al., 2004; 2006). Important bacterial loads are recovered from all these organs at 2–3 days post-infection (p.i.) in mice infected via the IG route (Bergmann et al., 2013; Bierne et al., 2002; Bou Ghanem et al., 2012; Chiba et al., 2011; Disson et al., 2008; Lecuit et al., 2001; Zhang et al., 2017b). From both the spleen and the liver, bacteria can

disseminate to the blood, either intracellularly in leukocytes or extracellularly (Bakardjiev et al., 2006; Drevets, 1999; Melton-Witt et al., 2012; Zhang et al., 2017b). Once in the blood, *Lm* can disseminate to the placenta and foetus, or the brain where entry occurs through the blood-brain or the blood-cerebrospinal fluid barrier, either directly or within an infected phagocyte (Banović et al., 2020, Drevets et al., 2008). Invasion of the brain can also occur independently of bacteraemia through retrograde axonal transport, either through the cranial nerves or the olfactory epithelium (Oevermann et al., 2010). The use of *Lm* clones differentially marked by their susceptibility to antibiotics (Bakardjiev et al., 2006) or genetically tagged (Melton-Witt et al., 2012; Zhang et al., 2017b) has allowed the identification of bottlenecks in the process of *in vivo* dissemination: the liver and spleen are seeded by a large number of clones (Bakardjiev et al., 2006; Melton-Witt et al., 2012; Zhang et al., 2017b), but few bacteria reach the brain (Zhang et al., 2017b) or the placenta and even fewer the foetus (Bakardjiev et al., 2006). The gall bladder, in which *Lm* replication is extracellular, was found to contain only one or very few clones that matched those isolated from faecal samples, suggesting that *Lm* shed in the faeces originate from the gall bladder (Zhang et al., 2017b) (Figure 6).

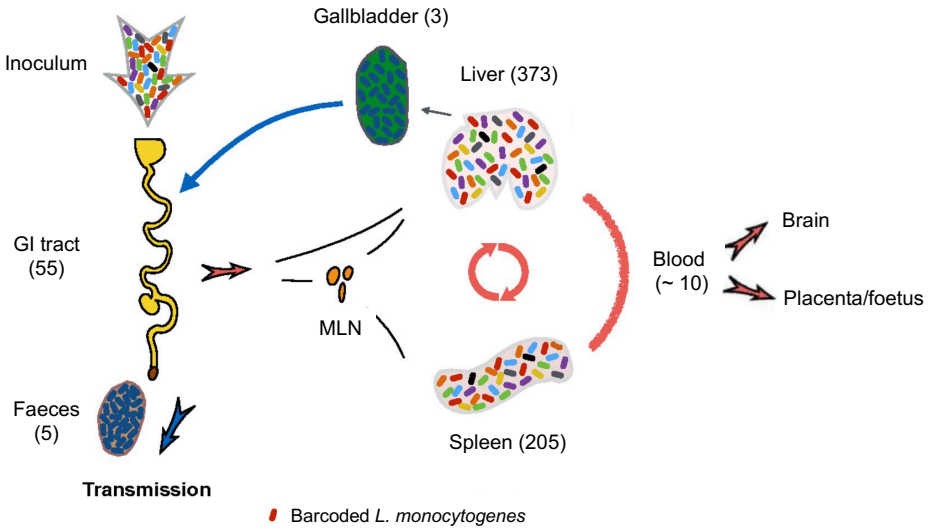

Figure 6 | Overview of the *Lm in vivo* infection process and population dynamics following intragastric inoculation of mice.
In vivo dissemination was mapped by orally infecting BALB/c mice with an inoculum of 3×10^9 *Lm* organisms from a library containing 200 barcoded clones and enumerating both total bacterial load and the frequency of each genetic barcode in different organs at 3 days p.i. to estimate the size of the founding population (Nb). *Lm* replicate in the GI tract and disseminate to the liver and spleen via MLNs or directly to the liver via the portal vein. From the liver and spleen only a small percentage of clones are found in the systemic circulation and fewer still go on to infect the brain or the placenta and foetus. Very few bacteria establish infection in the gall bladder, but the founder(s) ultimately replicate to very high numbers and become the principal source of *Lm* excreted in the faeces. Coloured rods represent single *Lm* clones; mean Nb values are shown in brackets. (Adapted from Zhang et al., 2017)

6.2. The intracellular infectious cycle of *Lm* in mammalian cells

A hallmark of *Lm* virulence is the bacterium's ability to invade and proliferate within almost all human cell types including intestinal epithelial cells, hepatocytes, fibroblasts, and endothelial cells (Cossart and Lebreton, 2014; Kayal et al., 1999) as well as phagocytic macrophages and DCs (Aoshi et al., 2009; Arnold-Schrauf et al., 2014). *Lm* invades non-phagocytic cells by specific receptor induced endocytosis through a zipper mechanism, as opposed to the bacterial secretion system-mediated trigger mechanism exploited by *Salmonella enterica* or *Shigella flexneri* that results in dramatic membrane ruffling and cytoskeletal rearrangements (Cossart and Sansonetti, 2004). *Lm* escapes from the internalisation vacuole through the concerted activity of several secreted virulence factors that disrupt the vacuole membrane (detailed in the following section). Another hallmark of *Lm* virulence is the bacterium's ability to spread from cell-to-cell through the exploitation of the host cell microfilament protein actin (Tilney and Portnoy, 1989), an ability that *Lm* shares with only three other unrelated bacterial genera (Weddle and Agaisse, 2018). The expression of actin assembly-inducing protein (ActA) on one bacterial pole stimulates the polymerisation of actin into "comet tails" that propel *Lm* into neighbouring cells in membrane protrusions (Tilney and Portnoy, 1989). Membrane protrusions are resolved into double membrane secondary vacuoles that are disrupted, in turn, and the infectious cycle reinitiated (Pizarro-Cerda et al., 2012) (Figure 7).

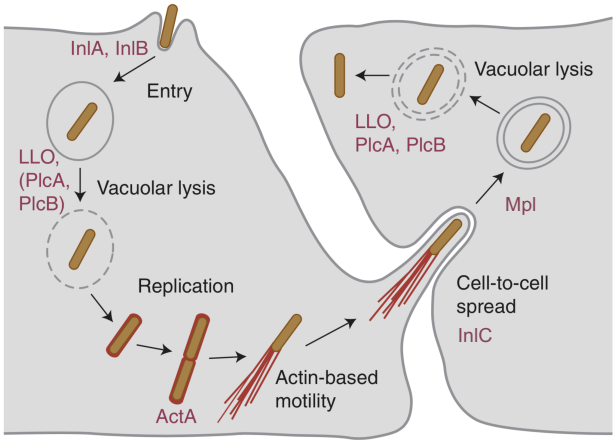

Figure 7 | *Lm* intracellular infection cycle in epithelial cells.
Lm induces its own internalisation into non-phagocytic epithelial cells through the specific interaction between two surface proteins (InlA and InlB) and their host cell receptors (E-cad and c-Met, respectively). *Lm* then rapidly escapes the internalisation vacuole (through the concerted action of LLO and phospholipases PlcA and PlcB) and replicates in the cytosol. Surface expression of ActA confers intracellular motility to *Lm* through host actin polymerisation and allows the bacteria to propel itself into neighbouring cells. With support from Mpl, membrane protrusions resolve into secondary vacuoles that are disrupted in turn, allowing a new infection cycle to start. (Adapted from Pizarro-Cerda et al., 2012)

6.3. Major virulence factors involved in the *Lm* infection process

The development of cell culture models and advances in molecular biology techniques in the 1980s allowed the identification of an important number of virulence factors necessary for the intracellular life of *Lm* (Gaillard et al., 1991; Portnoy, 1992). Comparative genomics between *Lm* and the non-pathogenic *L. innocua* at the turn of the century (Glaser et al., 2001) led to the identification and subsequent characterisation of additional proteins involved in *Lm* virulence (Dussurget et al., 2002; Faralla et al., 2016; Ghosh et al., 2018; Lebreton et al., 2011; Personnic et al., 2010), for review (Camejo et al., 2011; Pizarro-Cerda and Cossart, 2018; Radoshevich and Cossart, 2017). Of the ever-growing arsenal of *Lm* virulence factors that have been identified, only the major virulence factors essential to *Lm* intracellular life (Figure 7) are presented in this section. These genes are tightly regulated by positive regulatory factor A (PrfA), the master transcriptional regulator of *Lm* virulence genes (Cossart and Lecuit, 1998) and make up the core PrfA regulon (las Heras et al., 2011) (Figure 8).

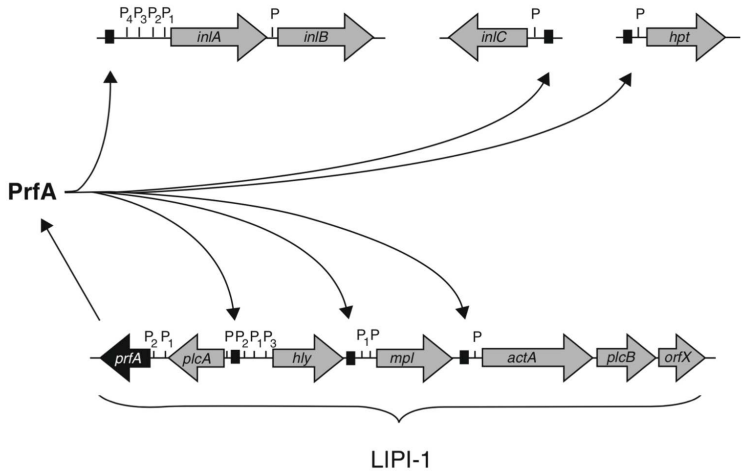

Figure 8 | The core *Lm* PrfA regulon.
The core PrfA regulon comprises LIPI-1 encoding PrfA, LLO (*hly* gene), ActA, PlcA, PlcB, Mpl and OrfX, plus three additional chromosomal loci: the *inlAB* operon and the *inlC* and *hpt* monocistrons. PrfA boxes are indicated by black squares, known promoters are indicated by 'P'. (Adapted from las Heras et al., 2011)

6.3.1. The "internalisation" locus *inlAB*

The *inlAB* locus was discovered using transposon mutagenesis to screen for mutants unable to enter Caco-2 cells (a human enterocyte-like cell line derived from a colon carcinoma), and the region into which the transposon had inserted in non-invasive mutants was termed *inl*, for internalisation (Gaillard et al., 1991). The two gene products from this prfA-regulated operon, InlA and InlB, encoded by the *inlA* and *inlB* genes respectively (Figure 8), are the two major

Lm virulence factors involved in cell invasion through host-cell receptor specific interactions. Both are surface proteins characterised by the presence of a N-terminal domain containing a peptide signal and adjacent leucine-rich repeats (LRRs) coupled to a region that has an Ig-like fold (Bierne et al., 2007; Schubert et al., 2001). Both InlA and InlB are sufficient to promote entry into non-phagocytic cells in an LRR domain-dependent manner (Braun et al., 1999; 1998; Lecuit et al., 1997). At least 35 distinct genes encoding structurally similar LRR domain containing proteins, all but four of which are surface proteins, have since been identified in the *Lm* pan genome and make up the large "internalin" family of *Lm* proteins (Bierne et al., 2007; Popowska et al., 2017). InlA is involved in intestinal barrier crossing (as described in section A.6.1), InlB in spleen and liver colonisation (Khelef et al., 2006), and both InlA and InlB are involved in an interdependent manner in the crossing of the placental barrier (Charlier et al., 2020; Disson et al., 2008; Gessain et al., 2015).

- Internalin A (InlA)

InlA is an 80 kDa *Lm* surface protein containing 15 LRR and is the prototypical member of the internalin family (Bierne et al., 2007; Gaillard et al., 1991). The InlA receptor is E-cad, a surface glycoprotein that forms adherens junctions through homophilic adhesion between neighbouring epithelial cells (Mengaud et al., 1996). Adherens junctions (and therefore E-cad), along with tight junctions, are essential at the intestinal, blood-brain, and placental barriers, where polarized epithelial cells are securely joined to form an impermeable barrier (Doran et al., 2013). The InlA/E-cad interaction mediates cell invasion by activating complex signalling pathways that lead to cortical actin polymerisation, plasma membrane reorganization, and the recruitment of clathrin endocytosis machinery (Pizarro-Cerda et al., 2012).

The InlA/E-cad interaction is species specific: human, guinea pig, rabbit, and gerbil E-cad interact with InlA, but a single amino acid substitution in mouse or rat E-cad precludes InlA recognition (Lecuit et al., 1999). The role of InlA in *in vivo* virulence has therefore been studied through the use of human E-cad (Lecuit et al., 2001, Nikitas et al., 2011) or humanised E-cad (Disson et al., 2008) expressing mice, in gerbils (Disson et al., 2008), guinea pigs (Lecuit et al., 2001), or using a *Lm* strain modified to express a "murinised" InlA that interacts with murine E-cad (Bergmann et al., 2013; Wollert et al., 2007). This modification of InlA has, however, been found to artifactually broaden the InlA receptor repertoire (Tsai et al., 2013). Epidemiological data also provides evidence that InlA plays a key role in human listeriosis as 96% of clinical strains (100% in the case of maternal–neonatal listeriosis) express full-length

inlA compared to 65% of strains recovered from food products (Charlier et al., 2020; Jacquet et al., 2004).

- Internalin B (InlB)

The second major *Lm* internalin to be identified (Dramsi et al., 1995; Gaillard et al., 1991), InlB has been found to mediate entry into a number of different cell types *in vitro*, including epithelial cells of various origins, notably murine and human hepatocyte derived cell lines and primary human hepatocytes (Braun et al., 1998; Brockstedt et al., 2004; Dramsi et al., 1995; Gessain et al., 2015; Pentecost et al., 2010; Quereda et al., 2019), as well as endothelial cells (Greiffenberg et al., 1998; Parida et al., 1998). The necessity of high InlB expression levels to mediate entry, and thus the physiological importance of InlB, has been questioned, however, as most studies have used the *Lm* EGD strain which displays a PrfA mutation leading to constitutive production of InlB (Bécavin et al., 2014; Phelps et al., 2018; Quereda et al., 2019). Virulence of InlB-deficient strains has been observed to be attenuated *in vivo* in a non-humanised E-cad murine model using both the EGD strain (Khelef et al., 2006) and an epidemic strain (Quereda et al., 2019). InlB cell invasion is mediated through the interaction of the hepatocyte growth factor (HGF) receptor (c-Met or HGFR) with InlB's seven N-terminal LRRs (Shen et al., 2000). c-Met is a tyrosine kinase receptor ubiquitously expressed at the surface of human cells, and its interaction with InlB, which mimics the interaction of c-Met with its natural ligand HGF, induces c-Met autophosphorylation and ubiquitination, and the subsequent recruitment of the clathrin endocytosis machinery and phosphoinositide 3-kinase (PI3K)-, serine/threonine kinase mammalian target of rapamycin (mTOR)-, and protein kinase C-α-mediated actin cytoskeleton rearrangements (Pizarro-Cerda and Cossart, 2018). As is the case of the InlA/E-cad interaction, the InlB/c-Met interaction is species specific: InlB interacts with human, gerbil and mouse c-Met but does not recognise the guinea pig or rabbit homologue (Disson and Lecuit, 2013; Khelef et al., 2006). Guinea pigs and rabbits are naturally susceptible to *Lm* infection, however (Hoelzer et al., 2012; Irvin et al., 2008). Common histopathological lesions include necrosis of the liver, suggesting that infection of this organ by *Lm* can occur in an InlB-independent manner.

6.3.2. Internalin C (InlC)

InlC is one of the four secreted members of the *Lm* internalin family (Bierne et al., 2007) and contributes to *Lm* virulence *in vivo* (Domann et al., 1997; Engelbrecht et al., 1996; Gouin et al., 2010; Leung et al., 2013). InlC is highly expressed and secreted by intracellular *Lm* and has

diverse functions (Gouin et al., 2019). InlC promotes bacterial cell-to-cell spread through its interaction with Tuba, a large scaffolding protein that is involved in maintaining epithelial cell apical junction tension. By inhibiting the interaction between Tuba and its binding partner neural Wiskott-Aldrich syndrome protein (N-WASP), apical cell junctions are slackened, thereby enhancing the ability of motile bacteria to deform the plasma membrane into protrusions (Leung et al., 2013; Rajabian et al., 2009). This effect of InlC was also found to impair chromosome alignment in mitotic cells thus delaying cell division (Costa et al., 2020). The role of InlC in the modulation of the host transcriptional response to *Lm* infection will be addressed in section C.4.3.

6.3.3. Virulence factors encoded by *Listeria* pathogenicity island-1

The main *Lm* virulence gene cluster – *Listeria* pathogenicity island-1 (LIPI-1) – encodes seven proteins that are essential to or contribute to *Lm* pathogenicity. These include listeriolysin O (LLO), two phospholipases and a metalloprotease (encoded by *hly*, *plcA*, *plcB*, and *mpl*, respectively) which contribute to vacuolar escape, an actin polymerising protein (ActA), a nucleomodulin (OrfX), and the global regulator of virulence gene transcription (PrfA).

- Listeriolysin O (LLO)

Listeriolysin O (LLO), encoded by the *hly* gene, was the first *Lm* virulence factor identified (Gaillard et al., 1986), leading to the discovery of the *hly* locus, now known as LIPI-1 (Dussurget, 2008). LLO is essential for *Lm* pathogenicity, mediating the escape of the bacterium from primary and secondary internalisation vacuoles and allowing its replication in the cytosol (Dussurget, 2008): LLO-deficient *Lm* are restricted to the internalisation vacuole (Gaillard et al., 1986) and are avirulent *in vivo* (Gaillard et al., 1986; Kathariou et al., 1987; Portnoy et al., 1988). LLO is a member of the cholesterol-dependent cytolysin (CDC) family of pore-forming toxins (PFTs), which contains more than 50 PFTs produced primarily by Gram-positive extracellular pathogens (Nguyen et al., 2019). CDCs require cholesterol for their pore-forming activity and are secreted as monomers that bind to cell membranes and oligomerise to form large transmembrane pores that can exceed 40 nm in diameter (Ruan et al., 2016).

LLO has structural and regulatory features unique to other CDCs that allow it to function intracellularly without causing host plasma membrane permeabilisation and consequent cell death. These distinctive features of LLO are essential for *Lm* pathogenesis: replacement of LLO

with other CDCs results in strains that are extremely cytotoxic and 10,000-fold less virulent *in vivo* (Nguyen et al., 2019; Schnupf and Portnoy, 2007). LLO's pore-forming activity is pH-dependent – its activity is optimal at acidic pH (≤5.6). At neutral pH at temperatures >30°C, the protein undergoes a rapid and irreversible denaturation of its structure (Ruan et al., 2016; Schuerch et al., 2005). The lack of cytotoxicity associated with intracellular LLO owes primarily to a unique 26-amino acid sequence near its N-terminus, however. This PEST-like sequence (i.e., rich in proline (P), glutamate (E), serine (S), and threonine (T)) limits LLO's activity in the cytosol by physically interacting with the host endocytic machinery to mediate the rapid removal of LLO from the plasma membrane by endocytosis (Chen et al., 2018).

- Phospholipases PlcA and PlcB

Two bacterial phospholipases C (PLC), a phosphatidylinositol-specific phospholipase C (PlcA) (Leimeister-Wächter et al., 1991; Mengaud et al., 1991) and a broad-range phospholipase C (PlcB) (Geoffroy et al., 1991; Vázquez-Boland et al., 1992), encoded by *plcA* and *plcB* respectively, act in synergy with LLO to mediate *Lm* escape from primary and secondary vacuoles. PlcA is secreted in an active state, whereas PlcB is secreted as an inactive proenzyme whose activation is mediated by a *Lm* metalloprotease (Mpl) (Marquis et al., 1997; Poyart et al., 1993). The roles of the two PLCs are overlapping: the single deletion of either plcA or plcB had little effect on cell to cell spread *in vitro* or on virulence *in vivo* but the double deletion mutant was severely diminished in its ability to escape from the primary vacuole and was 250- to 500-fold less virulent *in vivo* (Smith et al., 1995).

- Metalloprotease (Mpl)

The zinc metalloprotease encoded by the gene *mpl* (Domann et al., 1991) is, like PC-PLC, secreted as a pro-enzyme, with activation through self-cleavage initiated by low vacuolar pH (Forster and Marquis, 2012). Mpl is required for the maturation of PlcB (Marquis et al., 1997; Poyart et al., 1993) and the resolution of membrane protrusions into secondary vacuoles through an undefined mechanism (Alvarez and Agaisse, 2016).

- Actin-assembly-inducing protein (ActA)

ActA is the *Lm* virulence factor necessary and sufficient to trigger actin polymerisation and the formation of comet tails, conferring motility to *Lm* in the host cell cytosol (Domann et al., 1992; Kocks et al., 1992). Intracellular motility contributes significantly to *Lm* virulence as a means to evade autophagy and to disseminate within tissues. An in-frame *actA* deletion mutant

is around three orders of magnitude less virulent *in vivo* (Brundage et al., 1993). ActA recruits the actin-related proteins-2/3 (Arp2/3) complex through structural, functional and regulatory mimicry of the actin nucleation-promoting host cell proteins of the WASP/WASP-family verprolin-homologous protein (WAVE) family (Boujemaa-Paterski et al., 2001; Chong et al., 2009; Welch et al., 1997; 1998). In addition to conferring intracellular motility, this surface protein was shown to be involved in autophagy evasion independently of its ability to mediate bacterial motility (Yoshikawa et al., 2009), in biofilm formation (Travier et al., 2013), as well as entry into epithelial cells and vacuolar escape (Camejo et al., 2011).

6.4. Beyond the paradigm: the intravacuolar lifestyle of *Lm*

The intracellular lifecycle of *Lm*, as depicted above, has been extensively studied, and as such, *Lm* is considered a prototypical "cytosolic bacterium" as opposed to a "vacuolar bacterium", such as *Bartonella*, *Brucella*, *Chlamydia*, *Coxiella*, *Legionella*, *Mycobacteria* and *Salmonella* spp., that construct specific niches in vacuoles (Cossart and Helenius, 2014) (Figure 9). Recent studies, however, have highlighted the tenuous nature of this classification by providing evidence of intravacuolar stages in the life cycle of *Lm* in both phagocytic and epithelial cells (Bierne et al., 2018).

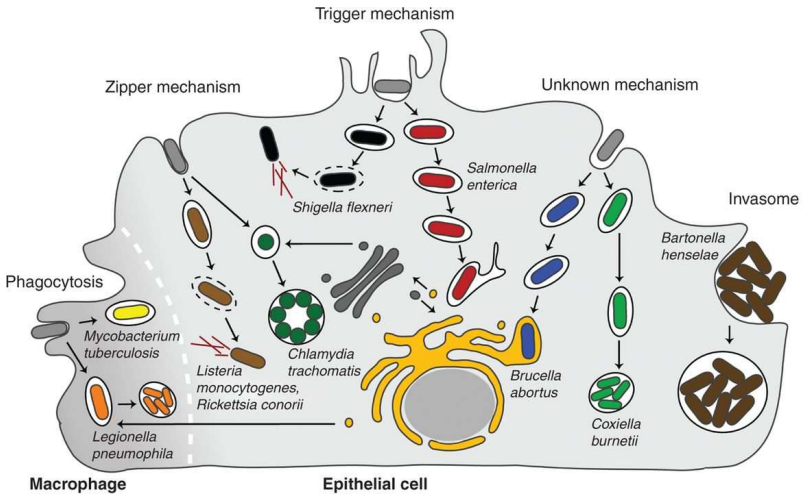

Figure 9 | Examples of various vacuolar lifestyles adopted by intracellular bacteria.
In epithelial cells, the obligate intracellular bacterium *Chlamydia trachomatis* resides in a vacuole closely associated with the Golgi complex. *Salmonella enterica* enters via a T3SS-mediated trigger mechanism and forms a replicative vacuole that acquires endosome and lysosome markers. The *Brucella abortus* endocytic vacuole matures into a replicative ER-derived vacuolar niche. *Coxiella burnetii* has evolved to survive and replicate in a lysosome-derived vacuole. *Bartonella henselae* can invade endothelial cells as a single bacterium or as a group, leading to the formation of an invasome which then constitutes an intracellular niche. In macrophages, *Legionella pneumophila* and *Mycobacterium tuberculosis* survive and replicate intracellularly in vacuolar compartments. (Source: Cossart and Helenius, 2014)

6.4.1. Vacuolar compartments derived from primary vacuoles: SLAPs and eSLAPs

- *Spacious* Listeria-*containing phagosomes (SLAPs)*

The first observation of *Lm* in vacuolar compartments was in mononuclear cells of liver granulomas in chronically infected severe combined immunodeficient (SCID) mice (Bhardwaj et al., 1998). These mice, deficient in lymphocyte immunity, are unable to clear *Lm* from the liver and spleen, but the majority do not succumb to the infection. At 21 days p.i., *Lm* were observed by electron microscopy to be located in membrane-bound vacuolar structures within liver granuloma macrophages, with the majority appearing intact (Bhardwaj et al., 1998). These vacuoles were later characterised in two subsequent *in vitro* studies and named spacious *Listeria*-containing phagosomes (SLAPs) (Birmingham et al., 2008; Lam et al., 2013). The primary internalisation vacuole (the phagosome) of the macrophage is thought to give rise to the SLAP through the LC3-associated phagocytosis pathway (Lam et al., 2013). Although the SLAP is positive for lysosomal-associated membrane protein 1 (LAMP1), it is negative for the lysosomal enzyme cathespin D and pH neutral indicating that the compartment does not mature into a phagolysosome (Birmingham et al., 2008). Acidification is thought to be prevented by LLO-dependent formation of small pores in the SLAP membrane that uncouples the pH gradient (Birmingham et al., 2008). They are thus non-degradative and permissive to *Lm* replication, albeit at a reduced rate (approximately 10-fold) compared to cytosolic replication (Birmingham et al., 2008).

- Epithelial SLAPs (eSLAPs)

As speculated by Brumell and colleagues, LC3-associated phagocytosis is capable of targeting *Lm* in other cell types (Birmingham et al., 2008; Lam et al., 2013). Peron-Cane et al. recently highlighted a subpopulation of *Lm*-associated internalisation vacuoles formed in LoVo intestinal epithelial cells, with molecular characteristics highly reminiscent of SLAPs: their single membrane is LC3- and LAMP1-positive, they are pH neutral, derive from primary internalisation vacuoles, and their formation requires the secretion of LLO (Peron-Cane et al., 2020). These compartments, named epithelial SLAPs (eSLAPs), however, allow efficient replication of *Lm* and are transient (several hours) (Peron-Cane et al., 2020). This is in contrast to SLAPs in which *Lm* reside as slow-growing forms over days or weeks (Bhardwaj et al., 1998; Birmingham et al., 2008) and which could serve as a persistent intravacuolar niche for *Lm* in macrophages (Bierne et al., 2018).

6.4.2. *Listeria*-containing vacuoles (LisCVs): a late-forming compartment for *Lm* persistence in epithelial cells

LisCVs are another type of single-membrane LAMP1-positive vacuoles that were identified in human hepatocytes and trophoblast cells as intracellular compartments in which bacteria enter a state of persistence (Kortebi et al., 2017). LisCVs are notably different from SLAPs and eSLAPs: they do not derive from primary vacuoles, are acidic, LC3-negative, and lysosomal marker cathepsin D-positive, suggesting fusion with lysosomes (Kortebi et al., 2017). They are partially degradative, but the majority of bacteria remain intact and enter a slow/non-replicative or a viable but non-culturable (VBNC) state within this vacuolar niche (Kortebi et al., 2017). Importantly, contrary to SLAPs and eSLAPs that form within the first hours of the intracellular infectious process (Birmingham et al., 2008; Peron-Cane et al., 2020), LisCVs are formed late (i.e., after 2–3 days), and essentially localise in perinuclear regions (Kortebi et al., 2017). The formation of LisCVs is concomitant to the loss of the actin-nucleating protein ActA and the arrest of actin polymerisation at the bacterial surface (Figure 10). At 72 h p.i. 60–80% of intracellular bacteria are observed in LisCVs. *Lm* thus switches its lifestyle from an active motile phase, to a non-motile quiescent phase in a subset of epithelial cells.

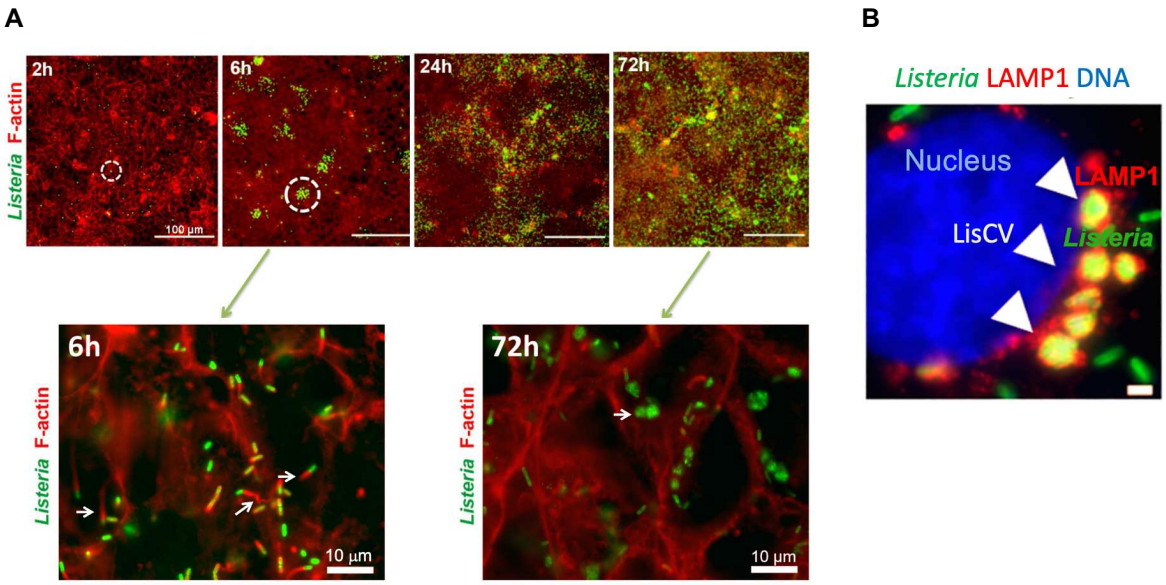

Figure 10 | *Lm* switches from actin-based motility to a vacuolar phase in human hepatocytes and trophoblast cells.
(**A**) Micrographs of placental JEG3 cells infected with *Lm* 10403S for different times and stained for *Lm* (in green) and F-actin (in red) (Bars: 100 µm). Below, higher magnifications of overlay images, at 6 h or 72 h, highlight the phenotypic switch between motile bacteria associated with filamentous actin (at 6 h), to clusters of actin-negative bacteria (at 72 h), as indicated by arrows (Bars: 10 µm). (**B**) At 72 h p.i., bacteria (in green) are included in LisCVs (indicated by triangles) marked by LAMP1 (in red), at the periphery of the nucleus (DNA, in blue) (Bars: 2 µm). (Adapted from Kortebi et al., 2017)

By opposition to primary and secondary vacuoles, involved in entry and intercellular dissemination, respectively, LisCVs are considered a "tertiary vacuole", corresponding to this new intracellular stage in the *Lm* lifecycle which occurs following cell-to-cell dissemination (Figure 11). *In vitro* data also suggest that *Lm* in LisCVs have the ability to return to an active phase of proliferation and dissemination (Kortebi et al., 2017). LisCVs are thought to be formed through a xenophagic process mediated by non-canonical autophagy pathways, as they are negative for the autophagy marker LC3. Akin to lysosomes, LisCVs remain intact in mitotic cells and are partitioned in daughter cells (Kortebi et al., 2017), evoking the possibility that non-replicating *Lm* could propagate during tissue regeneration following acute infection (Bierne et al., 2018).

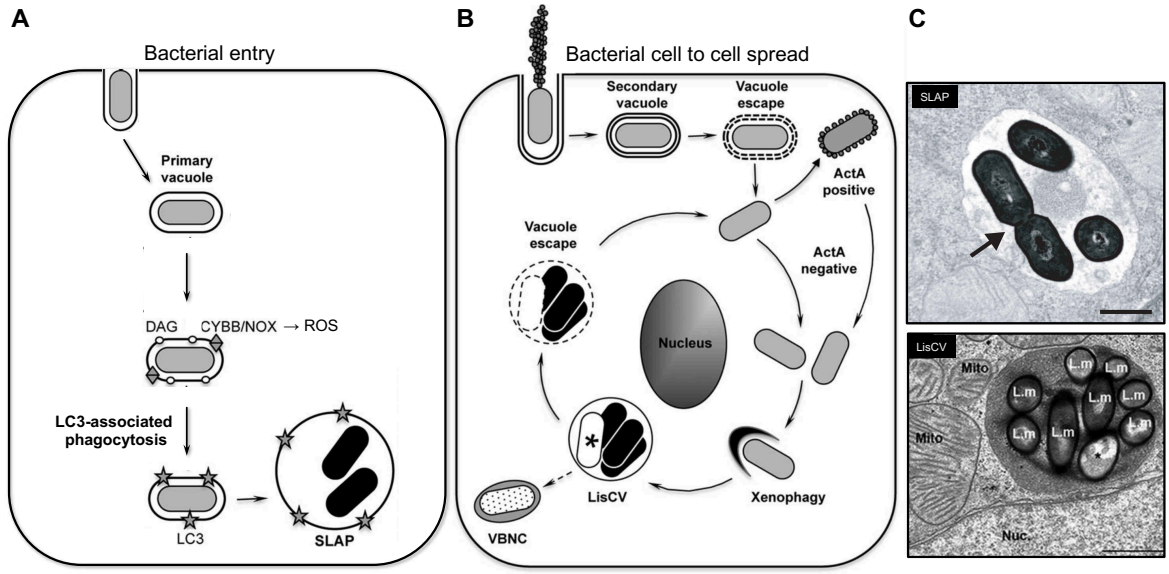

Figure 11 | The vacuolar stages in the intracellular lifestyle of *Lm*.
(**A**) Model of SLAP formation in murine macrophages. Bacteria are phagocytosed into a primary vacuole. In a subpopulation of phagosomes, in which bacteria secrete low amounts of LLO, diacylglycerol (DAG) accumulates to stimulate the activation of the CYBB/NOX2 NADPH oxidase and the production of reactive oxygen species (ROS). ROS recruits LC3 to the membrane, promoting the formation of non-acidic and non-degradative SLAPs, in which *Lm* replication occurs, but is reduced more than 10-fold (represented by black bacteria). (**B**) Model of LisCV formation in human hepatocytes and trophoblast cells. Following the active stage of bacterial motility and cell-to-cell spread, cytosolic bacteria arrest ActA expression and are captured through a xenophagy-like process that results in the formation of lysosome-like LisCVs. In these acidic compartments, while some bacteria are eliminated (white bacteria marked with an asterisk), a subpopulation resists degradation and enters a slow/non-replicative state (black bacteria). These surviving bacteria can either enter a VBNC state (punctuated bacteria) or can initiate a novel infectious cycle. (**C**) TEM images of *Lm* in SLAPs (top), or LisCVs (bottom), that enclose a mixed population of intact ("L.m") and damaged bacteria (indicated with a "*") and are localised close to the nucleus. Mito., mitochondria; Nuc., nucleus. (Adapted from Bierne et al., 2018)

Based on this work (Kortebi et al., 2017), and that of Brumell and colleagues (Birmingham et al., 2008; Lam et al., 2013), Bierne and colleagues hypothesized that residing as slow- to non-replicating forms in SLAPs (in macrophages) or LisCVs (in epithelial cells), could be a strategy employed by *Lm* to evade the immune system in the long-term and reside in the host in latent forms (Bierne et al., 2018). This property may play an important role in the asymptomatic carriage and transmission of this pathogen (Bierne et al., 2018). Indeed, these observations raise the question of the role of intravacuolar *Lm* in asymptomatic carriage, during the silent incubation phase of invasive listeriosis, or during asymptomatic listeriosis in healthy carriers, particularly animals. In these asymptomatic reservoirs, reactivation to replicative forms of *Lm* could allow for the release of *Lm* in the environment via the faeces, or lead to recurrent listeriosis (Bierne et al., 2018).

B. The liver and the hepatic phase of listeriosis

Decades of research using rodents to model human listeriosis have resulted in a well described infection scenario. After crossing the intestinal barrier and reaching systemic circulation, *Lm* is captured by phagocytic cells primarily in the liver in which potent innate immune responses take place. At the same time, *Lm* enters and actively replicates within hepatocytes, the parenchymal liver cells, until innate and adaptive immune cells mediate the total clearance of *Lm* from the organism. The liver is the major interface between the digestive system and the blood and performs a multitude of diverse functions crucial for maintaining physiological metabolic and immune homeostasis, including pivotal roles in both adaptive and innate immunity.

1. Structure and cellular composition of the liver

1.1. Structure of the liver

The liver is the human body's largest internal organ, with an average mass of 1.5 kg in adults, representing approximately 2% of body weight (Bogdanos et al., 2013). Located in the right upper quadrant of the abdomen just below the diaphragm, the human liver has four lobes: a major left and right lobe, and two smaller inferior lobes (Mescher, 2013). Its anatomical structure and unique vasculature allow maximal contact between circulating blood and hepatocytes, the main cells of the liver, and provide continuous opportunity for the exchange of metabolic and immunological information (Ben-Moshe and Itzkovitz, 2019). The liver has a dual blood supply; the portal vein supplies the majority (~80%) of oxygen-poor, nutrient- and antigen-rich blood from the GI tract. The remaining 20% of afferent blood is oxygenated blood from the systemic circulation entering via the hepatic artery (Mescher, 2013). The liver filters 1.5 L (~30% of total blood volume) each minute - the total volume of blood in the human body thus circulates through the liver approximately 360 times each day (Bogdanos et al., 2013; Nemeth et al., 2009). The liver consists of hundreds of thousands of repeating anatomical units termed liver lobules (Figure 12A), hexagonal columns approximately 1 mm in diameter (Teutsch, 2005). Rows of hepatocytes branch out from the central vein to the exterior portal nodes, where a branch of the bile duct, the hepatic portal vein and the hepatic artery form the portal triad, along with lymphatic vessels and nerve fibres (Figures 12B and 12C). Mixed venous and arterial blood enters the lobules from the portal nodes and flows radially inward through sinusoidal blood vessels towards the draining central vein and eventually to the inferior

vena cava. Bile secreted from hepatocytes flows outwards, in the opposite direction to blood flow, from the central to the portal zone through bile canaliculi that drain into the bile duct (Ben-Moshe and Itzkovitz, 2019; Mescher, 2013).

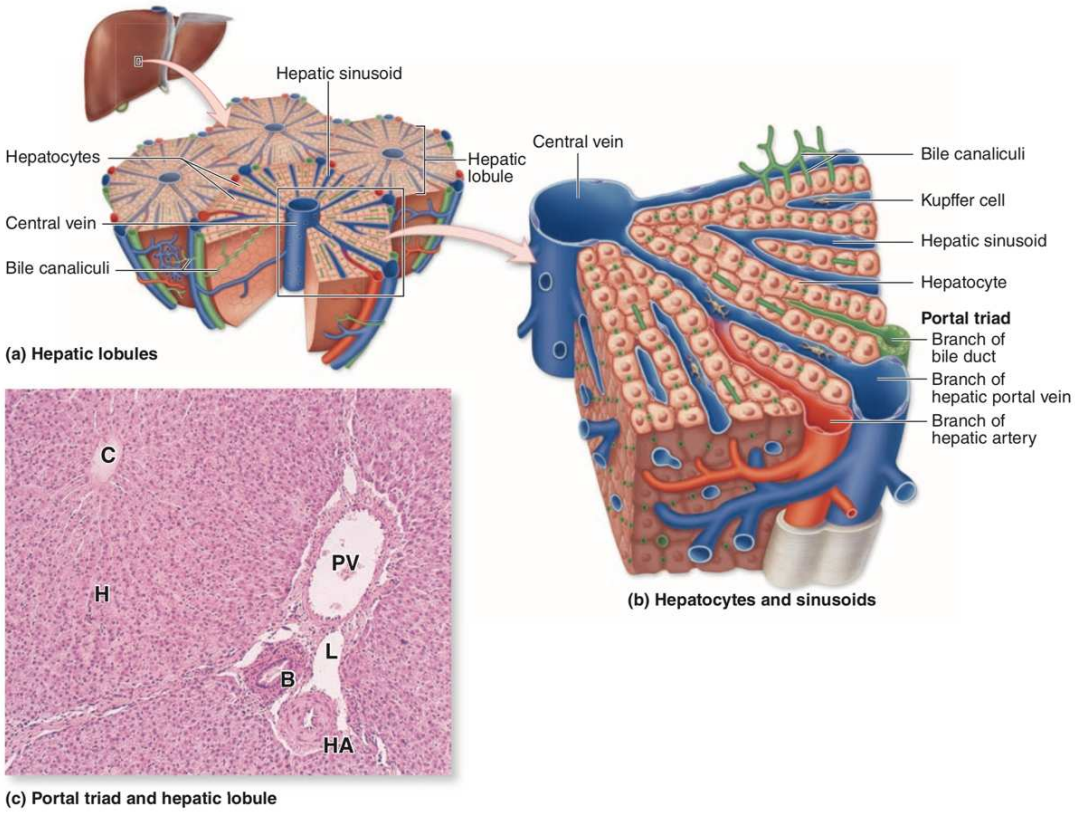

Figure 12 | Structure of the human liver.
(**a**) Hepatic lobules are the structural and functional unit of the liver. Each lobule is composed of a central vein, to which rows of hepatocytes and hepatic sinusoids converge. (**b**) The portal triad is composed of blood vessels and a branch of the bile duct: venous and arterial blood enter the hepatic lobule via branches of the portal vein and hepatic artery and mix in the hepatocyte-lined sinusoids through which blood flows, draining into the central vein. Bile is secreted by hepatocytes into bile canaliculi that drain into the bile ductule. (**c**) Micrograph of a hepatic lobule showing the central vein (C), plates of hepatocytes (H), and a portal node containing a portal venule (PV), hepatic arteriole (HA), a bile ductule (B), and lymphatic vessel (L) (220× magnification). (Source: Mescher, 2013)

1.2. Cellular composition of the liver

Hepatocytes are the primary parenchymal cells of the liver and among the most functionally diverse cells of the human body (Mescher, 2013). Non-parenchymal liver cells include liver sinusoidal endothelial cells (LSECs), cholangiocytes, Kupffer cells (KCs), hepatic stellate cells (HSCs), dendritic cells (DCs), and intrahepatic lymphocytes (IHLs) (Chen and Tian, 2021). Both hepatocytes and non-parenchymal hepatic cells interact to create an immunological micro-milieu that maintains tolerance to food-derived antigens on the one hand but that is also

capable of activating potent immune responses to invading pathogens on the other (Horst et al., 2016) (Figure 13).

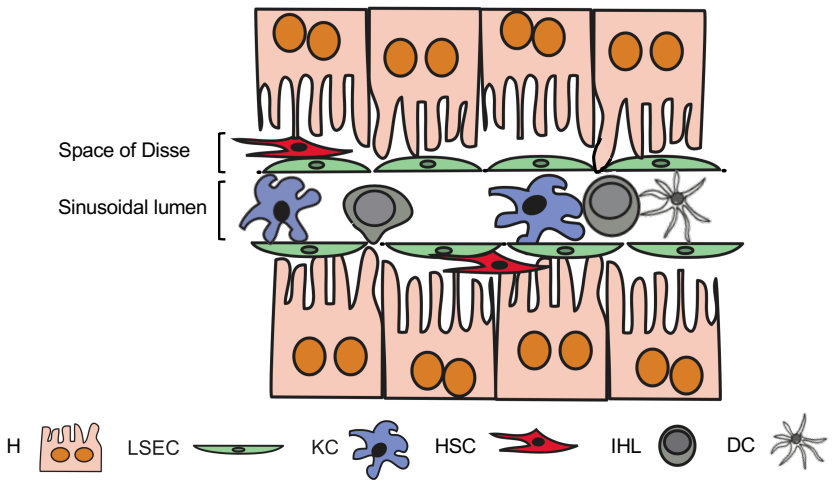

Figure 13 | Schematic representation of the cellular composition of a liver sinusoid.
Hepatocytes (H) form the bulk of the hepatic parenchyma and are separated from the sinusoidal blood flow by fenestrated liver sinusoidal endothelial cells (LSECs) that allow hepatocytes to interact directly with Kupffer cells (KCs), intrahepatic lymphocytes (IHLs), and dendritic cells (DCs) within the sinusoidal lumen. Separating LSECs from hepatocytes is the space of Disse, in which hepatic stellate cells (HSCs) reside. (Adapted from Bogdanos et al., 2013; Horst et al., 2016)

1.2.1. Hepatocytes

Hepatocytes are the primary parenchymal cells of the liver, constituting 80% of liver mass and 60–80% of its cellular composition (Gao, 2016). As such, they are responsible for performing most of the liver's diverse digestive, metabolic, endocrine, and exocrine functions (detailed below), and thus have great metabolic, protein production, and secretory capacity. Hepatocytes are large cuboidal or polyhedral epithelial cells, rich in mitochondria and abundant in rough and smooth endoplasmic reticulum (ER) where, respectively, plasma protein synthesis and enzymatic biotransformation takes place (Mescher, 2013). Like all epithelial cells, hepatocytes possess apical (bile canalicular) and basolateral (sinusoidal) plasma membrane domains composed of distinct surface proteins, channels, and receptors (Schulze et al., 2019). Mammalian hepatocytes are frequently binucleated and/or polyploid (up to 8N), the degree of which could play a role in the functional heterogenicity of hepatocytes, as does liver zonation, i.e., the position of the hepatocyte relative to the portal zone and thus the local blood oxygen, metabolite, and hormone concentration (Ben-Moshe and Itzkovitz, 2019; Donne et al., 2020). The presence of an extensive ER and Golgi network visible by TEM (Figure 14A), testifies to the role of the hepatocyte as a protein synthesis and secretion factory. It is within the ER and Golgi lumina that chaperone-assisted folding and (in the case of most secretory proteins)

glycosylation occur. Proteins are then packaged into Golgi-derived secretory vesicles for transport to and exocytosis at the basolateral membrane. The endocytic capacity of the hepatocyte is also evident by TEM, with clathrin-coated pits, although small (50 nm), occupying 2% of total hepatocyte surface (Schulze et al., 2019) (Figure 14B). Hepatocytes possess many irregular microvilli on their apical sides that are in direct contact with blood plasma and lymph in the narrow perisinusoidal space known as the space of Disse that separates hepatocytes from the hepatic sinusoids (Mescher, 2013). Hepatocyte microvilli can even extend through the fenestrated cells that compose the sinusoid walls to interact physically with immune cells located within the sinusoidal lumen and bathe directly in sinusoidal blood (Bogdanos et al., 2013; Li and Zeng, 2020; Warren et al., 2006) (Figure 14C).

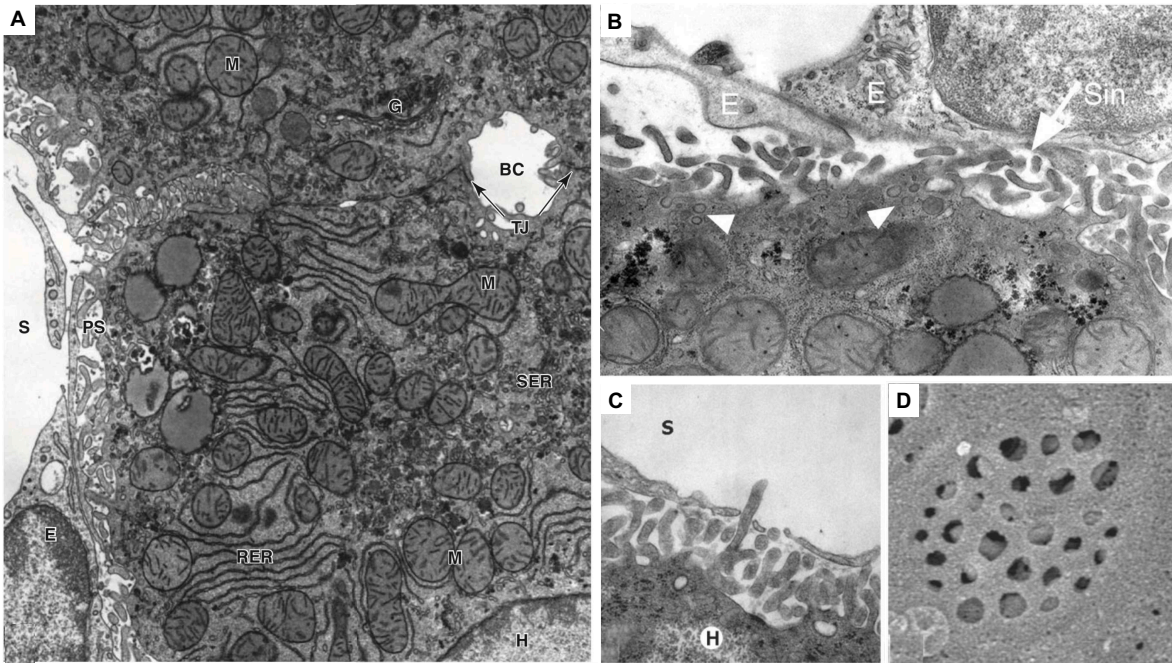

Figure 14 | Electron microscopy images of hepatocytes and LSECs.
(**A**) TEM image showing the crowded cytoplasm of the hepatocyte: a hepatocyte nucleus (H) in the lower right corner, is surrounded by smooth ER (SER), rough ER (RER), many mitochondria (M), Golgi complexes (G), and small electron-dense glycogen granules. Between the hepatocytes and the fenestrated LSECs (E) of the sinusoid (S) is the very small perisinusoidal space or space of Disse (PS) into which hepatocyte microvilli protrude. A bile canaliculus (BC), sealed by tight junctions (TJ), is formed by two adjacent hepatocytes. (**B**) TEM showing the basolateral hepatocyte membrane with its numerous microvilli that occupies much of the perisinusoidal space (Sin) delimited by LSECs (E). Arrowheads denote the abundant coated endocytic pits illustrating the endocytic capacity of hepatocytes. (**C**) TEM image of a hepatocyte microvillus which can be seen protruding from an endothelial fenestra into the sinusoidal lumen. (**D**) Scanning electron micrograph showing endothelium fenestrae and hepatic microvilli visible under the LSEC layer. (Source: Mescher, 2013 (A), Schulze et al., 2019 (B), Warren et al., 2006 (C,D))

Hepatocytes express a wide range of both surface and cytosolic pattern recognition receptors (PRRs), and are capable sensing and responding to pathogen invasion (Crispe, 2016). Constitutive and inducible expression of major histocompatibility complex class I (MHC-I) molecules as well as other intercellular adhesion molecules and ligands allow the direct targeting of hepatocytes by cytotoxic CD8 T and natural killer T (NKT) cells (Crispe, 2016; Gao, 2010; Nemeth et al., 2009). The role of hepatocytes as unconventional antigen presenting cells (APCs) and the fate of hepatocyte-activated T cells, however, remains open to debate (Bénéchet et al., 2019; Crispe, 2011; Klein and Crispe, 2006; Wong et al., 2015).

Hepatocyte turnover is slow in a healthy liver – the average life span of an adult hepatocyte is 200 to 300 days (Duncan et al., 2009) – but these epithelial cells have an unlimited and rapid regenerative capacity. After a typical two thirds partial hepatectomy in rodents, most of the liver mass is restored within 7–8 days, with complete restoration achieved within three weeks (Michalopoulos and Bhushan, 2021).

1.2.2. Cholangiocytes

Cholangiocytes or biliary epithelial cells are a morphologically and functionally heterogeneous, highly dynamic population of cuboidal or columnar epithelial cells that line the three-dimensional network of bile ducts known as the biliary tree that extends from the liver to the duodenum. Bile is secreted by hepatocytes into bile canaliculi that empty into the bile canals of Hering, then into the bile ductules, leaving the liver via the hepatic ducts to finally reach the gallbladder where bile is stored (Tabibian et al., 2013).

1.2.3. Liver sinusoidal endothelial cells

These fenestrated endothelial cells line the sinusoidal capillary channels of the liver and are the most abundant non-parenchymal hepatic cell population. LSECs lack a classical basement membrane and are fenestrated, increasing porosity and permeability and thus facilitating the movement of molecules and cellular mediators to and from the liver parenchyma (Chen and Tian, 2021; Mescher, 2013) (Figure 14D). LSECs possess very potent scavenger capabilities by virtue of the expression of many scavenger receptors coupled with high endocytic potential. Their important innate and adaptive immunological functions, including pathogen recognition and uptake, antigen presentation, cytokine secretion (i.e., tumor necrosis factor (TNF-α), interleukin (IL)-10), and regulation of leukocyte-recruitment, play a pivotal role in maintaining immune homeostasis (Bogdanos et al., 2013; Shetty et al., 2018).

1.2.4. Hepatic stellate cells

HSCs are located in the space of Disse, extending long dendritic-like processes that wrap around the sinusoids (Chen and Tian, 2021). In the absence of liver damage, they exist in a quiescent state and play a central role in vitamin A and lipid storage, storing 80–90% of total body vitamin A in intracytoplasmic lipid droplets, and in vasoregulation through their interactions with LSECs (Blaner, 2019; Horst et al., 2016). As a result of liver injury or infection, they become activated into myofibroblasts secreting extracellular matrix components such as collagen, laminin, and proteoglycans that form basement membrane-like structures, required for liver regeneration but the overproduction of which leads to liver fibrosis and eventually cirrhosis (Gupta et al., 2019). HSCs play a role in hepatic immunity by extensive chemokine, pro-inflammatory (i.e., IL-6, TNF-α) and immunoregulatory (i.e., transforming growth factor beta (TGF-β)) cytokine, and type I interferon (IFN-I) expression upon activation, promoting leukocyte chemotaxis, adherence, and activation (Gupta et al., 2019).

1.2.5. Kupffer cells

KCs are liver-resident immobile macrophages and represent ~35% of non-parenchymal liver cells and 90% of all tissue macrophages (Nguyen-Lefebvre and Horuzsko, 2015). Localized within the lumen of the liver sinusoids in direct contact with afferent blood derived from the GI tract, these very large intravascular cells with potent phagocytic capacity are the sentinels of innate liver immunity, detecting and clearing the majority of bloodborne bacteria within minutes to prevent systemic dissemination (Kubes and Jenne, 2018; Li and Zeng, 2020). KCs express an array of toll-like receptors (TLRs), scavenger receptors, complement receptors and antibody receptors, that allow the detection, binding and internalisation of pathogens and associated molecules. KCs are important APCs, expressing MHC-I, MHC-II, and co-stimulatory receptors necessary for T-cell activation. Under physiological conditions, however, the KC is a poor APC, exhibiting what has been termed a “tolerogenic” phenotype, and like LSECs, can actively promote T-cell tolerance (Kubes and Jenne, 2018). This is necessary to prevent undesired immune responses from immunoreactive molecules derived from the gut and present on dying or dead cells which are removed from circulation by KCs. Under pathological conditions, KCs are activated to differentiate into M1-like macrophages secreting pro-inflammatory mediators or M2-like (alternative) macrophages that secrete immunoregulatory cytokines (Nguyen-Lefebvre and Horuzsko, 2015; Tacke and Zimmermann, 2014). Unlike circulating macrophages that originate from bone-marrow derived monocyte precursors, KCs

have an embryonic origin and are self-renewing throughout adult life (Gomez Perdiguero et al., 2015; Schulz et al., 2012).

1.2.6. Hepatic dendritic cells

Hepatic DCs enter the liver as immature cells via the portal vein, and mature as they transit from the portal to the central vein or as they transmigrate through the LSECs to enter the space of Disse, eventually accessing the liver lymphatics (Kubes and Jenne, 2018). DCs are professional APCs, whose essential function is to internalise antigens derived from blood and tissue and to transport these antigens to a regional lymph node or the spleen for presentation to naive CD4 and CD8 T cells as well as B cells, inducing their antigen-specific activation and proliferation (Liaskou et al., 2012; Lurje et al., 2020). DC–T cell interaction and priming can also occur directly in the hepatic sinusoids, making the liver the only organ not traditionally considered to be a secondary lymphoid organ in which both CD4 and CD8 T cell priming can occur (Bénéchet et al., 2019; Lurje et al., 2020; Tay et al., 2014b). DCs also activate innate natural killer (NK) and NKT cells and secrete large amounts of IFN-I and other cytokines in response to pathogen-associated molecular pattern (PAMP) detection, thus contributing to both the innate and adaptive immune response (Crispe, 2014; Liaskou et al., 2012). Compared to their counterparts in non-hepatic tissue, however, liver-derived DCs are less efficient at priming T-cells and more likely to induce allogenic T-cell apoptosis (Bogdanos et al., 2013; Horst et al., 2016; Robinson et al., 2016; Tokita et al., 2008). As such, hepatic DCs, like KCs and LSECs, contribute both to the hepatic bias towards immune tolerance under physiological conditions, and to the robust pro-inflammatory responses elicited upon tissue damage or infection.

1.2.7. Intrahepatic lymphocytes

The human liver has an important innate and adaptive lymphocyte population resident in the sinusoidal lumen and scattered throughout the parenchyma (Bogdanos et al., 2013). Innate lymphocytes in the adult liver include NK cells, NKT cells, mucosal associated invariant T (MAIT) cells and γδ T cells. The highest proportion of IHL consists of innate lymphocytes NKT and NK cells: in both humans and mice, NKT cells and NK cells make up around half of the total IHL population (Chen and Tian, 2021; Mikulak et al., 2019). Liver resident NK cells continually scan for and rapidly eliminate cells displaying non-self signals and, upon activation, are major producers of type II interferon (IFN-II), like their circulating counterparts (detailed in section C.1.2.3) (Mikulak et al., 2019). Adaptive lymphocytes, including CD4 and

CD8 T cells as well as B cells represent about one third of total IHL in a healthy human liver (Chen and Tian, 2021). Circulating effector cytotoxic CD8 T cells have been shown to crawl along the sinusoids, actively patrolling and extending cell processes through the fenestrated sinusoidal endothelium to scan and kill any underlying hepatocytes that present cognate antigens, without the need to migrate into the liver tissue (Guidotti et al., 2015).

2. Functions of the liver

As the major interface between the digestive system and the blood, the liver is a metabolic hub, and the organ in which all substances of gastrointestinal origin – from nutrients to harmful xenobiotics or microbial toxins – are metabolised, processed, and stored before distribution or elimination. As residence to the largest population of tissue macrophages in the human body, the liver plays a major role, along with the spleen, in the clearance of pathogens from the blood. It is also the source of innate immune circulating plasma components known collectively as acute phase proteins.

2.1. Functions related to digestion, detoxification, and metabolic homeostasis

The main digestive function of the liver is the production of bile, a complex molecular soap composed primarily of bile acids, phospholipids, and cholesterol (Boyer, 2013; Mescher, 2013). Synthesised and secreted by hepatocytes, bile facilitates the emulsification, solubilisation and absorption of dietary fats within the intestinal lumen. It is also through bile secretion that the liver performs another of its many roles, the elimination of potentially harmful metabolic by-products and xenobiotics following their metabolic transformation and break down in hepatocytes in which more than 50 different cytochrome P450 enzymes (the major enzymes involved in drug metabolism) are embedded in the SER membrane (Boyer, 2013; Schulze et al., 2019).

The liver also plays a central role in maintaining carbohydrate and lipid metabolic homeostasis. When glucose and insulin levels are high, hepatocytes store glucose as glycogen in cytoplasmic granules or use it to synthesise fatty acids. Fatty acids are also absorbed from the blood, esterified with glycerol 3-phosphate to generate triglycerides, or with cholesterol to produce cholesterol esters, and stored in cytoplasmic lipid droplets – unique fat-storage organelles that serve as energy reserves during times of fasting – or packed into very low density lipoprotein particles to be shuttled to adipose or other extrahepatic tissue (Rui, 2014). In the fasting state,

when glucagon levels are high, the liver provides glucose through both glycogenolysis and gluconeogenesis to maintain euglycaemia, accounting for up to 90% of total endogenous glucose production after prolonged fasting (Petersen et al., 2017).

Cholesterol is a necessary component of vertebrate cell-membrane composition and the precursor of steroid hormones, but is also detrimental in excess. The liver is the major source of endogenous cholesterol *de novo* biosynthesis, which is esterified and stored in lipid droplets or secreted into the blood as constituents of lipoproteins, or converted into bile acids (Luo et al., 2019). The liver parenchyma is particulary important in its unique ability to uptake excess cholesterol from peripheral tissues and arterial macrophages in a process known as reverse cholesterol transport and to eliminate it though conversion into bile acids or through the direct excretion of free cholesterol into bile (Cohen, 2008; Nemes et al., 2016). The liver also regulates vitamin A homeostasis as the principal site of its uptake and storage (Blaner, 2019), as well as systemic iron homeostasis through the tightly regulated production and secretion of hepcidin, the peptide hormone that regulates iron absorption from the duodenum and its release from macrophages and other cells (Rouault et al., 2020). Other secreted signalling proteins of hepatic origin – termed hepatokines – regulate glucose and lipid metabolism and mediate crosstalk between organs in an endocrine manner (Jensen-Cody and Potthoff, 2021).

2.2. Immune functions

The key metabolic functions of the liver have long eclipsed its role in immunity. The concept of hepatoimmunology – or the liver as an immunological or lymphoid organ – emerged around 20 years ago (Mackay, 2002; O'Farrelly and Crispe, 1999). The liver is a key organ in immune function both in its role of clearing pathogens from the blood and in a secretory and regulatory capacity as the source of circulating immune system plasma components. Despite the major metabolic consequences of liver failure, the primary cause of death in patients with terminal cirrhosis is infection, and the severity of liver disease correlates with an increased risk of bacteraemia (Ashare et al., 2009; Bajaj et al., 2012; Fernández and Gustot, 2012).

The clearance of pathogens from the blood is carried out almost exclusively by the spleen and the liver, two highly vascularised organs, directly positioned within the systemic circulation (Benacerraf et al., 1959; Ganesan et al., 2011). The liver is uniquely positioned at the interface between the digestive system and the blood to form a vascular “firewall” that captures any invading microorganisms that have crossed the intestinal barrier (Balmer et al., 2014). While the spleen is particularly important in the capture of encapsulated bacterial pathogens, both

directly and through the production of opsonins, as well as an important site for antigen capture and lymphocyte priming (Dionne et al., 2017; Sharma et al., 2015), the liver alone clears blood of viruses (Ganesan et al., 2011), and the capacity of the liver in bacterial clearance is significantly greater than that of the spleen. Both Gram-negative and Gram-positive bacteria are eliminated within minutes from the blood of mice or rabbits following IV challenge: approximately 80% are recovered from the liver, compared to 20% from the spleen (Conlan, 1997; Parker and Franke, 1919). KCs, the most abundant resident tissue macrophages in the human body, effectuate bacterial clearance (Ebe et al., 1999; Wing and Gregory, 2002), whereas LSECs mediate viral clearance (Shetty et al., 2018). Under the right conditions, KCs, LSECs, as well as HSC and hepatocytes, can prime naive CD4 and CD8 T cells to differentiate into effective, fully functional helper and cytotoxic T cells, respectively, making the liver the only non-lymphoid organ in which T cell activation and differentiation can occur independently of secondary lymphoid tissues and DC involvement, challenging either the dogma that T cell priming occurs exclusively in secondary lymphoid organs, or the traditional view of the non-lymphoid nature of the liver (Bénéchet et al., 2019; Böttcher et al., 2013; Klein and Crispe, 2006; Tay et al., 2014b; Wuensch et al., 2006). On the other hand, T cell priming in the liver takes place in an environment biased towards tolerance and, in the absence of sufficient stimulatory signals, T cell activation can lead to long-term CD8 T cell dysfunction, apoptosis, and the absence of the development of immune memory (Bénéchet et al., 2019; Crispe, 2009; Wong et al., 2015). Tolerance to antigens presented in the liver can subsequently lead to systemic immune tolerance, which can be exploited by hepatotropic viruses and parasites, as well as malignant cells, leading to persistent infection or rapid cancer progression, respectively (Protzer et al., 2012; Zheng and Tian, 2019).

The liver also contributes to immune function as the major source of circulating plasma proteins that are involved in inflammatory responses and in pathogen recognition, opsonisation and elimination. Hepatocytes produce and secrete soluble PRRs, complement proteins, coagulation and fibrinolytic system components, as well as other crucial molecules of the systemic innate immune system (Gao et al., 2008).

2.2.1. The acute phase response and acute phase proteins

The inflammatory response triggered by infection, injury, ischemia, or intoxication is characterised, among other things, by fever, leukocytosis, behavioural changes such as anorexia, and an increase in the amount of circulating proteins that function to limit tissue injury and participate in host defence (Ehlting et al., 2021; Gabay and Kushner, 1999). These proteins were named "acute phase proteins" (APPs) as their increase was first observed in the plasma of patients during the acute phase of pneumococcal pneumonia. They can, however, accompany both acute and chronic inflammation (Bode et al., 2012; Gabay and Kushner, 1999). APPs have since been defined as proteins whose plasma concentration increases (positive APPs) or decreases (negative APPs) by at least 25% during inflammation (Bode et al., 2012; Gabay and Kushner, 1999). Positive APPs include complement proteins (detailed in the following section), opsonins (e.g., pentraxins, C reactive protein (CRP), serum amyloid A (SAA), serum amyloid P), coagulation and fibrinolytic system components (e.g., plasminogen, numerous coagulation factors, urokinase plasminogen activator, tissue plasminogen activator, fibrinogen, vitronectin, vitamin K-dependent protein S), protease inhibitors (e.g., α_2-macroglobulin, α_2-antiplasmin, α_1-antitrypsin, α_1-antichymotrypsin), iron and other metal transport proteins that scavenge for free haemoglobin and radicals (e.g., haptoglobin (HP), hemopexin, ferritin, ceruloplasmin), lipid metabolism- and transport-regulating apolipoproteins (e.g., APOA1, APOA4, APOA5, APOE) (Bode et al., 2012; Gabay and Kushner, 1999; Zhou et al., 2016) (Figure 15 and Table 4). Given their important physiological functions, most APPs are produced constitutively at a basal level by hepatocytes (Asselin and Blais, 2011), and upon stimulation, the production of positive APPs increases from about 50% in the case of ceruloplasmin and complement components such as complement component 3 (C3), to around 5-fold for HP, and to as much as 1000-fold in the case of CRP and SAA (Jain et al., 2011; Zhou et al., 2016).

The production of APPs by hepatocytes is induced by a variety of different cytokines that are released during inflammation, with IL-1- and IL-6-type cytokines being the major inducers. These two families of cytokines result in the production of distinct groups of APPs, which are classed as type I or type II depending on their induction by IL-1- or IL-6-type cytokines respectively (Baumann and Gauldie, 1994). The list of mediators involved in regulating APP production in the liver is extensive, however: IL-1-type cytokines include not only IL-1α and IL-1β, but also TNF-α and TNF-β, and the IL-6 cytokine group includes IL-6, leukemia inhibitory factor (LIF), IL-11, oncostatin M (OSM), ciliary neurotrophic factor (CNTF), and

cardiotrophin-1 (CT-1) (Baumann and Gauldie, 1994; Bode et al., 2012). Other cytokines involved in regulating APP-coding gene expression include C-X-C motif chemokine ligand 8 (CXCL8), IFN-γ, HGF, and epidermal growth factor (EGF) (Wigmore et al., 1997).

The hepatic expression of APP-coding genes is regulated primarily at the transcriptional level, although post-transcriptional mechanisms have also been reported for some APPs (Bode et al., 2012). Liver enriched transcription factors (TFs) such as hepatic nuclear factors (HNFs), and members of the CCAAT/enhancer-binding protein (C/EBP) family control basal expression levels. Transcriptional activation of APP-coding genes involves a variety of different transcriptional regulators including signal transducer and activator of transcription (STAT)3 and NF-κB (Asselin and Blais, 2011; Bode et al., 2012; Zhou et al., 2016) (Figure 15). Discordance between the plasma concentrations of different APPs in different inflammatory contexts is common and indicates that APPs are not uniformly regulated, with expression patterns depending on divergent cytokine environments (Gabay and Kushner, 1999; Slaats et al., 2016).

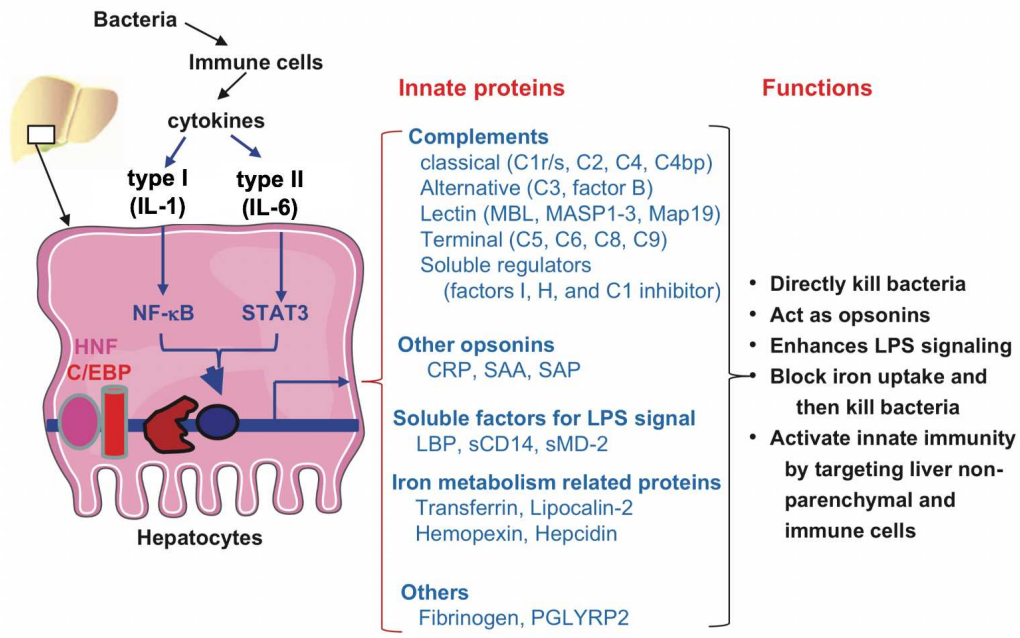

Figure 15 | Acute phase proteins and their role in innate immunity.
Complement proteins and other APPs important in innate immune function are secreted constitutively at a basal level by hepatocytes. Basal expression is controlled by liver-enriched transcription factors including HNFs and C/EBPs. In response to bacterial infection, pro-inflammatory cytokines secreted by immune cells induce hepatocytes to increase the production of type I and type II APPs through NF-κB and STAT3 activation, respectively. APPs have many and diverse antibacterial and immunostimulatory functions, some of which are listed on the right. (Adapted from Zhou et al., 2016)

Acute phase reactants mainly produced in hepatocytes	Major biological functions
Secreted pathogen recognition receptors (PRRs)	
C-reactive protein	Small pentraxin; recognizes phosphocholine and polysaccharides of bacteria such as the pneumococcal C polysaccharide and opsonizes microbes. Mediates complement activation and phagocytosis. Induces the release of ROS and cytokines from macrophages.
Serum amyloid P	Small pentraxin; Ca^{2+}-dependent binding of lipoprotein ligands (phosphoethanolamine) of bacteria. Interaction with complement and matrix components.
LPS binding protein	Interacts with LPS and transfers LPS to the LPS receptor complex (TLR4/MD2).
Soluble CD14	Binds LBP/LPS. Modulation of the myeloid cell response to LPS.
Peptidoglycan-recognition protein-2	N-acetylmuramoyl-L-Ala amidase; hydrolyzes the lactyl bond between the MurNAc and L-alanine in bacterial peptidoglycan; elimination of microbial peptidoglycans to prevent excessive inflammation.
Proteinase inhibitors	
α_1-Antichymotrypsin (ACT)	Serine proteinase inhibitor (serpin); inhibition of cathepsin G
α_1-Antitrypsin (AAT)	Like the ACT member of the serpin superfamily. Trypsin inhibitor. Inactivator of neutrophil elastase. Modulation of neutrophil chemotaxis and inhibition of neutrophil superoxide production.
α_2-Macroglobulin (α_2M)	Plasma protein. Sequence similarity with complement components C3 and C4 (all contain a unique type of reactive site: β-cysteinyl-γ-glutamyl thiol ester). Inhibits serine, cysteine, aspartate and metal proteases. Exhibits cytokine-, growth factor- and hormone-binding function. Modulation of mitogen- and antigen-driven T cell response.
Pancreatic secretory trypsin inhibitor	Specific trypsin inhibitor that prevents autoactivation of trypsinogen. Also called tumor-associated trypsin inhibitor (TATI).
α_1-Cysteine proteinase inhibitor	Cystatins are endogenous inhibitors of lysosomal cysteine proteinases.
Components of the complement system	
C3	Central component of the complement system, activated by the classical, the alternative and the lectin pathways. C3 is converted into C3a (anaphylatoxin) and C3b (interface between the innate and the adaptive immune system).
C4	Classical complement pathway. Activated C4 and C2 form a bimolecular complex, which can function as the C3-convertase of the classical pathway.
C9	Part of the lytic membrane attack complex (MAC). Formation of a stable transmembrane pore (Ø 10 Å) by binding of 10–18 C9 proteins on the membrane of the invading pathogen.
C4b-binding protein	Human complement inhibitor. Accelerates the decay of the C3-convertase.
Mannose-binding lectin (MBL)	Member of the collectin family. Opsonin. Activator of the classical pathway of complement and initiator of the mannose-binding lectin pathway. MBL-pentraxin heterocomplexes trigger a cross-activation of the complement system.
Factor B	Formation of C3-convertase by binding of factor B to C3b on microbial surfaces. Amplifier of the alternative pathway of complement or initiator of the MAC formation.
C1 inhibitor	Alpha-2-neuraminoglycoprotein. Serine protease inhibitor. Inactivator of the complement serine esterases C1r and C1s, kallikrein of the kinin system, and activated factors XI and XII of the coagulation system.
Iron homeostasis	
Hepcidin	Hepatic bactericidal protein. Main regulator of body iron homeostasis. Acts as an antimicrobial peptide by limiting iron availability.
Ferritin	Member of the ferritin-like superfamily. Provides iron homeostasis.
Metal chelating proteins or transport proteins	
Haptoglobin	Crucial role in heme-iron recovery. Binds free hemoglobin (Hb), thereby exerts bacteriostatic and antioxidant function. Reduces the loss of free Hb through glomerular filtration.
Ceruloplasmin	Plasma protein with a broad oxidase specificity. Oxidation of Fe^{2+} and Cu^{1+}. Regulator of copper and iron transport and homeostasis.
Hemopexin	Heme scavenging at systemic level. Glycoprotein with the highest binding affinity to heme, thereby exhibiting antioxidant functions.
Coagulation and fibrinolytic system	
Fibrinogen	Precursor of fibrin. Clotting factor. Central regulator of the inflammatory response (recruitment of leukocytes, association with cell surface receptors, growth factors and coagulation factors).
Plasminogen	Proenzyme. Plg is converted into the active serine protease plasmin (active fibrinolytic enzyme) through the action of a tissue-type plasminogen activator (tPA) or urokinase-type PA (uPA). Modulator of the inflammatory response by plasmin-mediated directional cell migration of hematopoietic cells.
Tissue plasminogen activator	Part of the plasminogen activation system. Highly specific serine proteinase – one of the two principal plasminogen activators. Acts as a fibrogenic cytokine. Major component in the matrix proteolytic network. Part of the plasminogen activation system. Serine protease which converts plasminogen to plasmin. Modulator of extracellular matrix (ECM) remodeling. Supports cell migration and invasion by multiple interactions with integrins, endocytosis receptors and growth factors.
Plasminogen-activator inhibitor 1	Member of the serpin protein family. Physiological inhibitor of the two plasminogen activators tPA and uPA.
Protein S	Vitamin K-dependent plasma glycoprotein. Acts as a nonenzymatic cofactor to activated protein C (APC) in the degradation of factors Va and VIIIa, thereby playing an important role in the protein C (PC) anticoagulant pathways.
Other APPs	
Angiotensinogen	Member of the non-inhibitory serpin family of protease inhibitors. Converted into angiotensin – part of the renin–angiotensin–aldosterone system (RAAS), which controls blood pressure.
IL-1 receptor antagonist (IL-1RA)	Member of the interleukin-1 family. IL-1Ra acts as a receptor antagonist, thereby inhibiting the activities of IL-1α and IL-1β.
α_1-Acid glycoprotein (AGP)	Inhibits activation of neutrophils and up-regulates the expression of IL-1RA by macrophages. Inhibitor of leukocyte extravasation.
Acute phase serum amyloid A (aSAA)	Apolipoprotein; major APP in most species. Associates with the third fraction of HDL and influences cholesterol metabolism. Free aSAA mediates the chemotaxis of monocytes, granulocytes or T-cells. Down-regulates fever and inhibits PGE2 release as well as oxidative burst. At high concentrations a prerequisite for the development of secondary amyloidosis.

Table 4 | Examples of APPs that are produced predominantly by hepatocytes during inflammation and a summary of their major biological function(s). (Source: Bode et al., 2012)

2.2.2. The complement system

The complement system is an evolutionary ancient and fundamental component of the innate immune system required for the detection and removal of invading pathogens (West et al., 2018). It was the first PRR system to be discovered: Jules Bordet was awarded with the Nobel prize in 1919 for his demonstration that there were two factors that conferred bactericidal properties to immune serum: heat-labile "alexin" (complement proteins) and heat-stable "sensitiser" (antibodies) (Cavaillon et al., 2020). As an integral part of the innate immune response, the complement also acts as a bridge between innate and acquired immunity (Beltrame et al., 2014; Nesargikar et al., 2012). The complement system is a network of more than 50 proteins that include secreted PRRs and effector molecules that circulate in the blood, the lymph, and the interstitial fluid, as well as receptors localised at the surface of immune cells. Liver-derived soluble complement components, most of which are APPs, constitute more than 15% of the globular fraction of plasma and circulate as inactive precursors (zymogens) (Dunkelberger and Song, 2010). Upon proteolytic activation, an enzymatic self-sustaining cascade is set in motion, producing molecules that can amplify the inflammatory response, opsonise targets for phagocytosis either directly or through the formation of immune complexes, initiate the formation of the membrane attack complex (MAC) that can directly lyse cells, promote B cell activation and differentiation, and tag apoptotic cells for removal (Verschoor and Langer, 2013, Beltrame et al., 2014) (Figure 16).

The complement system can be activated by various PAMPs or danger-associated molecular patterns (DAMPs) via the classical, lectin or alternative pathway: the classical pathway is activated through complement component 1q (C1q) binding to antigen-antibody complexes, and the alternative pathway through soluble PRR (mannose-binding lectin (MBL), ficolin, or collectin) detection of carbohydrate PAMPs; alternative pathway activation occurs via the spontaneous hydrolysis of C3 to C3(H_20) and its subsequent attachment to microbial surfaces (Beltrame et al., 2014). All pathways converge to trigger the cleavage of the central component, C3, producing the opsonin C3b which associates with C3 convertases to form the C5 convertase, which processes complement C5 into C5b, leading to the assembly of the MAC (Nesargikar et al., 2012) (Figure 17). MAC assembly can occur on different cell surfaces, such as those of bacteria, parasites, enveloped viruses, and aberrant host cells and forms a multimolecular structure that inserts into the membrane creating a functional pore that leads to the lysis of the cell (Dunkelberger and Song, 2010). While the MAC assembles on Gram-positive bacteria it is generally accepted that they are protected from MAC-dependent

lysis by their thick peptidoglycan layer (Berends et al., 2013).

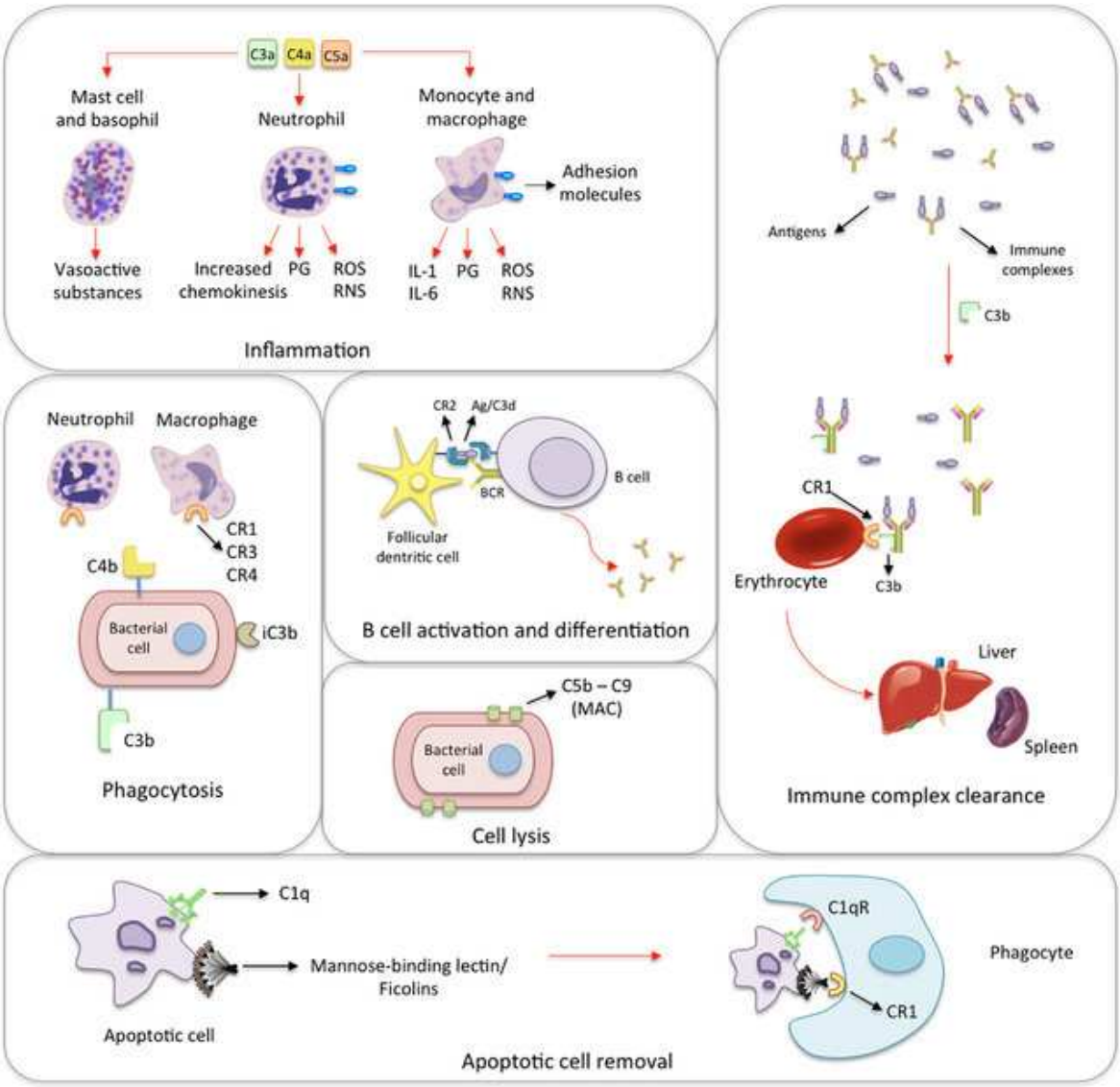

Figure 16 | Biological functions of the complement system.
Inflammation: the activation of the complement system generates the anaphylatoxins or complement peptides C3a, C4a, and C5a which mediate inflammatory responses by recruiting and inducing effector functions in mast cells, neutrophils, and macrophages. **Phagocytosis**: iC3b, C3b, and C4b bind to complement receptors on neutrophils and macrophages promoting phagocytosis. **B cell activation and differentiation**: the recognition of C3-tagged antigen by B cells promotes B cell activation and differentiation. **Cell lysis**: specific antibodies, MBL/ficolins, and spontaneous hydrolysis of C3 activate the complement on the surface of infectious microorganisms and lead to the formation of MAC and the lysis of the target cell. **Immune complex clearance**: immune complexes (antigen-antibody complexes) activate the complement system, C3b binds to the complexes and to complement receptor 1 (CR1) on erythrocytes, resulting in their removal by macrophages. **Apoptotic cell removal**: MBL, ficolins and C1q bind apoptotic cell debris, which is subsequently removed by phagocytic cells via C1q receptor (C1qR) and CR1 receptor binding. (Source: Beltrame et al., 2014)

C3b mediates the opsonisation and induces the subsequent phagocytosis of pathogens by neutrophils and macrophages that express receptors that bind C3 fragments, such as complement receptor 1 (CR1, or CD35), CR3 (CD11b-CD18), CR4 (CD11c-CD18), and complement receptor of the immunoglobulin superfamily (CRIg) (Helmy et al., 2006; West et al., 2018). Activation of C3 and C5 also generates small peptide-like products – C3a and C5a, respectively – known as anaphylatoxins, which mediate inflammatory responses by recruiting and inducing effector functions in neutrophils, macrophages, and mast cells via the engagement of their specific G protein-coupled receptors, C3aR, C5aR1 (CD88), and C5aR2 (Laumonnier et al., 2017) (Figures 16 and 17).

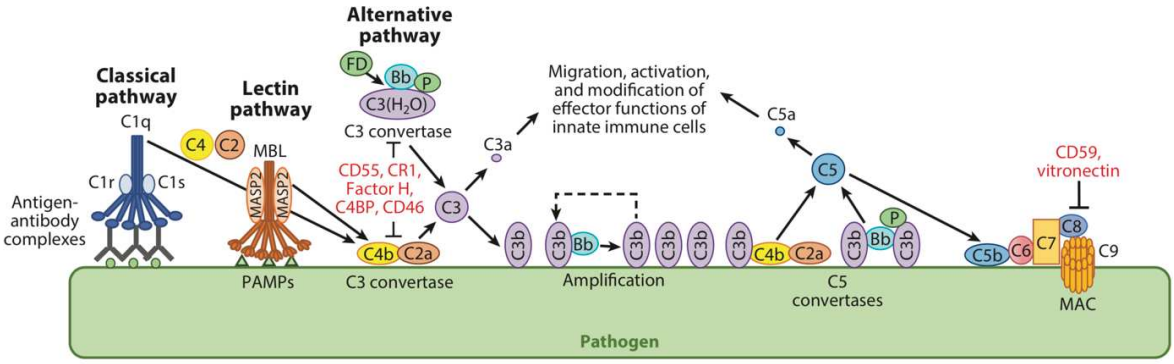

Figure 17 | **Activation of the complement cascade**.
Complement can be activated by three independent pathways: the classical pathway, the lectin pathway, or the alternative pathway. All three pathways converge to trigger the cleavage of C3 into C3a and C3b. C5 convertase is generated, C5b and C5a are produced, and surface bound C5b initiates the formation and insertion of the MAC into the target pathogen or host cell membrane. The generated anaphylatoxins C3a and C5a act as signalling molecules to mediate the inflammatory response. (Adapted from West et al., 2018)

While the innate immune properties of the complement system have long been known and studied, the role of complement components in the induction and regulation of the adaptive immune response is recent and increasingly appreciated. Complement fragment binding to complement and anaphylatoxin receptors on diverse immune cells, including B cells, T cells and DCs, provide costimulatory signals to induce proliferation, effector function and/or cytokine secretion, thus modulating the adaptive immune response (West et al., 2018). It is now also recognised that autocrine complement production in immune-privileged organs such as the brain and eye is the prime source of complement and that normal APC and T cell function depends on this locally produced and secreted C3 and C5 (West et al., 2018). Investigation into the endogenous production of C3 and C5 in T cells led to the discovery that complement activation is not confined to the extracellular space, as the dogma dictates, but also occurs

intracellularly (Arbore et al., 2016; Liszewski et al., 2013). Active intracellular complement – the "complosome" – has since been found to modulate T cell metabolism, driving T cell activation, differentiation, and contraction (West et al., 2018).

3. The hepatic phase of listeriosis

3.1. The hepatic phase in the murine listeriosis model

In the IV murine model of listeriosis, 60–70% of the initial inoculum can be recovered from the liver at just 10 minutes p.i. (Conlan, 1997; Gregory et al., 1996; Mackaness, 1962). This bacterial uptake is reduced to 15% in mice rendered deficient in KCs, illustrating the importance of these tissue macrophages in eliminating *Lm* from the blood (Ebe et al., 1999; Wing and Gregory, 2002). KCs very effectively capture and internalise *Lm* (Broadley et al., 2016; Li and Zeng, 2020) but are less microbicidal than their splenic counterparts *in vitro* (Filice, 1988; Lepay et al., 1985a; 1985b). Consistent with their role as sentinels in hepatic defence, KCs drive the influx of leukocytes to infection foci in the liver in both a chemokine- and adhesion molecule-dependent manner (Ebe et al., 1999; Serbina and Pamer, 2006; Shi et al., 2011; 2010). This uptake and elimination of extracellular *Lm* explains the reduction in bacterial load in the liver in the first 6–8 h p.i. (Conlan, 1999; Conlan and North, 1992), and correlates with an important influx of neutrophils into the liver which peaks at 2 h p.i. (Ebe et al., 1999; Gregory et al., 1996; 2002). Infiltrating neutrophils play an important role in controlling *Lm* infection in the liver by very effectively killing extracellular *Lm* and mediating the lysis and elimination of infected hepatocytes and KCs (Arnett et al., 2014; Rogers et al., 1996; Witter et al., 2016) (Figure 18A). Neutrophil-depletion prior to infection thus results in an important increase in bacterial load in the liver from as early as 6 h p.i. (Conlan and North, 1994; Gregory et al., 1996). KCs succumb massively and rapidly to necroptosis, however, to be replaced by infiltrating bone marrow-derived monocytes that proliferate and differentiate into tissue macrophages (Blériot et al., 2015). Indeed, the "monocytosis" (or increase in circulating monocytes) observed during listeriosis gave rise to the species name (as described in section A.1.1) (Murray et al., 1926).

Despite the early neutrophil and subsequent monocyte infiltrations into the liver, a surviving fraction of *Lm* establishes a highly productive infection in the liver parenchyma that accounts for the exponential increase in the hepatic bacterial burden from 6–8 h p.i., peaking at approximately 3 days p.i. (Conlan, 1999; Ebe et al., 1999; Gregory et al., 1992; Mackaness, 1962). *In vivo* observations of hepatic infection foci composed of heavily infected hepatocytes

(Conlan and North, 1991; 1992; Gaillard et al., 1996; Mandel and Cheers, 1980) (Figures 18B and 18C), *ex vivo* culture of hepatocytes isolated from infected mice (Gregory et al., 1992) (Figure 18D), and *in vitro* infections of hepatocyte cell lines in which *Lm* efficiently replicate and spread from cell to cell (Dramsi et al., 1995; Wood et al., 1993) provided evidence that hepatocytes constitute a replicative niche for *Lm*. Bacterial entry into hepatocytes is proposed to occur through either direct internalisation, via the InlA/B mediated pathways, or through cell-to-cell spread following initial infection of KCs (Appelberg and Leal, 2000; Dramsi et al., 1995; Gaillard et al., 1996). At 3–4 days p.i., the *Lm* burden in the liver plateaus then decreases, concomitant to the initiation of the adaptive immune response that peaks at 6–8 days p.i. and successfully clears the infection (Pamer, 2004).

The propensity of *Lm* to invade and damage the liver was at the origin of the original names proposed for this bacterium: *Bacillus hepatis* (by Hülphers, in 1911) and *Listerella hepatolytica* (by Pirie, in 1927), based on observations of liver necrosis in rabbits and gerbils (Gray and Killinger, 1966). In the murine model of listeriosis, isolated microabscesses are observed in the liver from as early as 24 h p.i., transforming into small granulomas from day 3 p.i. as neutrophils are replaced by macrophages and lymphocytes (Ebe et al., 1999; Gaillard et al., 1996; Mandel and Cheers, 1980; Rogers et al., 1996). The number and size of infected foci is proportional to the inoculum, and lethal doses of *Lm* are generally required to enable the visualisation of hepatic lesions (Gaillard et al., 1996; Rogers et al., 1996). In immunocompetent mice, only small increases, if any, in alanine aminotransferase (ALT) serum levels (an indication of liver damage) are observed, however, when sublethal doses are administered (Ng et al., 2005; Rogers et al., 1996). Once the infection is cleared no lesions remain and normal hepatic architecture is completely restored (Blériot et al., 2015).

In a more physiologically relevant IG inoculation model (murine, guinea pig, or rabbit), the extent and timing of liver colonisation is considerably more variable within experimental groups (due to the bottlenecks described in section A.6.1) and, in mice, between different genetic backgrounds (Hoelzer et al., 2012; Pitts and D'Orazio, 2018). *Lm* are detected in the liver from as early as 4 h to as late as 48 h p.i. in higher numbers and earlier than in the spleen (Drolia et al., 2018; Lecuit et al., 2001; Marco et al., 1992; Melton-Witt et al., 2012; Zhang et al., 2017b). Dissemination to the liver is thought to occur principally from bacterial translocation directly from the intestine via the portal vein, but also through the lymphatic system (Melton-Witt et al., 2012; Zhang et al., 2017b). Like in the IV model of murine listeriosis, the spleen and liver are the major organs colonised and the infection process and

bacterial growth curves in these organs subsequent to their invasion is similar in both IV and IG models (Gaillard et al., 1996; Pitts and D'Orazio, 2018). Murray *et al*. described that IV inoculation of rabbits differed from naturally acquired listeriosis uniquely in that focal necrosis, especially in the liver, was a constant and more dominant feature (Murray et al., 1926). This well-described infection scenario – the product of more than six decades of research since Mackaness' pioneering research in the 1960s – has, however, been constructed using only a few plent lineage II laboratory strains (notably EGD, EGDe, 10403S, and LO28) (Bécavin et al., 2014). The dogma that full clearance of *Lm* from the liver and spleen systematically occurs within 10 days has been questioned in recent publications following experiments in which clinical strains have been isolated from the liver at up to 21 days p.i. (Vázquez-Boland et al., 2020), and in which bacterial loads in the liver at 4–8 days p.i. exceed by several magnitudes that of laboratory strains (Hain et al., 2012; Maury et al., 2016; 2019).

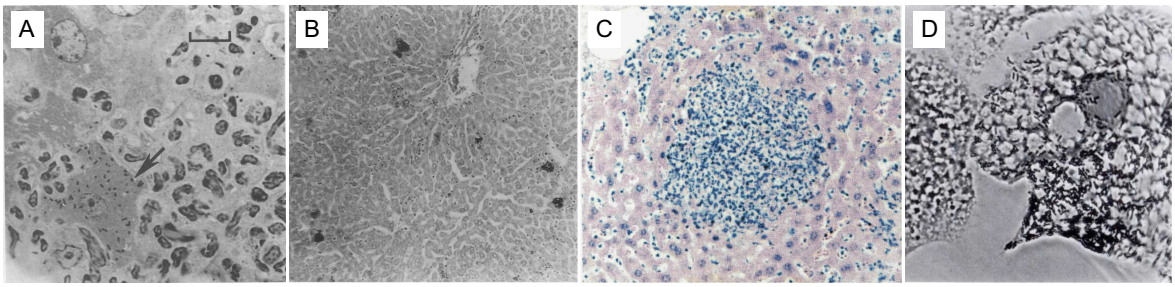

Figure 18 | Liver involvement in murine listeriosis.
(**A**) Liver section from a *Lm*-infected mouse at 24 h p.i. shows an infected hepatocyte undergoing dissolution by surrounding neutrophils (scale bar: 10 µm). (**B**) Gram-stained section of the murine liver at 24 h p.i. showing several infection foci (270×). (**C**) Haematoxylin and eosin-stained liver section from a sublethally *Lm*-infected mouse at 24 h p.i. shows neutrophilic microabscesses containing apoptotic cells surrounded by healthy parenchyma. (**D**) Gram-stained hepatocytes isolated from the liver of a mouse at 72 h post administration of a lethal dose of *Lm* (1000×). (Source: Conlan and North, 1992 (A), Mandel and Cheers, 1980 (B), Rogers et al., 1996 (C), Gregory et al., 1992 (D))

3.2. Liver involvement in human listeriosis

Little is known of the hepatic stage in human listeriosis, however. While transplacental transmission of listeriosis is known to cause disseminated abscesses and granulomas in the foetal liver (Schlech, 2019), liver involvement in adults is rarely documented. Rare reports describe the presence of granulomas, microabscesses, large solitary abscesses, or the clinical manifestations of acute hepatitis (Brönnimann et al., 1998; Gebauer et al., 1989; López-Prieto et al., 2000; Scholing et al., 2007; Yu et al., 1982). Again, surprisingly, few patients in which liver involvement is observed present significantly elevated ALT levels (Braun et al., 1993; Brönnimann et al., 1998; Scholing et al., 2007).

C. Host defences and transcriptional responses to *Lm* infection

The immune response to *Lm* depends on host recognition of pathogen-associated molecules both in the extracellular environment, the internalisation vacuole, and the cytosol. Immune and non-immune cells detect pathogens and respond by activating a transcriptional program that assists in combatting the invasion, either by direct antibacterial action or through cytokine and IFN secretion. Different cell types activate divergent transcriptional programs that allow the host to resolve infection. *Lm* infection induces a robust innate immune response that restricts bacterial growth in the intestine, liver and spleen until the adaptive immune response can be mounted to enable total bacterial clearance, as well as enhanced protection against future infections. *Lm*, on the other hand, has evolved specific mechanisms to counter or modulate this defence system via the secretion of virulence factors whose effects on the host cell contribute directly to the global transcriptional response observed.

1. Innate immune responses to *Lm*

Innate immunity is a first-line host defence mechanism that is rapid and non-specific and common to all animal species from primitive multicellular organisms to vertebrates (Hoffmann and Akira, 2013). The innate immune response depends on the recognition of pathogens and damaged or infected cells through the identification of foreign or danger-signalling molecular patterns by various receptors expressed by almost all immune and non-immune cells (Newton and Dixit, 2012). Depending on the cell type, cells respond by initiating anti-listerial mechanisms – including the production of reactive oxygen species (ROS) or reactive nitrogen species (RNS), inflammasome activation, the upregulation of autophagy-related pathways and activity, the secretion of cytokines and chemokines to signal help, the increased expression of receptors and cell adhesion molecules to better detect bacteria and infected cells as well as to enable the recruitment of immune cells to infection foci, and/or the setting in motion of either cellular survival or apoptotic programs (Lam et al., 2013; McDougal and Sauer, 2018; Radoshevich and Cossart, 2017; Stavru et al., 2011). Innate immune responses are rapidly triggered following *Lm* detection and are essential to host survival, as illustrated by the deleterious effects of the depletion of innate immune molecules, effector cell types, or innate immune cell receptors (Pamer, 2004; Stavru et al., 2011).

1.1. Innate immune sensing of *Lm*

Host recognition of bacterial pathogens is a critical component of the innate immune response, and multiple receptors and pathways capable of detecting and responding to extracellular, intravacuolar, or cytosolic *Lm* have been identified (Dussurget et al., 2014; Radoshevich and Cossart, 2017) (Figure 19). Upon infection, *Lm* can be sensed through secreted or bacterial surface PAMPs by PRRs on the host cell surface, within endocytic vesicles, or in the host cell cytosol (Radoshevich and Cossart, 2017). The principal PRRs involved in the recognition of *Lm* PAMPs include TLRs, retinoic acid-inducible gene I (RIG-I), stimulator of interferon genes (STING), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and the AIM2 inflammasome receptor (Dussurget et al., 2014; Theisen et al., 2018).

Of the TLRs, TLR1, TLR3, TLR10, and notably TLR2 (expressed at both the cell surface and in endosomes) are the most involved in *Lm* detection (Aubry et al., 2012; Regan et al., 2013; Wang et al., 2019). TLR binding to PAMPs such as *Lm* cell wall components or unmethylated CpG motifs in bacterial DNA leads to IκB kinase (IKK)-, serine/threonine-protein kinase (TBK1)- and mitogen-activated protein kinase (MAPK)-mediated activation of NF-κB, interferon regulatory factor (IRF)3, IRF7, and activator protein 1 (AP1) (Eldridge et al., 2020a). The nuclear translocation of these TFs induces the transcription of a large set of NF-κB–dependent pro-inflammatory cytokines, type I and type III IFNs, as well as a subset of interferon stimulated genes (ISGs) whose expression can be induced in an IFN-independent manner (Dussurget et al., 2014; Radoshevich and Cossart, 2017). With the exception of TLR3, all TLRs signal through the adaptor protein known as myeloid differentiation factor 88 (MyD88). TLR2 recruits the toll-interleukin 1 receptor (TIR) domain containing adaptor protein (TIRAP) adapter in addition to MyD88. TLR3 uses the TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent pathway (Dussurget et al., 2014).

STING detects cyclic dinucleotides in the form of cyclic di-adenosine monophosphate (c-di-AMP) secreted by cytosolic *Lm* or cyclic guanosine monophosphate–adenosine monophosphate (cGAMP) produced by host cyclic GMP-AMP synthase (cGAS) from *Lm* DNA following bacterial lysis in the cytosol. Activated STING recruits TBK1, which phosphorylates the TFs IRF3 and IRF7, resulting in IFN-I expression and NF-κB activation (Archer et al., 2014; Burdette et al., 2011; Woodward et al., 2010). The negative regulation of NF-κB signalling by RECON is inhibited by c-di-AMP (McFarland et al., 2017).

RIG-I binds double stranded 5′ triphosphate RNA (PPP-RNA) that is passively released upon bacterial lysis or actively secreted by intracellular *Lm* and signals via the mitochondrial antiviral signalling protein (MAVS) and STING (Abdullah et al., 2012b; Frantz et al., 2019; Hagmann et al., 2013; Pagliuso et al., 2019).

NLRs are cytosolic PRRs: NOD1 and NOD2 recognise conserved peptidoglycan motifs and signal via the adaptor protein RIP2 that recruits TGF-β-activated kinase 1 (TAK1) to activate NF-κB and the MAPK cascade (Mosa et al., 2009; Opitz et al., 2006). Other NLRs are involved in inflammasome activation (Regan et al., 2014). Inflammasomes are cytosolic innate immune surveillance systems that recognise a variety of PAMPs and DAMPs (Theisen and Sauer, 2016). *Lm* can activate a variety of inflammasomes via the detection of LLO, flagellin, or DNA secreted or released through bacteriolysis. Inflammasome receptors (NLRP3, NLRC4, or AIM2) interact with the adaptor protein apoptosis-associated speck-like protein (ASC) which recruits and activates caspase 1 (Theisen and Sauer, 2016). Caspase-1 activation leads to the processing and secretion of pro-inflammatory cytokines IL-1β and IL-18 as well as triggering the pyroptosis of *Lm*-infected cells (Eitel et al., 2010).

The PRRs described above play different roles in different cell types and tissues that have evolved divergent pathogen recognition strategies. IFN-I expression is independent of TLR2-MyD88 signalling in primary murine bone marrow derived macrophages (BMDM) (Leber et al., 2008; McCaffrey et al., 2004) and DCs (Feng et al., 2005; Pontiroli et al., 2012). In peritoneal macrophages, however, TLR2 and MyD88 are required for IFN-β expression (Aubry et al., 2012; Boneca et al., 2007). STING-detection of bacterial DNA appears to be largely restricted to immune cells; epithelial cells such as hepatocytes do not express STING and depend on RIG-I sensing of cytosolic bacteria (Hagmann et al., 2013; Jin et al., 2013; Luo et al., 2018; Thomsen et al., 2016). NOD2 appears to play a predominant role in *Lm* immune sensing in the intestine where TLR expression is low (Kobayashi et al., 2005; Price et al., 2018).

Intracellular sensors to detect cytosolic PAMPS are generally non-redundant at the level of the organism, however (Eldridge et al., 2020a). Deficiency in a single PRR renders mice more susceptible to *Lm* infection: *Tlr2* knock-out (KO) mice showed reduced survival to systemic infection (Torres et al., 2004), *Nod1* KO mice were less resistant to both IP and IV infection (Mosa et al., 2009), and *Nod2* KO mice showed increased susceptibility when challenged using the IG (but not the IV) route of infection (Kobayashi et al., 2005). Mice deficient in the general TLR adaptor protein MyD88 were highly susceptible to *Lm* infection, with completely

abrogated cytokine expression and neutrophil recruitment (Edelson and Unanue, 2002; Seki et al., 2002; Torres et al., 2004).

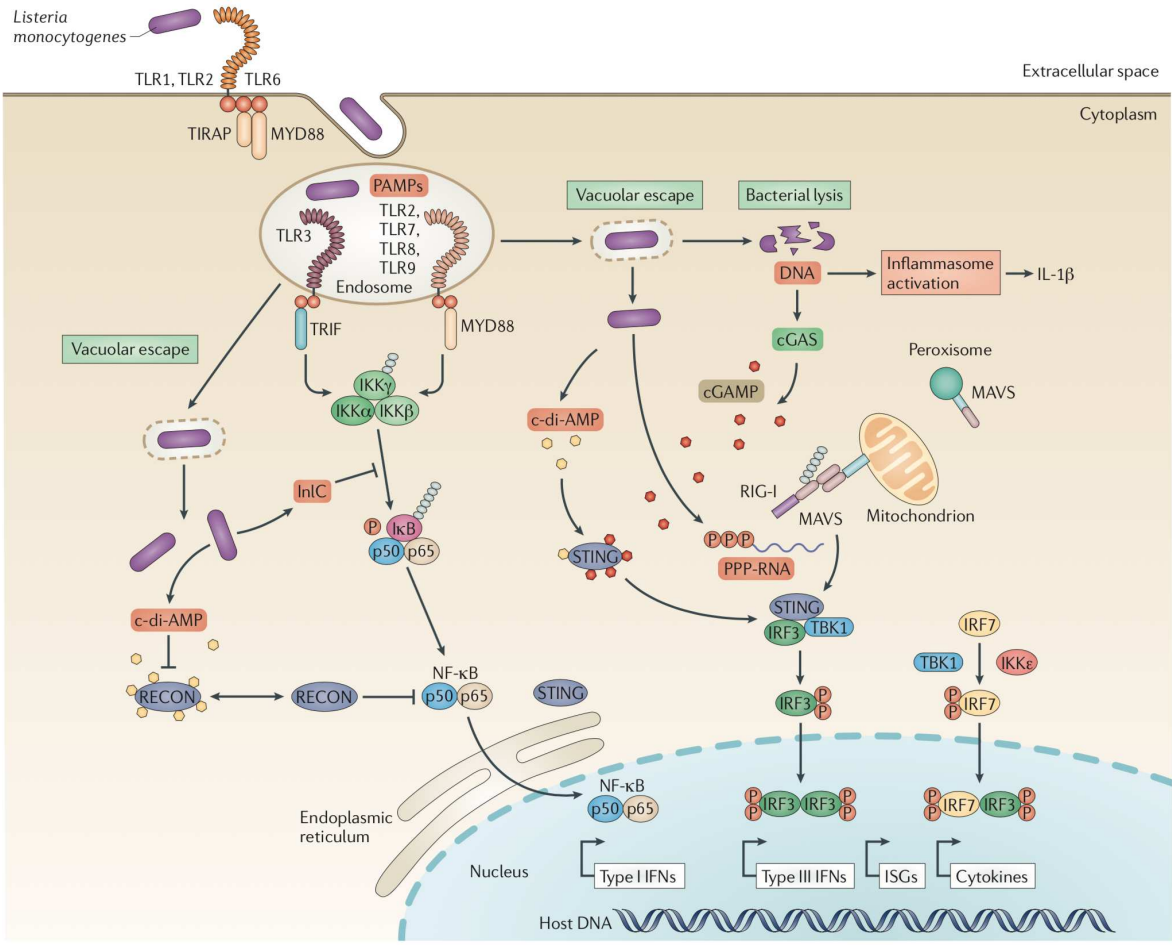

Figure 19 | Innate immune sensing of *Lm*.
Different PRRs detect extracellular, intra-endosomal, or cytosolic *Lm*. Membrane bound TLRs, as well as cytosolic PRRs such as NLRs (not shown), RIG-I, and STING detect listerial PAMPs including lipoteichoic acid, peptidoglycan motifs, and secreted or released nucleic acids in the form of c-di-AMP, cGAMP, or PPP-RNA. Detection results in the downstream activation of NF-κB and the nuclear translocation of IRF3 and IRF7, and the subsequent transcription of type I and III IFNs and other cytokines, as well as the IFN-independent transcription of a subset of ISGs. RECON inhibition of NF-κB is reversed in the presence of c-di-AMP. (Source: Radoshevich and Cossart, 2017)

1.2. Innate immune cells

Regardless of the infection model, macrophages and DCs are the first innate immune cells encountered by *Lm*. In the intestine, once translocated across the intestinal barrier into the lamina propria, *Lm* infects mainly macrophages (Disson et al., 2018). In the liver, KCs, the resident macrophages, are the front-line defence (Li and Zeng, 2020), as described in section B.3.1. In the spleen, several morphologically distinct macrophages and DCs rapidly internalise *Lm* (Aoshi et al., 2009; Conlan, 1996). Resident DCs, macrophages and mast cells at epithelial

barriers excrete inflammatory cytokines and chemokines upon *Lm* detection to recruit monocytes and neutrophils from the bone marrow and the circulation to infection foci (D'Orazio, 2019; Dietrich et al., 2010). Blocking CR3-expressing myeloid cell recruitment to infection foci using an anti-CR3 monoclonal antibody (mAb) results in a 1000-fold increase in bacterial load in the liver and spleen and renders mice susceptible to as few as ten colony forming units (CFU) (compared to 10^7 for untreated mice) (Rosen et al., 1989). Bacterial growth during the first few days of infection is controlled by various and diverse innate immune cells, including, macrophages, monocytes, neutrophils, NK cells, γδ T cells, and DCs that present a first line of defence (Stavru et al., 2011).

1.2.1. Monocytes and macrophages

Both lymphoid tissue resident CD169+ macrophages and liver resident KCs are essential in the initial clearance of *Lm* (Aoshi et al., 2009; Ebe et al., 1999; Gregory et al., 2002; Perez et al., 2017), and subsequent infiltrating monocytes are an equally vital component of the early innate immune response to *Lm* (Shi et al., 2010; 2011). Monocytes represent a heterogenous population of circulating blood leukocytes of myeloid origin that are pluripotent and can thus, upon recruitment to the infection site, differentiate into macrophages or other monocyte-derived cells such as monocyte-derived DCs (Grabowska et al., 2018). Emigration of monocytes from the bone marrow into the circulation during *Lm* infection is triggered by chemokines, while recruitment to infection foci is mediated through adhesion molecules (Shi et al., 2010). In the liver, intercellular adhesion molecule 1 (ICAM-1) on the surface of LSECs or KCs binds to CR3 on circulating monocytes (Serbina et al., 2012) (Figure 20). IFN-γ activated monocytes and macrophages phagocytose and kill *Lm* by the production of ROS or RNS and produce large amounts of inflammatory cytokines such as IL-12 and IL-18 (Shaughnessy and Swanson, 2007). Monocytes are also thought to act as Trojan horses in aiding *Lm* invasion, however. Monocytes can be infected in the bone marrow (Hardy et al., 2009), and then migrate to chemokine-expressing but uninfected tissue, transporting *Lm* to the brain or other peripheral tissue (Drevets et al., 2008; 2010).

1.2.2. Neutrophils

Polymorphonuclear leukocytes, or neutrophils, are the most abundant leukocytes in circulation and the first cells recruited to infected or injured tissue (Chaplin, 2010). Unlike monocytes, neutrophils are terminally differentiated into pre-programmed antimicrobial phagocytes and have a very short life span (Brostjan and Oehler, 2020; Witter et al., 2016). They play an

important role in controlling *Lm* infection in its early stages, especially in the liver (Carr et al., 2011; Witter et al., 2016). They are recruited in large numbers from the bone marrow in response to various chemokines and very efficiently and rapidly eliminate extracellular *Lm* and infected cells though phagocytosis and ROS/RNS production (Kobayashi et al., 2003; Witter et al., 2016). LLO is unable to mediate *Lm* phagosomal escape in these granulocytes, and instead enhances neutrophil phagocytic efficiency (Arnett et al., 2014). Another key contribution of neutrophils to the innate immune response is their important production of TNF-α, a cytokine at the top of the cytokine cascade essential in the innate immune response to *Lm* (Carr et al., 2011; Witter et al., 2016).

1.2.3. Natural killer cells

NK cells are innate cytotoxic lymphocytes that are able to recognise and kill malignant or infected cells by releasing cytolytic mediators such as perforin and granzymes or by expressing ligands able to trigger death receptors on target cells (Bernardini et al., 2012). Within the first few days of systemic *Lm* infection, NK cells are also the major source of the crucial pro-inflammatory cytokine IFN-γ (Clark et al., 2016; Humann et al., 2007; Kang et al., 2008).

1.2.4. Dendritic cells

DCs are professional APCs, central in the priming of T cells during *Lm* infection. Various DC subsets are important in both the pathogenesis and the host response to *Lm*. DCs can be divided into three major categories: plasmacytoid DCs (pDCs), conventional DCs (cDCs), and monocyte-derived DCs (mDCs or "inflammatory DCs") that arise in response to infection or inflammation (Chen et al., 2015; Edelson et al., 2011).

Splenic CD8α+ cDCs are potent APCs that traffic to T cell lymphoid tissue in the spleen or MLNs to prime naive T cells (Aoshi et al., 2008; Edelson, 2012). They also provide a replicative niche for *Lm*, however, and their migratory nature can promote listerial dissemination (D'Orazio, 2019; Waite et al., 2011). While macrophages contain and trap *Lm* in the marginal zone of the spleen, *Lm* survives in CD8α+ cDCs which migrate to the periarteriolar lymphoid sheath (the inner portion of the white pulp consisting mainly of T cells) where *Lm* numbers increase exponentially (Aoshi et al., 2009; Edelson et al., 2011; Liu et al., 2019; Neuenhahn and Busch, 2007). Mice depleted of this cDC subset are more resistant to *Lm* infection and have lower bacterial loads in both the liver and the spleen (Edelson, 2012; Perez et al., 2017). Intestinal CD103+ cDCs have also been implicated in the spread of *Lm* from

Peyer's patches to MLNs following GI invasion in another Trojan horse-like mechanism (Pron et al., 2001).

The prototypical mDC is the TNF- and inducible nitric oxide synthase (iNOS)-producing DC (Tip-DC) (Edelson, 2012). Tip-DCs are recruited to infection foci through CCR2 signalling and aid in controlling *Lm* replication through direct NOS-dependent bactericidal activity and indirectly by contributing to TNF-α production (Serbina et al., 2003b). Tip-DCs are also critical sources of IFN-I during infection (Dresing et al., 2010; Solodova et al., 2011).

1.2.5. Mast cells

Mast cells are tissue-resident myeloid cells strategically located in mucosal and epithelial barriers where antigen-pathogen encounters are frequent (Krystel-Whittemore et al., 2015). Mast cells do not internalise *Lm* but play a role in controlling infection by initiating neutrophil recruitment and mediating the inflammatory response through the secretion of pro-inflammatory cytokines and chemokines. Antibody-mediated depletion of mast cells results in increased bacterial burdens and reduced neutrophil infiltration in the IP murine listeriosis model (Dietrich et al., 2010; Gekara and Weiss, 2008; Soria-Castro et al., 2021).

1.3. Cytokines

The innate immune response to *Lm* critically depends on the rapid production and the non-redundant activity of a number of cytokines, including chemokines, pro-inflammatory cytokines, and IFNs, that, together, mediate the recruitment of circulating immune cells to infection foci, the activation of tissue-resident and recruited immune cells, the induction of anti-listerial activity and the production and secretion of innate immune effectors from both immune and non-immune cells (D'Orazio, 2019) (Figure 20).

1.3.1. Chemokines

Chemokines are among the first products of a common inflammatory response to infection, secreted in response to PRR sensing of PAMPs by both innate and adaptive immune cells, as well as epithelial cells such as hepatocytes and colon epithelial cells (Nau et al., 2002; Pontiroli et al., 2012; Serbina et al., 2003a; Tchatalbachev et al., 2010). Chemokines trigger the release of granulocytes and monocytes from the bone marrow and recruit bone marrow derived and circulating neutrophils, monocytes, and NK cells to infection foci (D'Orazio, 2019; Kang et al., 2008) (Figure 20).

- CXCL1, CXCL2, and CXCL8

C-X-C motif chemokine ligand (CXCL)1, CXCL2, and CXCL8 are neutrophil-attracting chemokines produced predominantly in the liver (Barsig et al., 1998; Ebe et al., 1999) that bind to C-X-C motif chemokine receptor 2 (CXCR2) expressed on neutrophils (Witter et al., 2016). Treatment with anti-CXCL2 and -CXCL8 mAbs severely ablated neutrophil recruitment and abscess formation in the liver (Ebe et al., 1999). CXCL8 has also been reported to induce the expression APPs in hepatocytes (as described in section B.2.2.1) (Wigmore et al., 1997).

- CCL2, CCL7, and CCL12

C-C motif chemokine ligand (CCL)2, CCL7, and CCL12 mediate monocyte recruitment in response to *Lm* infection through C-C chemokine receptor type 2 (CCR2) expression on mesenchymal stem cells in the bone marrow, monocytes, and a subset of NK cells (Serbina et al., 2012). CCR2 is necessary for the emigration of monocytes from bone marrow (but not to infection foci in the spleen or liver), and its deficiency markedly increases the susceptibility of mice to *Lm* infection (Blériot et al., 2015; Jia et al., 2008; Kurihara et al., 1997; Shi et al., 2010).

- CCL3, CCL4, and CCL5

CCL3, CCL4, and CCL5, are members of the monocyte chemotactic protein family and ligands of C-C chemokine receptor type 5 (CCR5) (Zhong et al., 2004). CCR5, like CCR2, is thought to play a role in NK cell recruitment to inflammatory sites (Bernardini et al., 2012; Kang et al., 2008).

1.3.2. Pro-inflammatory cytokines

- IL-6

IL-6 is a pleiotropic cytokine produced by a variety of cell types during *Lm* infection including KCs (Gregory et al., 1998) and other phagocytes of myeloid origin (Lücke et al., 2018) as well as mast cells (Soria-Castro et al., 2021). Mice treated with recombinant IL-6 prior to infection show enhanced bacterial clearance (Czuprynski et al., 1992; Liu et al., 1992). *Il6* KO mice or mice treated with a neutralising anti-IL-6 mAb, on the other hand, fail to control *Lm* infection and succumb to sublethal doses as both the inflammatory acute phase response (APR) and the recruitment of neutrophils are impaired (Dalrymple et al., 1995; Hoge et al., 2013; Kopf et al., 1994).

- IL-1α and IL-1β

IL-1 was first identified as a pyrogen that was subsequently found to be, in fact, two proteins, later named IL-1α and IL-1β, encoded by *IL1A* and *IL1B* genes, respectively. Both IL-1α and IL-1β bind the same cellular receptors (IL-1RI and the decoy receptor IL-1RII) and induce similar immunological effects (Fields et al., 2019). IL-1α is already active in its primary precursor form but IL-1β requires processing by caspase-1 cleavage before secretion (Fields et al., 2019). IL-1 receptor inactivation by mAb neutralisation or *Il1r1* gene KO increased bacterial loads by up to 1000- and 100-fold in the liver and spleen, respectively, but did not affect IL-6 or APP production (Havell et al., 1992; Labow et al., 1997). The combined mAb neutralisation of IL-1α, IL-1β, and IL-1RI in SCID mice increased mortality and bacterial load and impaired neutrophil migration and peripheral blood leukocytosis, and reduced MHC-II expression in macrophages (Rogers et al., 1992; 1994).

- IL-12 and IL-18

IL-12 (a heterodimeric cytokine encoded by *IL12A* and *IL12B* genes) and IL-18 (encoded by *IL18*) are produced early in infection mainly by activated macrophages and DCs. Both are important mediators in the immune response to *Lm*, primarily through their induction of IFN-γ secretion by NK and CD8 T cells (Berg et al., 2003; Edelson, 2012; Humann and Lenz, 2010; Kang et al., 2008; Tripp et al., 1994; 1993). While neither mAb neutralisation of IL-12 (Tripp et al., 1994) nor IL-12 gene deletion (Brombacher et al., 1999; Seki et al., 2002) significantly affected bacterial burden or susceptibility of mice to low infectious doses of *Lm*, at higher doses susceptibility was significantly increased. IL-12/IL-18 double KO mice were even more susceptible than the single IL-12 KO (Seki et al., 2002). IL-12 mediates the differentiation of CD4 T cells into Th1-type effector cells: the neutralisation of this interleukin during *Lm* infection promotes Th2-type CD4 T cell differentiation and the IL-4 production associated with the Th2 immune response (Hsieh et al., 1993; Lee et al., 2013).

- IL-23 and IL-17

IL-23 is a heterodimeric pro-inflammatory cytokine of the IL-12 family (Vignali and Kuchroo, 2012) and signals through its interaction with the heterodimeric IL-23 receptor (IL-23R), expressed on T cells, macrophages, and DCs (Sieve et al., 2010). IL-23 is secreted by activated macrophages and DCs and is involved in the generation and maintenance of IL-17-producing T cells and the induction of IL-17 secretion by γδ T cells (Indramohan et al., 2012; Meeks et

al., 2009). IL-17 promotes neutrophil and monocyte recruitment by inducing chemokine production (Indramohan et al., 2012) and is required for the generation of the Ag-specific CD8 T cell response to primary *Lm* infection (Xu et al., 2010). *Il23a* KO mice displayed decreased production of IL-17 that led to impaired monocyte and neutrophil infiltration in the spleen and liver, respectively, leading to increased bacterial burdens in both organs and reduced survival (Graham et al., 2011; Indramohan et al., 2012; Meeks et al., 2009). In the intestine, *Lm* invasion of Peyer's patches triggers the expression of IL-23 which in turn induces IL-17, IL-22, and IL-11 production which is necessary for the intestinal epithelium response to infection that blocks intestinal villus invasion by *Lm* (as described in section A.6.1) (Disson et al., 2018).

- TNF-α

Tumor necrosis factor (TNF-α) owes its name to its potent anti-tumour activity (Carswell et al., 1975), and is a pleiotropic cytokine critical for cell trafficking, inflammation, maintenance of lymphoid organ structure, and host defence against pathogens, including *Lm* (Grivennikov et al., 2005). TNF-α is secreted mainly by monocytes and neutrophils (Carr et al., 2011) and is responsible for the initial activation of innate immune cells by triggering the first wave of IFN-γ production by NK cells, and mediating the recruitment of DCs and inflammatory cells to infection foci through the induction of CCL2 production (Bancroft et al., 1991; Grivennikov et al., 2005; Humann and Lenz, 2010; Lee et al., 2013; Tripp et al., 1993). Mice deficient in TNF-α production (*Tnf* KO) (Li et al., 2017), lacking the TNF-α receptor (*Tnfr1* or *Tnfr2* KO) (Erickson et al., 1994; Pfeffer et al., 1993; Rothe et al., 1993), or treated with an anti-TNF-α mAb (Bancroft et al., 1991; Havell, 1989; Nakane et al., 1988) are highly susceptible to *Lm* infection and succumb to sublethal IV or IP doses. Increased risk of human listeriosis has also been identified in patients treated with anti-TNF-α mAbs (Li et al., 2017).

1.3.3. Anti-inflammatory cytokines

Anti-inflammatory cytokines inhibit the production of pro-inflammatory cytokines and antagonise their antimicrobial effects.

- IL-4

IL-4 favours the alternative activation of macrophages and a Th2 type immune response that increases susceptibility to *Lm* (Kaufmann et al., 1997; Lee et al., 2013, Blériot et al., 2015). Mice treated with an anti-IL-4 mAb showed increased resistance to *Lm* infection, decreased bacterial burdens and earlier clearance (Haak-Frendscho et al., 1992). IL-4 production by

basophils in the liver is necessary, however, for the subsequent return to hepatic homeostasis following infection and the induction of monocyte proliferation to replace KCs eliminated during infection (Blériot et al., 2015).

- IL-10

IL-10 inhibits the production of TNF-α, IL-12, and IFN-γ in murine splenocytes *in vitro* (Tripp et al., 1993) and reduces the listericidal capacity of splenic macrophages by delaying phagosome maturation and reducing iNOS production *in vivo* (Liu et al., 2019). Mice deficient in IL-10 are highly resistant to systemic *Lm* infection and show decreased bacterial burdens, earlier clearance and an enhanced pro-inflammatory cytokine response (Carrero et al., 2006; Dai et al., 1997; Foulds et al., 2006). IL-10 has also been reported, however, to play a protective role in a murine *Lm* meningoencephalitis model (Deckert et al., 2001) and is implicated in the generation of memory and effector CD8 T cells (Foulds et al., 2006).

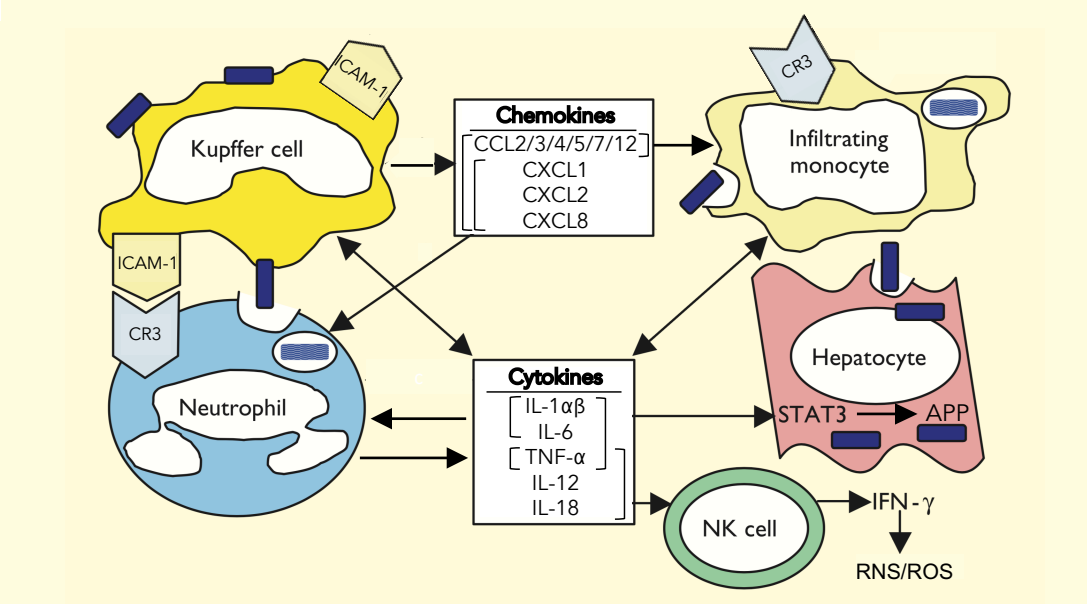

Figure 20 | A simplified scenario of the role of chemokines and pro-inflammatory cytokines in the innate immune response to *Lm* in the liver.
Liver resident macrophages – KCs – detect and capture *Lm* and secrete chemokines to recruit monocytes (e.g., CCL2) and neutrophils (e.g., CXCL1) to infection foci. Recruitment is facilitated by the interaction of the adhesion molecule ICAM-1 on the KC surface with CR3 expressed by infiltrating monocytes and neutrophils. Infiltrating and resident leukocytes secrete, in turn, various cytokines that induce the continued infiltration (e.g., IL-1α, IL-6) and activation (e.g., IL-1α, IL-1β) of effector cells, promote cytokine production (e.g., TNF-α, IL-12, IL-18 secreted by monocytes, macrophages, and neutrophils induce IFN-γ production by NK cells), and stimulate APP production in hepatocytes (e.g., IL-1β, IL-6, TNF-α secreted by KCs and monocytes). (Adapted from Cousens and Wing, 2000)

1.3.4. Interferons

The term “interferon” was coined by Isaacs & Lindenmann in 1957 to describe a substance produced by cells inoculated with inactivated virus that “interferes” with viral infection (Isaacs and Lindenmann, 1957). More than two decades later it became clear that IFN is not one, but a family of cytokines, grouped today into three types designated as type I, II, or III IFN (Borden et al., 2007). IFN-I was the first to be discovered and is the largest IFN group consisting of 13 or 14 IFN-α subtypes (in humans or mice respectively), IFN-β, -ε, -κ, -ω (humans) and -ζ, (mice). IFN-II has only one member: IFN-γ. Type III interferon (IFN-III) includes four IFN-λ subtypes: IFN-λ1 (IL-29), IFN-λ2 (IL-28A), IFN-λ3 (IL-28B), and IFN-λ4; in mice IFN-λ1 and IFN-λ4 are pseudogenes (Lasfar et al., 2006), and polymorphisms in human gene sequence have led to the loss of IFN-λ4 expression in several human populations (Hamming et al., 2013; Prokunina-Olsson et al., 2013).

- IFN signalling

IFN-I and IFN-III are produced by a wide range of immune and non-immune cells upon sensing of PAMPs by cellular PRRs (as described in section C.1.1). IFN-γ, on the other hand, is produced by certain immune cells primarily in response to other cytokines (as described in section C.1.3) (Alphonse et al., 2021). IFNs signal in an autocrine and paracrine manner through their respective receptors to activate janus activated kinase/signal transducer and activator of transcription (JAK/STAT) signalling cascades, resulting in the expression of hundreds of genes, known collectively as ISGs or the “interferome” (Samarajiwa et al., 2008). ISGs are best known for their broad-spectrum cell-autonomous anti-viral activity, for which IFNs were discovered, but they also play an important role in the immune response to intracellular bacteria and parasites, and their immunomodulatory functions continue to be elucidated (Hubel et al., 2019; Schneider et al., 2014; Tan et al., 2021). The number and nature of ISGs expressed depend on the IFN type and subtype as well as cell-type signalling variations (Alphonse et al., 2021; Lee and Ashkar, 2018). IFN receptors each contain two unique receptor chains, one with low affinity and one with high affinity for IFN binding (Schneider et al., 2014). Although IFN-I and IFN-III signal through unrelated receptors – IFNAR (composed of IFNAR1/IFNAR2 subunits) and IFNLR (IFNLR1/IL10R2), respectively, they trigger the same signal transduction cascade to initiate the transcription of a similar set of genes whose expression is dependent on IFN-stimulated response element (ISRE) promoter sequences (Lazear et al., 2019) (Figure 21).

IFN-γ signals through the IFN-γ receptor (IFNGR), composed of IFNGR1 and IFNGR2 chains, and leads to the transcription of ISGs containing a γ-activation sequence (GAS) in their promoter sequence (Dussurget et al., 2014). Each IFN receptor subunit is non-covalently and constitutively associated with a kinase of the JAK family: JAK1 in the case of IFNAR2, IFNRL1, and IFNGR1; tyrosine kinase 2 (TYK2) for IFNAR1 and IL10R2; JAK2 for IFNGR2 (Schneider et al., 2014). Upon IFN binding, JAK kinases form a functional complex that phosphorylate STATs resulting in STAT homo- (IFN-II) or hetero-dimerisation (IFN-I/III). Phosphorylated STAT1 homodimers form the IFN-γ activation factor (GAF) that translocates to the nucleus to activate of GAS-dependent gene expression, and STAT1/STAT2 associates with IRF9 to yield the IFN-stimulated gene factor 3 (ISGF3) complex that activates the transcription of ISRE-dependent genes (Schneider et al., 2014) (Figure 21). The IFN signalling cascade described above is referred to as canonical, but non-canonical signalling pathways have also been described, and involve non-classical modifiers of STATs which include, among others, the MAPK and PI3K/mTOR pathways (Majoros et al., 2017; Mazewski et al., 2020).

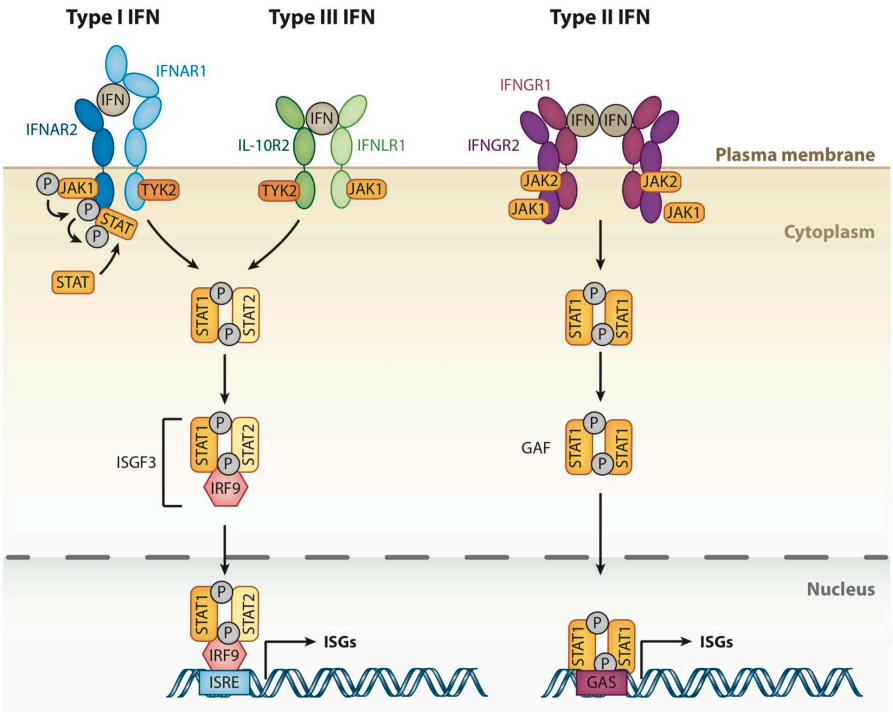

Figure 21 | Signal transduction by type I, II, and III IFNs.
Engagement of IFNAR1/IFNAR2 and IFNLR1/IL10R2 receptors by their respective ligands IFN-I and IFN-III, triggers the phosphorylation of JAK1/TYK2 kinases that activate STAT1 and STAT2. Phosphorylated STAT1/STAT2 heterodimers bind IRF9 to form the ISGF3 complex which translocates to the nucleus where it induces the expression of ISGs regulated by ISRE-dependent promoters. IFN-II binding to the IFNGR1/IFNGR2 receptor induces phosphorylation of JAK1/JAK2 kinases that activate STAT1. Phosphorylated STAT1 homodimers form GAF that translocates to the nucleus to induce the expression of ISGs regulated by GAS-dependent promoters. (Source: Schneider et al., 2014)

- Type II interferon: IFN-γ

IFN-γ is perhaps the most critical cytokine for controlling primary *Lm* infection and is involved in both the innate and adaptive phases of the immune response (D'Orazio, 2019; Zenewicz and Shen, 2007). Resistance to systemic listeriosis is severely impaired in mice treated with a neutralising anti-IFN-γ mAb (Bancroft et al., 1987; Buchmeier and Schreiber, 1985) or in mice with targeted disruption of one of the IFNGR-coding genes (*Ifngr1* or *Ifngr2*) (Dai et al., 1997; Huang et al., 1993; Lee et al., 2013) or the gene encoding IFN-γ (*Ifng*) (Harty and Bevan, 1995; Seki et al., 2002). In an IG murine listeriosis model using humanised E-cad mice (Disson et al., 2008), mAb-mediated neutralisation of IFN-γ and the use of *Ifng* KO mice illustrated the essential role of this cytokine in *Lm*-induced enterocyte proliferation, in controlling the bacterial burden in intestinal tissue and in limiting the dissemination of *Lm* to MLNs and the spleen (Disson et al., 2018; Reynders et al., 2011). IFN-γ activates macrophages and amplifies their listericidal activities through increased expression of ROS- and RNS-producing enzymes (Dussurget et al., 2014) and is essential for inducing the production of inflammatory cytokines by various innate immune cells (Dai et al., 1997; Kiderlen et al., 1984; Lee et al., 2013).

- Type I interferons

IFN-α and IFN-β are produced by both immune and non-immune cells in response to *Lm* infection *in vitro* (Bierne et al., 2012b; O'Riordan et al., 2002; Stockinger et al., 2009), and *in vivo* (Bergmann et al., 2013; Dresing et al., 2010; Solodova et al., 2011; Stockinger et al., 2009). IFN-β expression is highest in the spleen, peaking at around 24 h, and Tip-DCs are the major source (Dresing et al., 2010; Solodova et al., 2011). While type I IFNs are generally potent antiviral mediators, their contribution to the outcome of bacterial infection is disparate due to their pleiotropic effects in distinct cell environments and at different stages in the infectious process (Boxx and Cheng, 2016; Carrero, 2013).

Numerous studies have shown that IFN-I expression is detrimental to the host during systemic *Lm* infection. Mice deficient in IFN-I signalling, either through IFNAR1, IRF3, IRF7, or IRF9 KO are more resistant to infection and exhibit bacterial burdens in the liver and spleen several orders of magnitude lower than their wild type (WT) counterparts at 3–4 days p.i. (Auerbuch et al., 2004; Carrero et al., 2004; Demiroz et al., 2021; O'Connell et al., 2004; Stockinger et al., 2009). Multiple mechanisms have been proposed to explain the deleterious effect of IFN-I in *Lm* infection. IFN-I signalling enhances *Lm* infection-induced macrophage apoptosis (O'Connell et al., 2004; Stockinger et al., 2002; Zwaferink et al., 2008) as well as splenic

lymphocyte apoptosis (Carrero, 2013; Carrero and Unanue, 2006). IFN-I signalling has also been linked to the impairment of neutrophil recruitment during *Lm* infection (Dietrich et al., 2010), due both to the induction of bone marrow neutrophil apoptosis (Dietrich et al., 2010; Navarini et al., 2006) and to IFN-I-mediated negative regulation of IL-17A+ γδ T cell expansion and IFN-I (and IL-12) antagonisation of IL-17 production by these cells (Curtis et al., 2009; Henry et al., 2010). IFN-I has also been implicated in impeding IFN-γ signalling by downregulating IFNGR1 expression at the transcriptional level (Eshleman et al., 2017; Kearney et al., 2013; Rayamajhi et al., 2010b), and, more recently, the ISG and negative regulator of IFN-I signalling ubiquitin-specific protease 18 (USP18) was shown to promote bacterial replication by inhibiting TNF-α signalling and subsequent ROS production in DCs (Shaabani et al., 2018). Another ISG, IFN-induced transmembrane protein 3 (IFITM3), was found to suppresses the proteolysis of ActA and LLO in macrophages, leading to increased *Lm* cell-to-cell spread (Tan et al., 2021). This is consistent with the results of a previous study by the same team in which the authors demonstrated the ability of IFN-I signalling to promote the cell-to-cell spread of *Lm* through enhanced ActA polymerisation (Osborne et al., 2017).

It is of note, however, that several recent studies have, in contrast, highlighted a beneficial role for IFN-I during *Lm* infection, pointing to the infection route and the timing of IFN-I production as determinative factors. When the natural route of infection was employed, using *Lm* strains expressing murinised InlA, IFNAR KO mice showed either decreased or equivalent survival, and had similar bacterial loads in the liver and spleen compared to their WT littermates (Kernbauer et al., 2013; Pitts et al., 2016). Moreover, administering IFN-β to mice shortly after a lethal IV inoculation significantly prolonged their survival, suggesting a beneficial effect for the host during the early phase of *Lm* infection (Pontiroli et al., 2012).

- Type III interferons: IFN-λ

As IFN-I and IFN-III induce the same JAK/STAT signalling pathway, early studies suggested that IFN-III were functionally redundant with IFN-I, although it is now clear that IFN-I and IFN-III have distinct functions (Hemann et al., 2017; Stanifer et al., 2020). This is partly due to the differential expression of receptors in different tissues and cell types. IFNAR is ubiquitously expressed, albeit at highly variable levels, with binding sites ranging from 200 to up to 250,000 depending on the cell type (Zanin et al., 2021). IFNLR expression, on the other hand, is restricted to epithelial cells (Ank et al., 2008; Dickensheets et al., 2013; Lin et al., 2016; Mordstein et al., 2010; Pott et al., 2011; Sommereyns et al., 2008; Zhou et al., 2007) and

a subset of immune cells, notably human and mouse neutrophils (Blazek et al., 2015; Broggi et al., 2017; Espinosa et al., 2017; Galani et al., 2017), macrophages (de Groen et al., 2015; Hou et al., 2009; Liu et al., 2012; Read et al., 2019; Wang et al., 2017), pDCs (Ank et al., 2008; Kelly et al., 2016; Yin et al., 2012), and human mDCs (Dolganiuc et al., 2012; Mennechet and Uze, 2006; Read et al., 2019). The kinetics and the magnitude of the IFN-I and IFN-III response also differ, contributing to producing the distinct physiological responses of these two IFN types. Whereas IFN-I induces the rapid and transient expression of ISGs with high amplitude, IFN-λ mediates long-lasting expression of ISGs with lower amplitude (Forero et al., 2019; Jilg et al., 2014; Marcello et al., 2006; Pervolaraki et al., 2018; Pulverer et al., 2010).

The use of *Ifnlr1* and double *Ifnl2/3* KO mice and cell lineage-specific *Ifnlr1* deletion has contributed greatly to the identification of non-redundant functions for IFN-III in immunity, and has highlighted the particular importance of this IFN type at epithelial barriers and mucosal surfaces (Lee and Baldridge, 2017; Stanifer et al., 2020; Walker et al., 2021; Ye et al., 2019). IFN-III, and specifically IFN-λ1, is, for example, the predominant IFN type produced by human hepatocytes in response to hepatitis C virus (HCV) infection and is associated with ISG upregulation and the inhibition of viral replication (Marukian et al., 2011; Park et al., 2012; Thomas et al., 2012). In response to influenza A virus, respiratory epithelial cells primarily produce and respond to IFN-III (Jewell et al., 2010; Mordstein et al., 2010). Intestinal epithelial cells express low levels of IFNAR and respond poorly to IFN-I but are potent producers of IFN-III in response to enterovirus infection and respond strongly to this IFN (Hernández et al., 2015; Lin et al., 2016; Mahlakõiv et al., 2015; Su et al., 2020). In murine rotavirus and norovirus gastroenteric infection models, IFN-III is the predominant IFN type responsible for eliciting ISG expression that mediates viral clearance (Mahlakõiv et al., 2015; Nice et al., 2015; Pott et al., 2011).

IFN-III has been shown to have immunomodulatory properties and to exert similar anti-viral activity to IFN-I without the tissue-damaging inflammatory response associated with IFN-I signalling (Blazek et al., 2015; Broggi et al., 2017; Davidson et al., 2016; Forero et al., 2019; Galani et al., 2017). Recent studies have, however, highlighted IFN-III mediated aggravation of lung pathology and inflammation including reduced epithelial barrier integrity during respiratory viral infection, resulting in increased susceptibility to bacterial superinfections (Broggi et al., 2020a; Major et al., 2020; Read et al., 2021).

While the expression of IFN-III genes is known to be induced by multiple bacterial pathogens, including *Lm*, in a variety of human and murine cell types, including epithelial cells, *in vitro* (Bierne et al., 2012b; Lebreton et al., 2011; Odendall et al., 2014; 2017; Peignier et al., 2020), few studies have examined the contribution of IFN-λ to bacterial clearance or pathogenesis *in vivo* (Cohen and Parker, 2016; Kotenko et al., 2019). Both *Staphylococcus aureus* and *Pseudomonas aeruginosa* were found to activate IFN-III signalling in the lungs, and clearance of both bacteria was improved in IFNLR KO versus WT mice (Cohen and Prince, 2013; Pires and Parker, 2018). IFNLR deficiency also enhanced bacterial clearance from the lungs and protected mice from bacteraemia following *Klebsiella pneumoniae* intranasal inoculation (Ahn et al., 2019). Although the role of IFN-III in *Lm* infection has not been characterised, the modulation by a *Lm* virulence factor of ISG expression downstream of IFN-III signalling in intestinal epithelial cells suggests a role for IFN-λ in the host response to infection by this pathogen (see section C.5) (Lebreton et al., 2011; 2012).

2. The adaptive immune response to *Lm*

Early studies using mice that lack the lymphocytes or the MHC-I molecules necessary for an adaptive immune response highlighted the necessity of adaptive immunity in the total clearance of *Lm* (Bhardwaj et al., 1998; Emmerling et al., 1975; Ladel et al., 1994). These mice mount an efficient response in the early stages of infection and do not succumb to infection but are unable to clear the bacteria within the duration of the studies (up to 60 days). Conventional mice, on the other hand, mount an adaptive immune response at around 4 days p.i., and not only clear the infection within 7–10 days but become highly resistant to a subsequent lethal challenge (Pamer, 2004).

Sterilising and long-term immunity was later found to rely on cell-mediated rather than antibody-mediated immunity, and, in particular, on the expansion and maintenance of antigen-specific CD8 T cells (Harty et al., 1996; Qiu et al., 2018). CD8 T cells have bactericidal activity through direct cytolytic activity on infected host cells and, along with CD4 T cells, participate in cytokine production (notably TNF-α and IFN-γ) (Grivennikov et al., 2005; Zenewicz and Shen, 2007). The acquisition of *Lm*-derived antigens by DCs in the spleen is important for the priming of CD8 T cells (Broadley et al., 2016; Neuenhahn and Busch, 2007). Most of these antigens derive from virulence factors secreted by *Lm* into the host cell cytosol and are presented in the context of MHC-I molecules that tag the infected cell for targeted destruction by CD8 T cells (Chávez-Arroyo and Portnoy, 2020; Witte et al., 2012).

3. The transcriptional response to *Lm* infection

Regulation of gene expression at the transcriptional level is essential in order for each cell to respond to infection upon PRR sensing of PAMPs by activating the signalling pathways described in section C.1.1. The transcriptional landscape is reshaped upon infection not only through the activity of TFs, but also by epigenetic factors that remodel chromatin (DNA packaged histone complexes called nucleosomes) by means of histone modifications to render DNA more or less accessible to transcription (Bierne and Hamon, 2020; Eldridge et al., 2020a).

Since the turn of the century, transcriptional profiling of virus- or bacteria-infected cells or tissue using microarrays, and more recently RNA-sequencing (RNA-seq), has been widely employed to reveal large-scale changes upon infection to the host genetic program. The effects of pathogens on host cell gene expression include a common "core host response" independent of cell-type or pathogen as well as cell-type and pathogen specific patterns (Jenner and Young, 2005; Kidane et al., 2013; Singhania et al., 2019; Tran Van Nhieu and Arbibe, 2009). The transcriptomic response to *Lm* infection has focused mainly on innate immune cells, notably primary human or murine macrophages or monocytes (Abdullah et al., 2012a; Herskovits et al., 2007; Leber et al., 2008; McCaffrey et al., 2004; Nau et al., 2002; 2003; Perez et al., 2017; Rayamajhi et al., 2010a; Tchatalbachev et al., 2010) or macrophage-like cell lines (Carrasco-Marín et al., 2012; Cohen et al., 2000), primary DCs (Pontiroli et al., 2012; Popov et al., 2006; 2008), mast cells (Dietrich et al., 2010), or neutrophils (Kobayashi et al., 2003). In regard to the transcriptional response in unique cell types, four studies have been published in which the transcriptional response to *Lm* in epithelial cells was examined (Baldwin et al., 2002; Besic et al., 2020; Eskandarian et al., 2013; Johnson et al., 2021), two of which were published in the last 12 months. One study whose focus was on the host response to *Lm* infection of the brain microvascular endothelial cell line HBMEC has also been published (Wang et al., 2011).

In addition to analysis in unique cell types, the global transcriptional response to *Lm* has been examined in murine blood (Dieterich et al., 2008; Ng et al., 2005; Pitt et al., 2016; Singhania et al., 2019) or tissue, including the small intestine (Archambaud et al., 2012; Lecuit et al., 2007), the liver (Demiroz et al., 2021; Dieterich et al., 2008; Joseph et al., 2004; Ng et al., 2005; Pitt et al., 2016), the spleen (Pitt et al., 2016), and the brain (Drevets et al., 2008) (Table S14, Descoeudres et al., 2021). While the number of publications is by no means insignificant, only a handful of the cited studies have dissected in detail the transcriptional response, for example (Cohen et al., 2000; Johnson et al., 2021; Leber et al., 2008; Lecuit et al., 2007), with

others providing little more than a short list of marker genes observed at a single timepoint, for example (Dieterich et al., 2008; Ng et al., 2005).

These global transcriptomic analyses have generally revealed vastly reshaped host gene expression upon infection, with temporal and cell-type specific signatures. Transcriptomic profiling of the infected host cell using both WT and mutant *Lm* strains and transgenic mice has contributed greatly to establishing the roles of the different PRRs involved in *Lm* sensing in different cell types, and their associated signalling pathways, and the transcriptional response is largely the consequence of these signalling events. Various studies have also highlighted novel aspects of host-pathogen interactions, including the role of nuclear receptors involved in lipid metabolism (Abdullah et al., 2012a; Joseph et al., 2004), as well as epigenetic modifications (Eskandarian et al., 2013) in shaping the host response to *Lm* infection.

3.1. Signalling pathways activated in response to *Lm* infection

Global transcriptional analyses in different cell types have highlighted a number of signalling pathways that are commonly activated in all cell types in response to intracellular invasion by *Lm*, and result in both common and divergent gene expression programs. The host transcriptional response to *Lm* infection in both immune and non-immune cells is dominated by rapid NF-κB and MAPK pathway activation. Subsequent IRF3/IRF7 dependent gene expression profiles are observed involving IFN expression and downstream ISG induction upon cytosolic detection of *Lm* (Leber et al., 2008; O'Riordan et al., 2002) (Figure 23).

3.1.1. Nuclear factor κB (NF-κB) signalling pathways

The host transcriptional response to *Lm* infection is mediated largely through NF-κB–dependent pathways. NF-κB activation occurs very rapidly in macrophages upon infection and does not require cytosolic detection (Hauf et al., 1997; 1994; Reimer et al., 2007). Transcriptomic studies have almost systematically highlighted the importance of NF-κB activation in the genetic reprogramming observed during infection (Baldwin et al., 2002; Besic et al., 2020; Cohen et al., 2000; Johnson et al., 2021; Leber et al., 2008; Lecuit et al., 2007; Nau et al., 2002).

NF-κB is a family of inducible TFs, composed of five structurally related members – p50, p52, RELA/p65, RELB and c-REL – which mediate transcription of target genes by binding to a specific DNA element – κB enhancer – as various hetero- or homodimers. NF-κB proteins are normally sequestered in the cytoplasm by members of the IκB family of inhibitory proteins,

the best characterised being IκBα (encoded by the gene *NFKBIA*). NF-κB members p50 and p52 are synthetised as large precursors, NF-κB1/p105 (encoded by *NFKB1*) and NF-κB2/p100 (*NFKB2*), respectively, from which the C-terminal region containing ankyrin repeats is post-translationally cleaved. These C-terminal regions are structurally and functionally analogous to IκB inhibitory proteins. The primary mechanism for canonical NF-κB activation is via the ubiquitin-dependent degradation of IκBα following its phosphorylation by the trimeric IκB kinase (IKK) complex composed of two catalytic subunits (IKKα and IKKβ) and a regulatory subunit NF-κB essential modulator (NEMO or IKKγ). Proteasomal degradation of IκBα results in rapid and transient nuclear translocation of NF-κB and the transcription of target genes. The non-canonical pathway involves the activation of NF-κB inducing kinase (NIK, or MAP3K14), which leads to the proteolytic processing of NF-κB2 to p52, and the nuclear translocation of the noncanonical NF-κB complex p52/RelB (Sun, 2017; Zhang et al., 2017a) (Figure 22). Non-canonical NF-κB signalling occurs upon stimulation of a subset of the tumour necrosis factor superfamily receptors (TNFRs) and is slower and longer-lasting than canonical signalling that is triggered by a large variety and number of signals (Dorrington and Fraser, 2019).

The importance of NF-κB signalling in *Lm* infection is illustrated by the defective clearance of bacteria in mice deficient in the p50 subunit of NF-κB, with bacterial loads in the spleen several log superior to that of WT mice (Sha et al., 1995). *MAP3K14*, the gene encoding NIK, was one of only six genes (as was *MYD88*, encoding the TLR signalling adaptor protein) identified in a screen of over 350 ISGs found to restrict *Lm* infection in fibroblasts (Perelman et al., 2016). *MAP3K14* was observed to be highly upregulated in infected colon epithelial cells (Baldwin et al., 2002) and other genes encoding both canonical and non-canonical NF-κB family members and inhibitory proteins are often upregulated by *Lm* infection (Baldwin et al., 2002; Johnson et al., 2021; Kobayashi et al., 2003; Nau et al., 2002; Tchatalbachev et al., 2010). The manipulation of NF-κB signalling by *Lm* (detailed in section C.4) also attests to the importance of these TFs.

Although NF-κB is a fairly simple regulatory system, it responds to a large variety and number of signals that are interpreted into different patterns of gene expression in different cell types and biological contexts, depending on the NF-κB dimer and the presence of other TFs at specific promotor or enhancer sequences (Zhang et al., 2017a). The expression of IFN-β is, for example, the result of the cooperation of NF-κB with other TFs: the *IFNB1* promoter contains NF-κB binding sites as well as two ISREs (Iwanaszko and Kimmel, 2015), thus requiring both IRF3 and NF-κB for expression, as observed *in vitro* (Leber et al., 2008; Reimer et al., 2007).

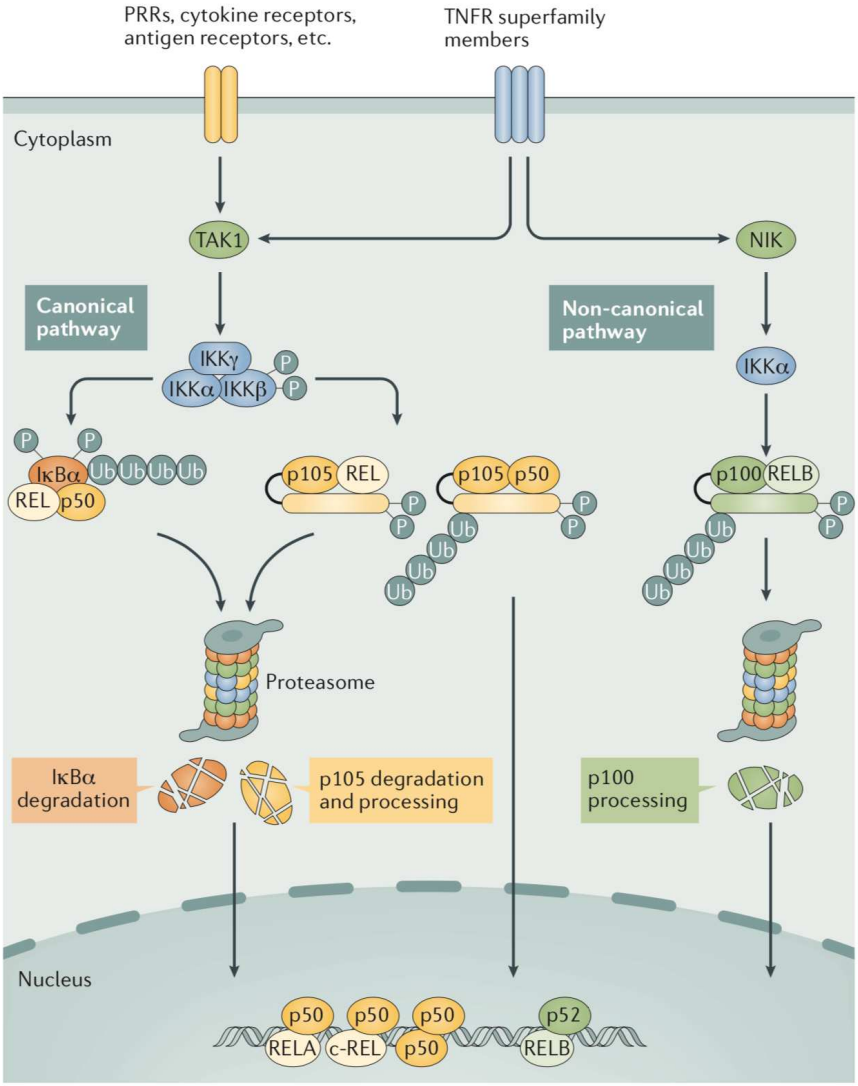

Figure 22 | Canonical and non-canonical NF-κB pathways.
The canonical NF-κB pathway is triggered by signals from a large variety of immune receptors, which activate the kinase TAK1. TAK1 then activates the trimeric IKK complex via phosphorylation of IKKβ. The activated IKK complex phosphorylates, in turn, members of the IκB family (such as the prototypical IκBα and the IκB-like molecule p105) which sequester NF-κB members in the cytoplasm. Upon phosphorylation by IKK, IκBα and p105 are targeted for ubiquitin (Ub)-dependent degradation in the proteasome, resulting in the nuclear translocation of canonical NF-κB family members, which bind as various dimeric complexes to specific DNA elements, termed κB enhancers, of target genes. The non-canonical NF-κB signalling pathway, on the other hand, activates the NF-κB inducing kinase (NIK). NIK phosphorylates and activates IKKα, which, in turn, phosphorylates p100, triggering selective degradation of its C-terminal IκB-like structure and leading to the generation of p52 and the nuclear translocation of p52 and RELB. (Source: Sun, 2017)

3.1.2. Mitogen-activated protein kinase (MAPK) signalling pathways

MAPK pathways are activated upon PRR sensing of PAMPs and consist of a series of at least three kinases: a MAPK kinase kinase (MAP3K) that activates a MAPK kinase (MAP2K), which in turn activates a MAPK. Three major MAPK families exist: the extracellular signal-regulated kinase (ERK), mitogen-activated protein kinase 14 (p38), and Jun N-terminal kinase (JNK) (Arthur and Ley, 2013). The phosphorylation of MAPKs activates the TF activator protein 1 (AP-1) (composed of FOS/JUN family hetero- or homodimers) which contributes to the host transcriptional response to *Lm* (Eldridge et al., 2020a) (Figure 23).

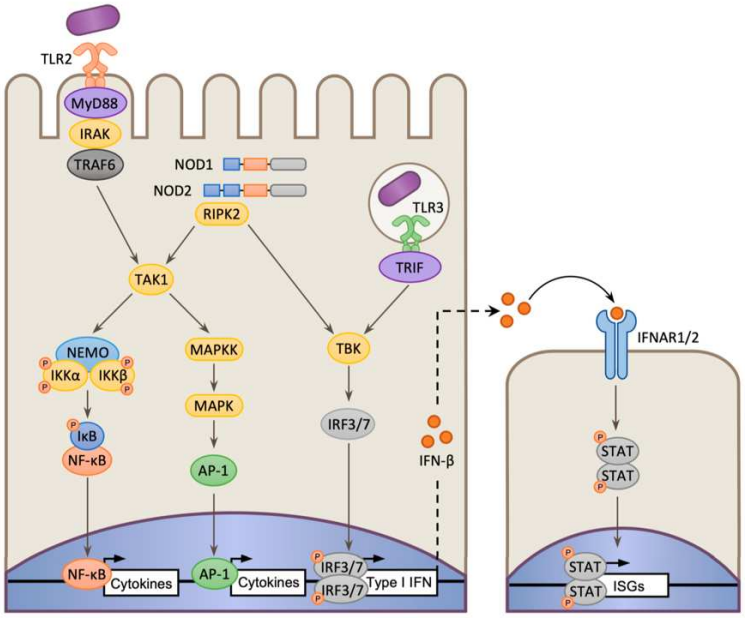

Figure 23 | The host transcriptional response to *Lm* infection common to both immune and non-immune cells.
The host transcriptional response to *Lm* infection in both immune and non-immune cells is dominated by rapid NF-κB and MAPK pathway activation upon PRR sensing of extracellular or intracellular bacteria, followed by IRF3/7 dependent IFN signalling and downstream ISG expression upon *Lm* detection by intracellular PRRs. (Source: Eldridge et al., 2020a)

3.2. Gene expression signatures in immune, endothelial, and epithelial cells

The early host transcriptional response to *Lm* infection is generally dominated by transcriptional activation, with gene repression increasing as the infection progresses (Besic et al., 2020; Cohen et al., 2000; Drevets et al., 2008; Pontiroli et al., 2012; Wang et al., 2011). This early transcriptional activation includes the upregulation of cytokine – especially chemokine – as well as adhesion molecule expression, and is common to all immune (Abdullah

et al., 2012a; Cohen et al., 2000; Dietrich et al., 2010; Herskovits et al., 2007; Leber et al., 2008; McCaffrey et al., 2004; Nau et al., 2002; Pontiroli et al., 2012; Tchatalbachev et al., 2010) and non-immune cells including epithelial (Baldwin et al., 2002; Besic et al., 2020; Eskandarian et al., 2013; Johnson et al., 2021) and endothelial cells (Wang et al., 2011). This signature is observed in the intestine (Archambaud et al., 2012; Lecuit et al., 2007), the liver (Demiroz et al., 2021; Joseph et al., 2004; Pitt et al., 2016), and the brain (Drevets et al., 2008) and reflects the importance of cell signalling to induce the chemotaxis of circulating neutrophils and monocytes, essential in controlling early infection, and to activate both resident and infiltrating immune cells.

3.3. The host transcriptional response to *Lm* infection in immune cells

3.3.1. Macrophages

The first transcriptomic data of the host response to *Lm* infection was obtained in the human monocyte cell line THP-1 at 2 h p.i. (Cohen et al., 2000). Higher fold changes that were more reproducible in the upregulated gene set resulted in a final tally of 74 upregulated and 23 downregulated genes. A dramatic upregulation of chemokines, of the pro-inflammatory cytokines IL-1 and TNF-α, as well as surface proteins involved in the recruitment and activation of immune effector cells was observed. Genes involved in iron sequestration and storage, as well as 11 antiapoptotic genes were upregulated (Cohen et al., 2000).

A subsequent seminal research work using primary murine BMDM to assess the host transcriptional response at four different timepoints over 8 h showed *Lm* to induce two basic categories of genes: an "early/persistent" cluster consistent with NF-κB-dependent responses downstream of TLRs, and a distinct "late response" cluster largely composed of ISGs and including IFN-β gene upregulation (McCaffrey et al., 2004). While the early/persistent cluster was strongly enriched for activated genes, approximately one third of the genes in the late response cluster were repressed. These included many genes involved in cell cycle regulation (including cell proliferation activator MYC proto-oncogene (MYC)), DNA replication, and nucleic acid metabolism, consistent with the role of IFNs as physiological growth inhibitors that affect the cell cycle. McCaffrey et al. also compared the BMDM response to WT *Lm*, able to access the host cell cytosol, to that of a LLO-deficient strain unable to escape from the primary internalisation vacuole (*LmΔhly*), as well as heat-killed *Lm*, and found the late response cluster to be unique to WT *Lm*, with *LmΔhly* and heat-killed *Lm* inducing only the universal early/persistent response. The distinct "late response" was not, however, unique to

Lm, as it was also triggered by a strain of LLO-expressing *Bacillus subtilis*, able to access the macrophage cytosol (McCaffrey et al., 2004). A subsequent study by the same group demonstrated that IFN-γ activation of BMDMs prior to *Lm* infection enhanced their bactericidal activity, resulting in phagosomal-degraded bacteria that induced IFN-β expression characteristic of cytosolic bacteria, even when the vacuole-restricted Δ*hly* strain was employed (Herskovits et al., 2007). Leber et al. went a step further and, using both host and pathogen mutagenesis in a "genetics-squared" approach (Persson and Vance, 2007), elegantly demonstrated that the vacuolar "early/persistent" response was MyD88 dependent, and the cytosolic IFN/ISG response was MyD88-independent but IRF3-dependent (Leber et al., 2008). While pro-inflammatory cytokines IL-1α, IL-1β, and TNF-α and chemokines such as CXCL1 and CXCL2 were expressed in response to the Δ*hly Lm* strain in WT BMDM, IFN-β and ISG expression was unique to WT *Lm* in MyD88 KO BMDM (Leber et al., 2008). In subsequent studies, both the ligand (c-di-AMP) and the PRR (STING) were identified as being at the origin of cytosolic sensing of *Lm* and subsequent IFN-I expression (Burdette et al., 2011; Woodward et al., 2010) (Figure 19).

The transcriptional response to *Lm* observed in cultured murine BMDM at 10 h p.i. also revealed striking differential expression of genes involved in IFN-γ signalling (Rayamajhi et al., 2010b). The genes encoding the TF STAT1 and the kinase JAK2, involved in both IFN-I/III and IFN-II signalling were upregulated 10-fold in infected macrophages while *Ifngr1*, specific to IFN-γ signalling, was downregulated almost 7-fold. This transcriptional downregulation was confirmed *in vivo* and corresponded with decreased cell-surface IFNγR in splenic myeloid cells and BMDMs. Secreted IFN-α/β was found to regulate the MyD88-independent downregulation observed, revealing a new facet of the antagonistic crosstalk between type I and type II IFNs (Rayamajhi et al., 2010b).

The gene expression profile of splenic CD169+ macrophages isolated from infected mice was compared to those isolated from non-infected mice at 12 h p.i. (Perez et al., 2017). *Lm* infection resulted in CD169+ macrophages displaying an inflammatory macrophage (M1) signature characterised by an upregulation of genes related to immune system responses, defence responses, inflammatory responses, innate responses, and response to bacterium. A total of 795 differentially expressed genes (DEGs) were identified in infected CD169+ macrophages compared to cells from uninfected mice and included many of the genes previously identified by *in vitro* infection of the human macrophage cell line THP-1 or of primary murine

macrophages, including genes encoding pro-inflammatory cytokines as well as accessory molecules of inflammatory cytokine receptors (Perez et al., 2017).

As is the case in *Mycobacterium* spp.- or *Salmonella*-infected macrophages, the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ) was found to be upregulated in primary human peripheral blood mononuclear cell (PBMC)-derived CD14+ monocytes within 1 h p.i. with *Lm* (Abdullah et al., 2012a). PPARγ is best known as the master regulator of fatty acid storage and glucose metabolism in adipose tissue but is increasingly recognised as an important regulator of lipid metabolism and immune function in leukocytes (Hernandez-Quiles et al., 2021). Transcriptomic analysis of the *Lm*-infected primary human monocytes at a later timepoint (24 h p.i.) revealed that around 15% of deregulated genes are known to be regulated by PPARγ. The authors went on to characterise the role of PPARγ in *Lm* infection in the murine listeriosis model and found that myeloid-specific PPARγ deletion increased survival, resulted in decreased bacterial loads in the liver and spleen, increased the expression of essential inflammatory mediators (i.e., IFN-γ, TNF-α, IL-12), and led to improved monocyte recruitment. Increased bactericidal function of PPARγ KO compared to PPARγ WT macrophages was also observed *in vitro* (Abdullah et al., 2012a). PPARγ activation in macrophages is known to drive the alternative M2 macrophage phenotype, and can repress inflammatory cytokine expression through the transrepression of NF-κB and the stabilisation of co-repressor complexes on promoters of pro-inflammatory genes (Hernandez-Quiles et al., 2021). Interestingly, liver X receptor (LXR), another member of the nuclear receptor family, can transrepress NF-κB target genes in the same manner as PPARγ (Schulman, 2017), yet LXR ablation renders mice more susceptible to *Lm* (Joseph et al., 2004) (detailed in section C.3.6.2).

3.3.2. Dendritic cells

CD8α+ cDC are primary targets of *Lm* in systemic listeriosis (detailed in section C.1.2), and the DC transcriptional response to *Lm* infection has been examined in both D1 cells, a spleen-derived murine DC line (Pontiroli et al., 2012), and in primary human immature DCs generated from blood-derived monocytes (Popov et al., 2006; 2008). D1 cells were exposed to *Lm* for 2–24 h and their gene expression profiles broadly grouped into “early” (2–4 h p.i.), “middle” (8–12 h p.i.) and “late” (24 h p.i.) responses as described by Portnoy and colleagues (McCaffrey et al., 2004). The highest proportion of upregulated compared to downregulated DEGs was observed at the earliest timepoints, with “middle” and “late” DEGs equally up- and downregulated. The number of DEGs progressively increased between the early and the late

time points. A large number of genes encoding signalling molecules, receptors and adhesion molecules, and TFs, as well as genes encoding enzymes and anti-apoptotic molecules and genes involved in tissue remodelling were identified as differentially regulated by *Lm* infection. In particular, gene clusters corresponding to the early response were enriched in genes involved in the "cytokine-cytokine receptor interaction", "chemotaxis", and "locomotion" Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. The late cluster was characterised by the presence of IFN-I-coding genes and ISGs. IFN-β and IFN-α transcript upregulation was first detected at 4 h and 8 h p.i., respectively, but peak expression was observed at 24 h p.i. (Pontiroli et al., 2012). This late upregulation suggests that IFN-I expression requires cytosolic detection of *Lm* in DCs, as has been reported previously for macrophages and other cells (Feng et al., 2005; O'Riordan et al., 2002; Stockinger et al., 2002).

3.3.3. Neutrophils

Kobayashi et al. uncovered a common pathogen-induced transcription profile in polymorphonuclear cells (PMNs or neutrophils) following phagocytosis of the Gram-positive bacteria *Lm*, *S. aureus*, and *Streptococcus pyogenes*, and the Gram-negative bacteria *Borrelia hermsii* and *Burkholderia cepacia* (Kobayashi et al., 2003). Neutrophil gene deregulation increases with time following phagocytosis. With the exception of *S. pyogenes*, few pathogen-specific changes in neutrophil gene expression after phagocytosis were observed. Genes upregulated from 90 min p.i. included *TNF*, consistent with recent studies that highlight the importance of neutrophils in the production of this inflammatory cytokine that is key in mediating *Lm* clearance (Carr et al., 2011). Genes encoding proteins involved in the activation and recruitment of immune effector cells as well as components of the TLR2, MAPK, and NF-κB signal transduction pathways were upregulated. The authors observed that the phagocytosis of bacteria initiates an apoptosis differentiation program in human PMNs that facilitates neutrophil programmed cell death, with almost 20% of total DEGs classed as apoptosis or cell fate related. The phagocytosis of bacteria was indeed found to accelerate PMN apoptosis (Kobayashi et al., 2003), as has been confirmed in more recent studies (Brostjan and Oehler, 2020; Fox et al., 2010).

3.3.4. Mast cells

Dietrich et al. compared the transcriptional response to *Lm* and *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) at 6 h p.i. of primary bone marrow derived mast cells (BMMCs) to that of BMDM (Dietrich et al., 2010). *Lm* and *S.* Typhimurium adhere to mast

cells, but they are not internalised, as opposed to the rapid phagocytosis of these bacteria by macrophages. A much larger number of genes was upregulated by both the Gram-positive and Gram-negative bacteria in macrophages compared to mast cells (1,421 versus 248 genes) (Dietrich et al., 2010). Bacterial activation of mast cells by *Lm* and *S.* Typhimurium was found to result in the upregulation of pro-inflammatory cytokine gene expression (i.e., *Tnfa*, *Il1a*, *Il1b*, *Il6*) but not *Ifnb1* or downstream ISG expression, as was observed in macrophages. This is consistent with the dogma that cytosolic detection is not required to activate the expression of pro-inflammatory cytokines but is required for IFN-I gene and downstream ISG expression (Dietrich et al., 2010), as previously described in macrophages (Aubry et al., 2012; Leber et al., 2008).

3.4. The host transcriptional response to *Lm* infection in endothelial cells

Neurolisteriosis involves the invasion of human brain microvascular endothelial cells (HBMEC) that constitute (together with astrocytes and pericytes) the blood-brain barrier and the first line of defence against CNS infection (Banović et al., 2020; Ghosh et al., 2018; Wang et al., 2011). Cultured HBMEC cells infected for 4 h showed a large-scale reprogramming of gene expression, with twice as many activated versus repressed genes (Wang et al., 2011). The most highly activated pathway at this early timepoint was the N-formyl-methionyl-leucyl-phenylalanine (fMLP) pathway. As N-formylated peptides such as fMLP play a key role in host defence as potent chemoattractants for phagocytic leukocytes (He and Ye, 2017), the upregulation of the fMLP pathway, in addition to the upregulation of chemokines, highlights the importance of the recruitment of neutrophils, NK cells, and T cells to the blood brain barrier during early *Lm* infection (Wang et al., 2011). Primary human umbilical vein endothelial cells (HUVEC) were also found to respond to *Lm* infection by producing essentially pro-inflammatory cytokines and chemokines and by upregulating the expression of adhesion molecules such as ICAM-1 and E-selectin, as well as increasing nitric oxide synthesis (Drevets, 1997; Krüll et al., 1997; Rose et al., 2001; Schmeck et al., 2005; 2006).

3.5. The host transcriptional response to *Lm* infection in epithelial cells

The earliest research work employing microarray technology to examine the host response to *Lm* infection in epithelial cells employed the human colon epithelial cell line Caco-2. Baldwin et al. infected Caco-2 cells for 30 min to 8 h and examined the transcriptional response at five different timepoints to discover a predominantly NF-κB pathway-dominated transcriptomic signature (Baldwin et al., 2002). Of the 50 most highly induced genes, 20% encoded proteins

that are either involved in NF-κB signal transduction or activated by NF-κB such as the chemokines and adhesion molecules IL-6, CXCL1, CXCL2, ICAM-1, as well as MAPK-activated transcription factors FOS and JUN (AP-1 subunits), MYC, and the PRRs TLR2 and CD14. Surprisingly, the same signature was observed in Caco-2 cells infected with *Lm*Δ*actA* or Δ*prfA*, suggesting the absence of a transcriptomic signature specific to the presence of motile or cytoplasmic bacteria that was later observed in macrophages (Baldwin et al., 2002; Leber et al., 2008; McCaffrey et al., 2004).

The infection of the human epithelial cervix carcinoma cell line HeLa with either WT *Lm* or a *Lm* strain that remains vacuolar (*Lm*Δ*hly*Δ*plc*), however, provided the first evidence that the cytosol-specific transcriptomic response observed in macrophages (Leber et al., 2008; McCaffrey et al., 2004) also exists in epithelial cells (O'Riordan et al., 2002). The presence of *Lm* in the cytosol was necessary to activate the expression of IFN-β and CXCL8 in HeLa cells as in non-activated BMDMs (O'Riordan et al., 2002). On the other hand, the induction of IFN-λ expression was only partially reduced upon infection with a vacuole-confined LLO-deficient *Lm* strain, compared to the WT strain, in the human epithelial colon carcinoma cell line LoVo (Bierne et al., 2012b).

In LoVo cells, host gene expression in the first 5 h p.i. is dominated by transcriptional activation (121 upregulated versus 49 downregulated DEGs) (Besic et al., 2020), as observed by Baldwin et al. in the human colorectal adenocarcinoma cell line Caco-2 (Baldwin et al., 2002). The early transcriptional activation led to a pronounced induction of genes associated with pro-inflammatory and IFN-I responses to *Lm* invasion, with chemokines and other cytokines (CXCL1, CXCL2, CXCL8, CCL2, granulocyte-macrophage colony-stimulating factor (GM-CSF), lymphotoxin-beta (LTB), TNF-α) upregulated from the earliest timepoint examined (2 h p.i.). At 10 h p.i., the up- or downregulation of other pathways emerged, including protein catabolic response, chromatin silencing, histone modification, and mitochondrial metabolism (Figure 24A). The promoter regions of upregulated genes were analysed for the enrichment of TF binding sites. As expected, NF-κB binding sites dominated and had high normalised enrichment scores, highlighting the early induction of NF-κB-dependent signalling during *Lm* infection of epithelial cells (Besic et al., 2020).

Transcriptomic analysis of primary human trophoblasts at 5 h p.i., revealed a transcriptomic signature dominated by gene activation with 359 upregulated genes and only 26 genes significantly downregulated (Johnson et al., 2021) (Figure 24B). 41 of the upregulated DEGs have transcriptional activity and include NF-κB subunits, FOS/JUN family TFs, IRFs, and MYC. Genes encoding pro-inflammatory cytokines (TNF-α, IL-1α/β, GM-CSF, IL-23A) and anti-inflammatory molecules (IL-10, IL-1RA) were upregulated as well as chemokines CCL2, CCL3, CCL4, CCL20, CXCL1, CXCL2, CXCL3, IL-6, CXCL8, and adhesion molecules CD40 and ICAM-1 and T cell ligand CD80. All three IFN-λ isoform transcripts (*IFNL1*, *IFNL2*, *IFNL3*), but no IFN-I-coding genes, were upregulated. KEGG pathway analysis revealed that *Lm* infection upregulated genes encoding proteins involved in TLR2, NLR, and cytosolic DNA sensing pathways, as well as in downstream signalling pathways NF-κB, MAPK, PI3K-protein kinase B (Akt), and JAK-STAT. Gene Ontology of Biological Processes (GOBPs) enriched in the upregulated gene set include terms related to the inflammatory and immune response, transcriptional regulation, cell-cell signalling and signal transduction, and the negative regulation of apoptosis and cell proliferation (Johnson et al., 2021).

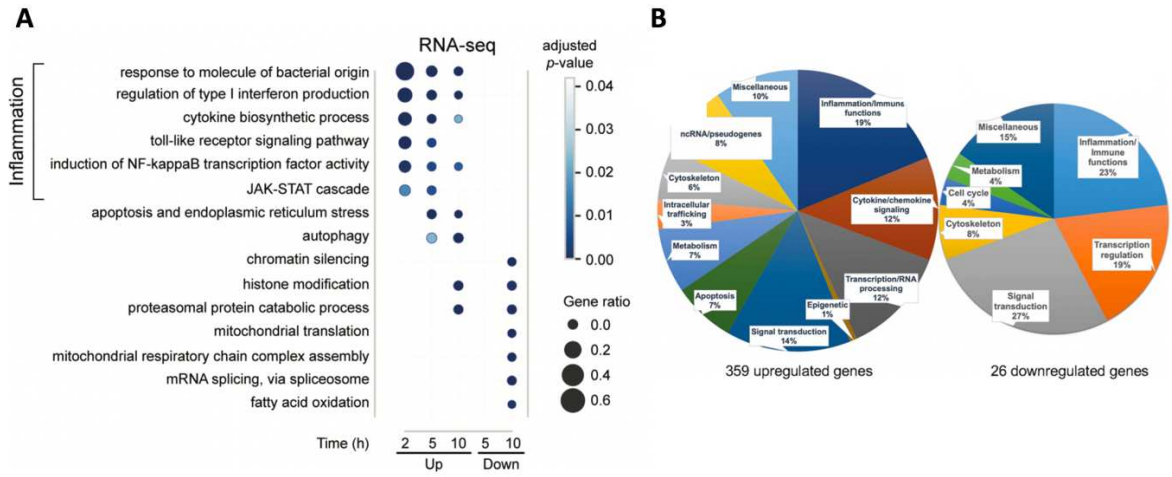

Figure 24 | The transcriptional response to short term *Lm* infection in epithelial cells.
(**A**) Enrichment analysis of GOBP terms for genes up- or downregulated (relative to non-infected cells) at 2, 5, or 10 h p.i. in LoVo cells. (**B**) DEGS identified at 5 h p.i. in primary human trophoblasts were classed based on their known function. These divergent means of analysing and representing RNA-seq data obtained from *Lm*-infected epithelial cells converge to portray the domination of gene activation versus gene repression in the early host transcriptional response. Functions related to the immune response, signal transduction (notably NF-κB and IFN signalling), and apoptosis are enriched in the upregulated gene sets. (Source: Besic et al., 2020 (A); Johnson et al., 2021 (B))

Contrary to the predominantly upregulated gene signature observed in other epithelial cell lines in the first 8 h of infection (Baldwin et al., 2002; Besic et al., 2020; Johnson et al., 2021), the transcriptomic response at 5 h p.i. in the human cervical adenocarcinoma cell line HeLa was

dominated by gene repression (Eskandarian et al., 2013), with the repression of 272 genes and the activation of 158. With the exception of one gene, all downregulated genes and 128 of the upregulated genes were found to be regulated by the histone deacetylase (HDAC) sirtuin 2 (SIRT2) (Eskandarian et al., 2013). *Lm* was thus one of the first examples of an invasive bacterium reported to modify host gene expression through the induction of histone modifications (Dong and Hamon, 2020; Hamon et al., 2007; Schmeck et al., 2005). HDACs are generally associated with gene repression, as they remove the acetyl groups from histones that maintain chromatin in a more relaxed structure permissive to transcription (Eskandarian et al., 2013) (detailed in section C.5.1). Functional annotation of the 271 SIRT2-dependent downregulated DEGs revealed an enrichment in genes encoding DNA binding proteins and/or implicated in transcriptional regulation (Eskandarian et al., 2013).

3.6. The host response to *in vivo* infection in the murine listeriosis model

3.6.1. The intestine

Two studies from the same laboratory investigated the host response to *Lm* in the intestine, in which germ-free transgenic mice expressing either human E-cad (Lecuit et al., 2001) or humanised E-cad (Disson et al., 2008) were infected via the IG route (Archambaud et al., 2012; Lecuit et al., 2007). Both report an intestinal response characterised by an upregulation of genes involved in immune responses and a downregulation of genes involved in lipid, amino acid, and energy metabolism.

In Lecuit et al., the authors report of an intestinal response to *Lm* infection at 72 h (the timepoint that corresponds to peak levels of *Lm* in intestinal tissue and MLNs) that is dominated by transcriptional upregulation, with four times more upregulated than down regulated genes. Gene networks created from upregulated genes in the top functional categories revealed the activation of, among others, JAK/STAT, TLR, antigen presentation, NF-κB, GM-CSF, ERK/MAPK, PI3K-Akt, and apoptotic signalling pathways. Interestingly, this transcriptional signature was attenuated but generally maintained in the absence of InlA or of both InlA and InlB expression by *Lm*. An LLO-deficient strain, however, did not produce a similar transcriptional signature, and neither disseminated to the spleen nor induced lymphocyte recruitment to the lamina propria. This reflects both the necessity of cytosolic detection of *Lm* for the MyD88-dependent expression of certain chemokines and ISGs (Dolowschiak et al., 2010; Leber et al., 2008; Serbina et al., 2003b) as well as the necessity of LLO in the infection process (as described in section A.6.3.3), and the importance of LLO in eliciting the host cell

response through the activation of a large number of signalling pathways (Lecuit et al., 2001) (as described in section C.4.1).

In Archambaud et al., the intestinal transcriptional response was examined at an earlier timepoint (24 h p.i.) and found *Lm* infection to significantly affect the transcription of almost 1,000 genes, with an almost equal number of up- and downregulated DEGs identified. Functional annotation of DEGs revealed deregulated pathways involved in immune response, intracellular signalling, nuclear receptor signalling, and xenobiotic metabolism as well as amino acid, carbohydrate, and lipid metabolism. The most significantly induced genes were involved in immune responses and included many ISGs, whereas genes related to host metabolism were highly repressed (Archambaud et al., 2012).

3.6.2. The liver

Research into the host transcriptomic response to *Lm* infection in the liver has highlighted the crosstalk between metabolism and the innate immune response. Increased susceptibility to systemic *Lm* infection and increased hepatic bacterial burden in mice deficient in LXR signalling led to the identification of the genes regulated by this lipid-metabolism associated nuclear receptor during infection (Joseph et al., 2004). In the liver of WT mice, 2-day *Lm* infection induced the expression of inflammatory cytokines and chemokines, including the monocyte chemotactic proteins CCL2, CCL7, and CCL12, as well as IL-5, IL-6, IL-12, and IFN-γ. The hepatic transcriptional response to *Lm* in LXR KO compared to WT mice revealed LXRs to be macrophage-specific negative regulators of many NF-κB-dependent inflammatory genes such as iNOS, IL-6, and IL-12 yet also direct positive regulators of CD5L (also known as SPα), an anti-apoptotic protein highly induced during *Lm* infection and more recently described as a regulator of lipid metabolism and inflammation (Sanchez-Moral et al., 2021). This work (Joseph et al., 2004) and associated work by Tontonoz and colleagues (Castrillo et al., 2003; Joseph et al., 2003) established a link between lipid metabolism and macrophage immune function and the inflammatory response, and is among the pioneering work at the origin of the emerging field of immunometabolism (Cardoso and Perucha, 2021; Mathis and Shoelson, 2011; Schulman, 2017).

The crosstalk between metabolism and the innate immune response to *Lm* infection was further highlighted in a very recent publication in which the authors describe the transcriptomic and metabolomic signature of liver tissue obtained from mice infected via the IP route (Demiroz et al., 2021). 1390 genes were found to be significantly deregulated at 24 h p.i., and, in addition

to inflammatory and IFN pathways, gene set enrichment analysis (GSEA) also identified numerous metabolic pathways including oxidative phosphorylation (OXPHOS), bile acid and fatty acid metabolism, glycolysis, and adipogenesis related pathways. The profound metabolic changes suggested by this gene deregulation were investigated by metabolomic analyses that indicated increased OXPHOS and fatty acid oxidation and decreased glutaminolysis in the liver of infected mice and in infected BMDMs *in vitro*. Furthermore, this metabolic reprogramming was partially mediated by IFN-I signalling activated upon infection, as evidenced by comparing the response of *Ifnar1* and *Irf9* KO mice to that of WT mice (Demiroz et al., 2021).

3.6.3. The brain

Lethal systemic *Lm* infection was shown to modify gene expression in the brain even in the absence of CNS invasion (Drevets et al., 2008). Gene expression was analysed at daily intervals over a four-day infection time course. The number of significant DEGs was highest at 24 h p.i. (339 DEGs whose expression was modified at least 2-fold) and decreased progressively during the infection time course (37 DEGs at 96 h p.i.). At 24 h p.i. five times more activated than repressed genes were identified, whereas from 48 h p.i. the proportion of downregulated DEGs increased progressively to compose an almost exclusively downregulated gene signature at 96 h p.i.

Serum IFN-γ was found to be a critical trigger of the transcriptional response, although IFN-γ expression was not found to be upregulated in brain tissue. Functional annotation of DEGs revealed largely unique sets of signalling pathways at each infection timepoint: the only two pathways significantly deregulated at more than one timepoint were “NF-κB signaling” and “apoptosis signaling”. The activation of the NF-κB pathway was illustrated by the upregulation of genes encoding IFN-β1, IL-1α, IL-1β, and TNF-α, and ISGs were among the most highly upregulated DEGs. The genes encoding the chemokines CXCL9 and CXCL10, as well as E-selection were highly upregulated and are thought to mediate monocyte recruitment to the brain (Drevets et al., 2008). The *Lm*Δ*hly* deletion mutant failed to elicit the same response, confirming the role of LLO in promoting phagosomal escape to allow cytosol PAMP sensing, and in triggering the release of pro-inflammatory cytokines from infected cells (Dewamitta et al., 2010; Hamon and Cossart, 2011). It is interesting to note, however, the upregulation of IFN-I genes by cells that are not directly infected by *Lm*.

4. *Lm* virulence factors involved in the host transcriptional response

As described above, the host transcriptional response to *Lm* infection is triggered by the detection of PAMPs, often bacterial structural components or metabolites that are common to different pathogens. In a simplified scenario of host-pathogen interactions, the transcriptional response is either initiated by the host and in favour of host survival and/or pathogen elimination (response to conserved bacterial PAMPs, for example) or instigated by specific pathogen virulence factors to subvert host protective responses or to modify host cell metabolism to ensure pathogen survival. In reality, however, the distinction is hazy, and the line hard to draw between pro-host and pro-pathogen responses. This is illustrated by the manipulation of host transcription by *Lm* virulence factors that often have pleotropic and opposing effects, and for which the physiological consequences are difficult to evaluate. Detailed here are the major *Lm* virulence factors described as playing a role in the transcriptomic response to *Lm* infection. Virulence factors can modify host cell transcription either through very specific interactions with host cell components (e.g., LntA), or through the initiation of a multitude of signalling pathways upon host cell membrane disruption that are common to PFTs and membrane damage in general (e.g., LLO).

4.1. Listeriolysin O (LLO)

LLO, the pore-forming toxin that mediates the escape of *Lm* from internalisation vacuoles, like other PFTs, is a potent regulator of cellular signalling and immunity (Cajnko et al., 2014). Extracellular LLO causes an influx of calcium ions and an efflux of potassium, initiating the activation of a large number of signalling pathways including the NF-κB pathway and the ERK1/2, p38, and JNK MAPK pathways (Gonzalez et al., 2011; Seveau, 2014) that contribute largely to the early transcriptional program initiated by *Lm* infection, as described in section C.3.1. In addition, LLO-induced signalling cascades can also result in epigenetic modifications – histone deacetylation and dephosphorylation – as observed in HeLa cells, resulting in the subsequent downregulation of key inflammatory genes (Hamon and Cossart, 2011; Hamon et al., 2007). Histone deacetylation and dephosphorylation is dependent on LLO-induced potassium efflux, and this ion efflux is also necessary for the cleavage and secretion of mature IL-1α and IL-1β from activated macrophages (Dewamitta et al., 2010; Hamon and Cossart, 2011; Meixenberger et al., 2010) (Figure 25A).

4.2. Internalin B (InlB)

Through its interaction with c-Met, as described in section A.6.3.1, InlB mediates the rapid and sustained activation of NF-κB in macrophages through a transient degradation of IκBα and the prolonged degradation of IκBβ (Mansell et al., 2001). NF-κB activation correlates with an increase in TNF-α and IL-6 production in an InlB dose-dependent manner and occurs via cell signalling that requires the small G-protein Ras-dependent activation of PI3K and downstream Akt phosphorylation (Mansell et al., 2001; 2000). Akt has been proposed to directly phosphorylate IKKα (Ozes et al., 1999), but the mechanism behind the involvement of the PI3K-Akt pathway in NF-κB activation remains controversial (Oeckinghaus et al., 2011). HGF activation of NF-κB is conversely independent of PI3K-Akt (Müller et al., 2002), consistent with the observation that InlB does not bind c-Met with the same affinity nor produce the same effects upon stimulation compared to HGF (Copp et al., 2003).

The InlB-associated signalling pathway also contributes to the host transcriptional response to infection, by triggering the translocation of the NAD+ dependent deacetylase enzyme SIRT2 to the nucleus, and the subsequent loci-specific deacetylation of histone H3 at lysine 18 (H3K18) (Eskandarian et al., 2013). This process is dependent on host importin 7 (IPO7) for nuclear translocation (Eldridge et al., 2020b) and on protein phosphatases 1A/1B (PPM1A/PPM1B) which dephosphorylate SIRT2 at serine 25 (S25), allowing its association with chromatin (Pereira et al., 2018). SIRT2 activity during *Lm* infection causes widespread transcriptional changes (Eskandarian et al., 2013) (as described in section C.3.5). SIRT2-mediated deacetylation of H3K18 appears to play an important role during infection, as chemical inhibition or siRNA knock-down (KD) of either SIRT2 or PPM1A/PPM1B reduces the number of intracellular bacteria *in vitro* without affecting host cell entry (Eskandarian et al., 2013; Pereira et al., 2018). Similarly, SIRT2 KO also restricts *Lm* survival during *in vivo* infection (Eskandarian et al., 2013). Certain SIRT2-repressed genes, such as lymphoid enhancer binding factor 1 (*LEF1*) and *CXCL12*, have roles in regulating immune responses. The modulation of SIRT2 activity by *Lm* may thus serve as a strategy to block or downregulate the induction of immune and antimicrobial responses at both the cell-intrinsic and systemic levels.

Both InlB and LLO illustrate how signalling cascades induced by *Lm* virulence factors have a broad impact on the host transcriptional program through several mechanisms, including histone modifications (Figures 25A and 25B).

4.3. Internalin C (InlC)

InlC counteracts the host immune response to *Lm* infection by inhibiting NF-κB activation in both macrophages and epithelial cells. InlC binds to IκB kinase α (IKKα), inhibiting IκBα degradation and thus the nuclear translocation of NF-κB, resulting in decreased pro-inflammatory cytokine gene expression in macrophages *in vitro* and reduced chemokine expression and neutrophil recruitment to the peritoneal cavity *in vivo* (Gouin et al., 2010) (Figure 25C). At later stages during infection, InlC is post-translationally modified by the host cell to increase the inflammatory response in neutrophils. Monoubiquitinated InlC interacts with and stabilizes the alarmin S100A9, resulting in increased ROS production by neutrophils, restricting infection and promoting host survival (Gouin et al., 2019).

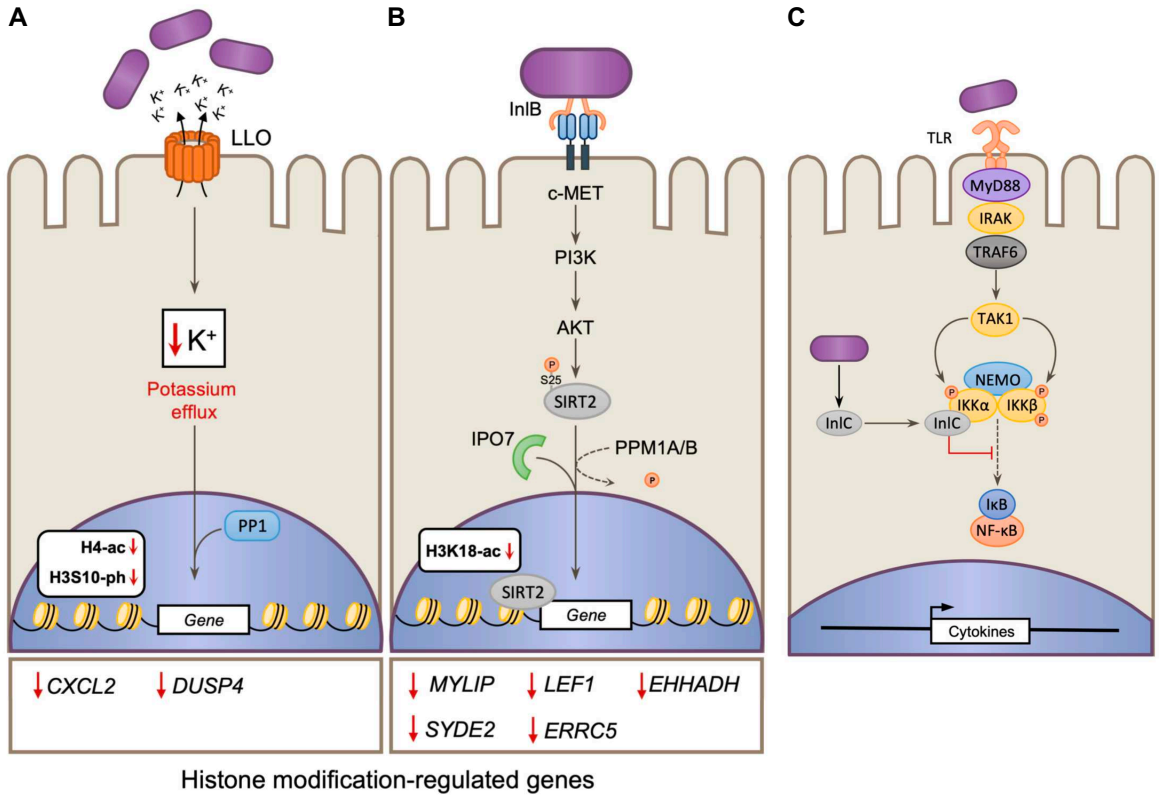

Figure 25 | *Lm* virulence factor-mediated alteration of host gene expression.
(**A**) *Lm* infection or purified LLO alone induces a global reduction in histone H4 acetylation (H4-ac) and histone H3 phosphorylation (H3S10-ph) which correlates with the downregulation of CXCL2 and DUSP4 genes. (**B**) InlB induced c-Met/PI3K-Akt signalling triggers the redistribution of SIRT2 to host chromatin, resulting in the deacetylation of H3K18 and gene repression. (**C**) The virulence factor InlC is produced and secreted by cytosolic *Lm*. InlC binds IKKα and blocks IκB phosphorylation and degradation, thereby inhibiting NF-κB nuclear translocation and the transcription of NF-κB-regulated genes such as cytokine-coding genes. (Adapted from Eldridge et al., 2020a)

4.4. Internalin H (InlH)

InlH, another member of the *Lm* internalin family, is, like InlC, involved in dampening the immune response by downregulating the production of IL-6 *in vivo*. Inactivation of *inlH* led to increased IL-6 levels (but not those of other cytokines) in the liver (Personnic et al., 2010), and reduced bacterial load in the blood, liver, and spleen of IV-infected mice (Personnic et al., 2010; Schubert et al., 2001). However, the mechanisms by which InlH impacts IL-6 production are not yet elucidated.

4.5. *Listeria* adherence protein (LAP)

NF-κB activation can also be induced by LAP, a *Lm* surface protein that plays a role in *Lm'*s ability to breach the intestinal barrier, as described in section A.6.1. The interaction of LAP with Hsp60 on enterocytes activates NF-κB signalling, increasing CXCL8, IFN-γ, and especially TNF-α and IL-6 expression at both the transcript and protein levels, which contributes to polymorphonuclear and mononuclear cell infiltration of the ileum as well as MLCK-mediated opening of the epithelial barrier (Drolia et al., 2018).

4.6. *Listeria* nuclear targeted protein A (LntA)

The *lntA* gene, encoding the *Listeria* nuclear targeted protein A (LntA), is one of 22 genes identified in the genome of *Lm* strain EGDe that encode proteins with a secretory signal peptide and are absent in the genome of the non-pathogenic *L. innocua* as uncovered by comparative genomics in 2001 (Glaser et al., 2001). The characterisation of this secreted effector by Bierne and colleagues led to the identification of the first *Lm* virulence factor to target the host cell nucleus thus modulating the transcriptional response of the infected cell by directly manipulating epigenetic machinery (Lebreton et al., 2011), as detailed below.

5. *Lm* manipulation of ISG expression through the targeting of host epigenetic machinery: the LntA-BAHD1 paradigm

A cell microbiology approach to search for *Lm* secreted proteins targeting intracellular organelles identified LntA as a nuclear-targeting bacterial protein (Lebreton et al., 2011). Such bacterial effectors exerting their functions in the host cell nucleus have been defined by Bierne and Cossart as "nucleomodulins" and have been discovered in several other intracellular pathogens (Bierne and Pourpre, 2020; Bierne et al., 2012a). The human protein targeted by

LntA in the nucleus has been identified as bromo adjacent homology domain-containing 1 (BAHD1) (Bierne et al., 2009; Lebreton et al., 2011; 2014).

5.1. The epifactor bromo adjacent homology domain-containing 1 (BAHD1)

In each human cell two meters of DNA are compacted in the nucleus, wrapped around histone proteins to form nucleosomes that are packed into chromatin fibres (Bierne and Hamon, 2020; Ou et al., 2017). The state of chromatin compaction plays a major role in regulating gene expression by controlling the accessibility of DNA to the transcriptional machinery. The regulation of chromatin structure is a dynamic process that involves DNA methylation, and a wide variety of histone post-translational modifications (PTMs) such as methylation, acetylation, phosphorylation, and ubiquitination, and the remodelling of nucleosomes (Bierne, 2017; Bierne and Hamon, 2020). These different mechanisms function in concert to regulate the formation of chromatin structures that are either loosely packed and transcriptionally active (known as euchromatin), or highly condensed and transcriptionally silent (heterochromatin). Histone PTMs and DNA methylation patterns control transcription not only by modifying chromatin structure, but also by the direct recruitment of DNA-binding TFs (Bierne, 2017; Bierne and Hamon, 2020). Chromatin-based transcriptional regulation is mediated by macromolecular complexes that are composed of scaffold proteins, epigenetic “readers”, “writers”, “erasers”, and remodelers. These proteins have been dubbed “epifactors” (Medvedeva et al., 2015) and specifically recognise (“read”), add (“write”), or remove (“erase”) histone PTMs or DNA methylation, or have remodelling activities (Bierne, 2017). The combinatorial assembly of epifactors with DNA-binding TFs activates or represses transcription and affects cell-specific gene expression in response to developmental, physiological or environmental stimuli (Bierne, 2017). In turn, cell signalling pathways influence the expression levels, localisation and assembly of epifactors into complexes. Chromatin-remodelling complexes control key pathways during embryonic development and adult life and their deregulation can lead to a wide range of pathological processes, including those involved in infectious disease (Bierne, 2017).

The characterisation of the BAHD1 protein revealed it as a central component of a novel HDAC1/2-associated complex. Biochemically, a series of experiments involving yeast two-hybrid (Y2H), co-immunoprecipitation and colocalization (Bierne et al., 2009), as well as tandem-affinity purification of BAHD1-associated proteins (Lakisic et al., 2016; Lebreton et

al., 2011) showed that BAHD1 forms a complex with HDAC1/2, histone lysine methyltransferases (KMT, e.g., G9a), readers of methylated histone H3 and methylated DNA (heterochromatin protein 1 (HP1) and methyl-CpG-binding domain protein 1 (MBD1), respectively), as well as other components (e.g., chromodomain Y like (CDYL) and KRAB-associated protein 1 (KAP1)). Importantly, the major partners of BAHD1 were identified as mesoderm induction early response (MIER) proteins (MIER1/2/3), in particular MIER1 and MIER3 (Fan et al., 2021; Lakisic et al., 2016; Lebreton et al., 2011).

Extensive characterisation of MIER1 by Gillespie and colleagues showed that this transcriptional and chromatin regulatory protein interacts with HDAC1/2, the KMT G9a, and CREB-binding protein (CREBBP) (Blackmore et al., 2008; Ding et al., 2003; Gillespie and Paterno, 2012; Wang et al., 2008), and shows structural and functional similarities with members of the metastasis-associated protein (MTA) family of the nucleosome remodelling and deacetylase (NuRD) complex, due to its EGL-27 and MTA1 homology (ELM2) and switching-defective protein 3 (Swi3), adaptor 2 (Ada2), nuclear receptor co-repressor (N-CoR), transcription factor (TF)IIIB (SANT) domains (Derwish et al., 2017; Ding et al., 2003; 2004; Paterno et al., 1997). The ELM2 domain of MIER1 is responsible for interaction with HDACs (Derwish et al., 2017; Ding et al., 2003). MIER2 and MIER3 show homology to MIER1, especially in their ELM-SANT domains (Derwish et al., 2017). MIER2 has been shown to recruit HDACs, but less efficiently than MIER1 (Derwish et al., 2017). MIER3 does not recruit HDACs (Derwish et al., 2017), but is associated with histone H3 and H4 deacetylation through histone acetyltransferase (HAT) p300 inhibition (Zhang et al., 2020).

BAHD1, on the other hand, shares with MTA proteins a bromo adjacent homology (BAH) domain (Bierne et al., 2009), which is known to promote protein-protein interactions and nucleosome binding (Yang and Xu, 2013) (Figure 26A). Bierne and colleagues have proposed the model in which BAHD1 and MIER proteins cooperate to fulfil a scaffolding function similar to that of MTA in the NuRD complex (Lakisic et al., 2016) (Figure 26B). The BAHD1-MIER scaffold bridges histone methylation on lysine 9 (meK9), histone deacetylation and DNA methylation, all of which are epigenetic marks that promote gene silencing (Figure 26C).

The functional study of BAHD1 is consistent with this biochemical characterization. BAHD1 triggers chromatin compaction into heterochromatin and induces gene silencing (Bierne et al., 2009). BAHD1 KD alters histone acetylation, histone methylation and DNA methylation patterns at target genes (Lakisic et al., 2016) and BAHD1 overexpression remodels the DNA

methylome in human cells (Libertini et al., 2015). The genes inhibited by BAHD1 differ between cell types and the specific stimuli. Full KO of the *Bahd1* gene in mice results in phenotypic defects in the placenta and brain (Lakisic et al., 2016; Pourpre et al., 2020) as well as metabolic defects, particularly in cholesterol metabolism (Lakisic et al., 2016). BAHD1 has been shown to regulate inflammation in the colon during ulcerative colitis (Zhu et al., 2015), and during *Lm* infection, BAHD1 represses the expression of ISGs downstream of IFN-I/III signalling in LoVo epithelial cells (Lebreton et al., 2011; 2014).

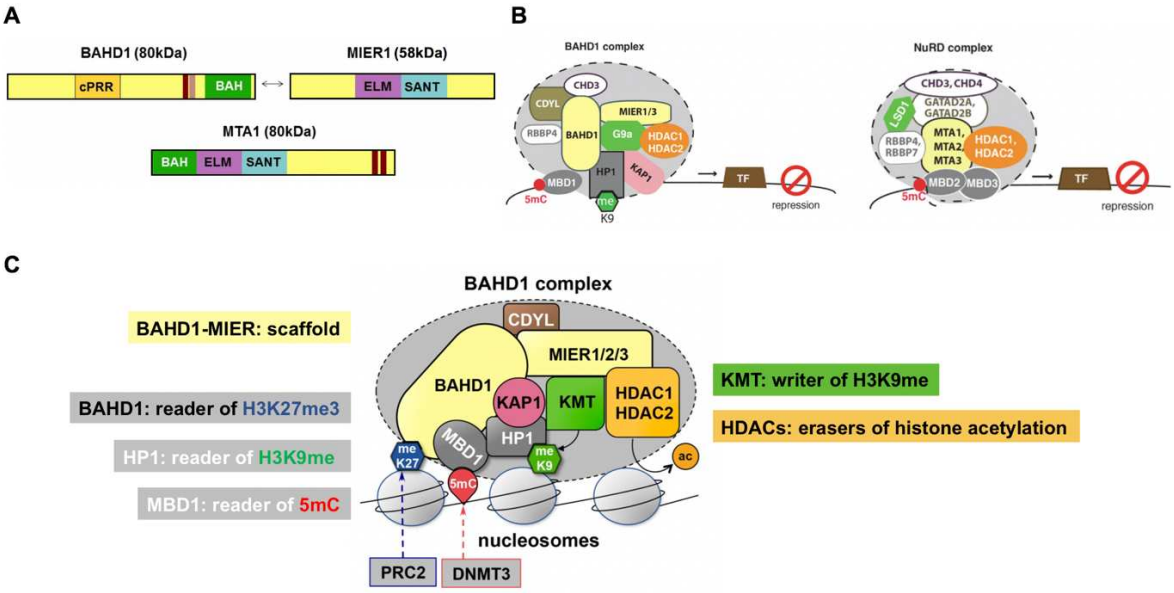

Figure 26 | The BAHD1-MIER chromatin-repressive complex.
(**A**) Schematic representation of BAHD1 and MIER1 (top) and MTA1 (bottom) scaffold proteins. Coloured boxes represent domains or motifs (orange: central proline-rich region (cPRR); green: BAH domain; purple: ELM2 domain; bleu: SANT domain); brown boxes are nuclear localization sequences. (**B**) Schematic representation of the BAHD1-MIER (left) and NuRD (right) complexes. The similar colour codes in the two complexes highlight subunits sharing a common function. (**C**) Schematic representation of the BAHD1-MIER complex: BAHD1 and MIER1/2/3 act as scaffolding proteins; KMT mediates the methylation of H3K9 ("meK9"), and HDACs remove acetyl groups (abbreviated "ac") on histones; MBD1, HP1, and BAHD1 are readers of the epigenetic marks 5mC, meK9, and H3K27me3 ("meK27"), respectively. (Adapted from Lakisic et al., 2016, Pourpre et al., 2020)

5.2. LntA, a BAHD1 inhibitor that stimulates interferon responses

When *Lm* produces LntA, the protein is secreted into the host cell cytosol and then enters the nucleus, where it impedes the binding of BAHD1 to the promoters of ISGs. By preventing the recruitment of BAHD1 and HDACs to ISG promoters, LntA triggers the acetylation of histones rendering chromatin accessible and thus activating ISG expression (Lebreton et al., 2011) (Figure 27).

Both *lntA* deletion and overexpression results in decreased bacterial loads in the blood, liver, and spleen following IV infection in mice. On the host side, haplo-deficiency for *Bahd1* in mice similarly impacts the efficiency of *Lm* infection (Lebreton et al., 2011). This suggests that the regulated secretion of LntA by *Lm* manipulates the activity of BAHD1 during infection to fine tune the IFN response for optimal survival and colonisation of the host (Lebreton et al., 2012).

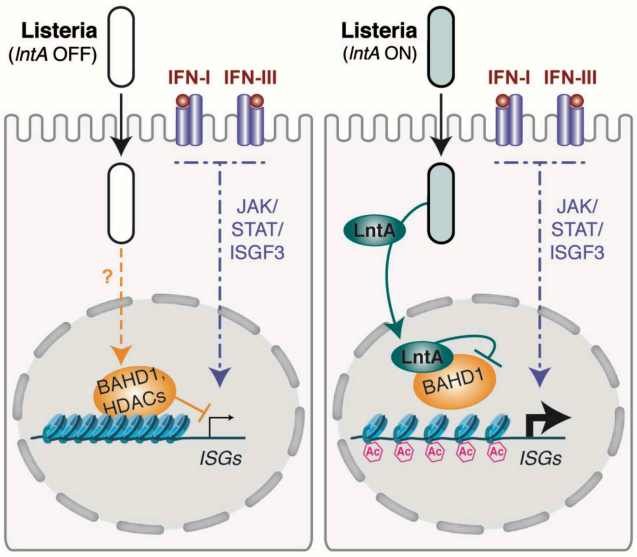

Figure 27 | LntA inhibition of BAHD1-mediated repression of ISGs.
Lm infection drives the BAHD1 complex to repress IFN-I/III activated ISG expression in epithelial cells via an unknown mechanism (left); LntA expressed by cytosolic *Lm* inhibits the BAHD1-mediated repression of ISGs (right). (Source: Lebreton et al., 2012)

THESIS OBJECTIVES

Lm is one of the best characterised intracellular pathogenic bacteria. More than 40 bacterial effectors, as well as numerous cellular processes, have been identified as contributing to efficient infection in mammalian cells (Lebreton et al., 2016; Radoshevich and Cossart, 2017). In addition, several studies have analysed the transcriptional response of host cells to cellular invasion by *Lm*. These studies have employed either cultured cell lines, or the murine listeriosis model to examine the host response at the organ level or in primary cells. However, studies of the transcriptional response of host cells to *Lm* infection remain fragmentary, as they are often limited to the early stages of the infection process (from the first hour to one day). The long-term fate of intracellular *Lm* and the infected host cell reprogramming upon long-term infection, has not been studied. Furthermore, research has generally been restricted to a limited number of cell types, predominantly phagocytic cells.

The *Epigenetics and Cellular Microbiology* team, my host laboratory, was the first to study long-term infection of *Lm* in human epithelial cells. The team identified a new phase of the *Lm* life cycle in human hepatocytes and trophoblast cells. They showed that after the early phases of entry and cytosolic replication, bacteria progressively cease to polymerise actin during intercellular dissemination and, after 2–3 days of infection, are captured in membrane structures forming "*Listeria*-containing vacuoles" (LisCV) (Kortebi et al., 2017). In this vacuolar niche, the bacteria enter a non-replicative life phase, known as the "persistence" phase. The team proposed that these persistent *Lm* could parasitise cells in the long term, in a dormant state, which could play a role during the asymptomatic incubation period of listeriosis or in asymptomatic carriage (Bierne et al., 2018).

My thesis project aimed to determine the consequences of this persistent infection on the transcriptional program of host cells, in order to identify deregulated processes that could allow the bacteria to persist in the long term, without being eliminated by host defence systems. The hepatocyte response, in particular, was chosen for several reasons:

(*i*) The liver is a major target organ of *Lm*, both essential for the elimination of bacteria during the acute phase of infection and as an important replicative niche for *Lm* in hepatocytes.

(*ii*) The immune responses elicited in the liver during invasive listeriosis have been well characterised in the mouse. However, there is no data on the specific response of hepatocytes (murine or human) to intracellular infection with *Lm*. While several studies have examined the transcriptional response of the liver in the murine listeriosis model

(Demiroz et al., 2021; Dieterich et al., 2008; Joseph et al., 2004; Ng et al., 2005; Pitt et al., 2016), none have explored the specific contribution of hepatocytes versus immune cells.

(*iii*) Hepatocytes are epithelial cells with a long life span (Duncan et al., 2009). *Lm* could persist long term within these cells with lasting effects on hepatocyte gene expression.

(*iv*) Asymptomatic colonisation of humans or animals could include a hepatic phase.

(*v*) The liver possesses immuno-tolerogenic properties that render hepatocytes propitious as a persistent niche for numerous hepatotropic virus and parasites (Protzer et al., 2012), and could thus favour *Lm* persistent infection.

My first objective was to optimise or to develop new protocols in order to obtain an efficient and homogeneous persistent infection in two human hepatocyte cell lines (HepG2 and Huh7 cells) and in primary murine hepatocytes.

My second objective was to analyse the transcriptional changes occurring in hepatocytes after three days of *Lm* infection. In particular, after the identification, by RNA-seq, of genes differentially regulated between infected and non-infected cells, my goals were (*i*) to analyse the cellular processes disrupted in each model, (*ii*) to deduce a common signature of persistent *Lm* infection, (*iii*) to study the "strain effect" and the "time effect" on these transcriptional deregulations.

My third objective was to investigate mechanisms that could explain the effect of persistent *Lm* infection on hepatocyte gene deregulation, focusing on the role of epigenetic regulators. Preliminary work had identified three genes (*BAHD1, MIER1* and *MIER3*) encoding subunits of the BAHD1-MIER repressive chromatin-remodelling complex (Lakisic et al., 2016; Lebreton et al., 2011) as being specifically overexpressed in infected HepG2 cells. BAHD1 has a role in repressing the IFN response during *Lm* infection of intestinal cells (Lebreton et al., 2011) and in repressing lipid metabolism genes (Lakisic et al., 2016). MIER1 has been extensively characterised by Gillespie and collaborators in the context of its role as a tumour suppressor in breast carcinoma (Clements et al., 2012; Gillespie and Paterno, 2012; Li et al., 2013; 2015). My goal was thus to explore the role of the BAHD1-MIER complex during persistent *Lm* infection, in the context of a collaborative project between the laboratories of H. Bierne and L. Gillespie.

RESULTS

Part 1. An immunomodulatory transcriptional signature associated with persistent *Lm* infection in hepatocytes

1.1. Published results

These results were published in the journal *Frontiers in Cellular and Infection Microbiology* (Descoeudres et al., 2021) and comprise the majority of the results obtained during my thesis. The original research article presents the results that respond to my first two thesis objectives – with the exception of the time-dependence of the transcriptomic signature (addressed in section 1.2) – notably:

(*i*) The establishment of long-term, LisCV-stage infection models in two human hepatocyte cell lines (HepG2 and Huh7 cells) and in primary murine hepatocytes.

(*ii*) An overview of the transcriptional reprogramming that occurs in hepatocytes after three days of *Lm* infection ("long-term infection") and the identification of a gene expression signature associated with intracellular bacterial persistence common to all three models.

(*iii*) The examination of cellular processes disrupted by long-term infection that revealed:

(*a*) cholesterol metabolism-associated gene downregulation in long-term infected human hepatocytes;

(*b*) the reduced expression of hepatic innate immunity genes – or APPs – that translated to reduced APP secretion by infected hepatocytes. We further provide evidence that both the constitutive and cytokine-driven expression of APPs were repressed by long-term *Lm* infection.

(*iv*) The characterisation of a major facet of the epithelial cell response to *Lm* infection, the IFN and ISG response, including the quantification of IFN expression at both the transcript and protein levels over the infection time course in all three models.

(*v*) The independence of the transcriptomic and proteomic signatures to the strain of *Lm* employed that was evidenced through the acquisition of similar results regardless of the laboratory or epidemic strain employed.

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ORIGINAL RESEARCH
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An Immunomodulatory Transcriptional Signature Associated With Persistent *Listeria* Infection in Hepatocytes

*Natalie Descoeudres[1], Luc Jouneau[2], Céline Henry[1], Kevin Gorrichon[3], Aurélie Derré-Bobillot[1], Pascale Serror[1], Laura Lee Gillespie[4], Cristel Archambaud[1], Alessandro Pagliuso[1] and Hélène Bierne[1]**

[1] Université Paris-Saclay, INRAE, AgroParisTech, Micalis Institute, Jouy-en-Josas, France, [2] Université Paris-Saclay, INRAE, Virologie et Immunologie Moléculaires, Jouy-en-Josas, France, [3] Université Paris-Saclay, Institut de Biologie Intégrative de la Cellule, CEA, CNRS UMR 9198, Université Paris-Sud, Gif-sur-Yvette, France, [4] Terry Fox Cancer Research Laboratories, Division of BioMedical Sciences, Faculty of Medicine, Memorial University of Newfoundland, St. John's, NL, Canada

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Changyong Cheng, Zhejiang A & F University, China

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***Correspondence:**
Hélène Bierne
helene.bierne@inrae.fr

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Listeria monocytogenes causes severe foodborne illness in pregnant women and immunocompromised individuals. After the intestinal phase of infection, the liver plays a central role in the clearance of this pathogen through its important functions in immunity. However, recent evidence suggests that during long-term infection of hepatocytes, a subpopulation of *Listeria* may escape eradication by entering a persistence phase in intracellular vacuoles. Here, we examine whether this long-term infection alters hepatocyte defense pathways, which may be instrumental for bacterial persistence. We first optimized cell models of persistent infection in human hepatocyte cell lines HepG2 and Huh7 and primary mouse hepatocytes (PMH). In these cells, *Listeria* efficiently entered the persistence phase after three days of infection, while inducing a potent interferon response, of type I in PMH and type III in HepG2, while Huh7 remained unresponsive. RNA-sequencing analysis identified a common signature of long-term *Listeria* infection characterized by the overexpression of a set of genes involved in antiviral immunity and the under-expression of many acute phase protein (APP) genes, particularly involved in the complement and coagulation systems. Infection also altered the expression of cholesterol metabolism-associated genes in HepG2 and Huh7 cells. The decrease in APP transcripts was correlated with lower protein abundance in the secretome of infected cells, as shown by proteomics, and also occurred in the presence of APP inducers (IL-6 or IL-1β). Collectively, these results reveal that long-term infection with *Listeria* profoundly deregulates the innate immune functions of hepatocytes, which could generate an environment favorable to the establishment of persistent infection.

Keywords: *Listeria monocytogenes*, liver, acute phase response, interferon, persistence, innate immunity, cholesterol, transcriptomics

INTRODUCTION

The liver has essential roles in metabolism and detoxification. It also acts as a barrier to systemic infections, by its major role in the detection, capture and clearance of pathogens present in the blood (Protzer et al., 2012; Kubes and Jenne, 2018). Certain pathogens, including viruses (*e.g.*, Hepatitis A, B, C, D, E viruses), parasites (e.g., *Plasmodium falciparum*, *Toxoplasma gondii*, *Entamoeba histolytica*) and bacteria (e.g., *Listeria monocytogenes*, *Salmonella Typhimurium*, *Francisella tularensis*, *Brucella abortus*, *Streptococcus pneumoniae*) are able to invade hepatocytes, the parenchymal cells of the liver. Among these pathogens, *L. monocytogenes* (hereafter referred to as *Listeria*) is a bacterial food contaminant capable of reaching and multiplying in the liver after crossing the intestinal barrier. In the majority of individuals, invasion of *Listeria* is successfully cleared, but if the infection is not controlled by an adequate immune response, the proliferation of *Listeria* can lead to the release of intracellular bacteria into the circulatory system and invasion of other sites, such as the brain in immunocompromised individuals, and the placenta and the fetus in pregnant women, leading to sepsis, meningo-encephalitis, miscarriages and neonatal infections (Schlech, 2019). These severe clinical manifestations make listeriosis one of the most lethal foodborne infections (European Food Safety, 2021).

Most of our knowledge of the liver phase of listeriosis comes from experimental infections in animal models, mainly mice, resulting in a very well described infection scenario, although some differences may arise from the different routes of inoculation and/or bacterial dose used. After intravenous inoculation, more than 60% of the bacteria are cleared from the bloodstream by the liver within 10 minutes. Bacteria are bound to Kupffer cells (KC, the resident liver macrophages) and subsequently eliminated through a complex interaction between KC and neutrophils that migrate rapidly to the liver in response to infection (Conlan and North, 1991; Gregory et al., 1996; Gregory et al., 2002; Witter et al., 2016). Six hours (h) after infection, about 90% of liver bacteria are associated with hepatocytes, within which bacterial replication takes place for 2-3 days. Thus, *Listeria* loads in the liver increase exponentially before reaching a plateau after 3-4 days of infection, and then decrease with the development of specific immunity (Cousens and Wing, 2000). Bacterial invasion of hepatocytes is proposed to occur *via* two routes: direct internalization or cell-to-cell spread from KC (Dramsi et al., 1995; Gaillard et al., 1996; Appelberg and Leal, 2000). Histology (Conlan and North, 1991) and electron microscopy observations (Gaillard et al., 1996) suggest that bacteria spread in the liver parenchyma using the actin-based motility process described in cellular models *in vitro* (Pilgrim et al., 2003; Kortebi et al., 2017). In line with this, *Listeria actA* mutant strains, which do not produce the actin-polymerization factor *actA*, are three orders of magnitude less virulent compared to wild-type strains in murine models (Domann et al., 1992). The direct passage of bacteria from one hepatocyte to another is proposed to generate infectious foci in which *Listeria* disseminates through the parenchyma, without coming into contact with the humoral effectors of the immune system.

Following the phase of active growth in hepatocytes, the bacterial burden strongly decreases in the liver as a result of potent innate immune responses (Cousens and Wing, 2000). Several cell types contribute to the defense of the infected liver against *Listeria* infection, in particular neutrophils, natural killer (NK) cells, dendritic cells (DC) (Conlan and North, 1991; Gregory et al., 1996; Cousens and Wing, 2000; Arnold-Schrauf et al., 2014; Witter et al., 2016) and KC, whose necroptotic death triggers the recruitment of infiltrating monocytes, which proliferate and differentiate into macrophages at the site of infection (Bleriot et al., 2015). These immune cells work together *via* cell to cell contacts and the secretion of cytokines and chemokines to kill bacteria or inhibit their replication, and to lyse infected hepatocytes. The hepatocytes themselves actively participate in the innate immune response by constitutively producing and secreting a variety of proteins that play an important role in innate immunity, such as complement factors and proteins involved in hemostasis (Zhou et al., 2016). These proteins, whose production increases rapidly and substantially in response to inflammatory stimuli are known as acute phase proteins (APPs) (Gabay and Kushner, 1999; Zhou et al., 2016). Production of pro-inflammatory cytokines by KC, monocytes and neutrophils, in response to *Listeria* infection, stimulates APP production by hepatocytes (Kopf et al., 1994; Kummer et al., 2016). This first wave of non-specific defenses is essential to host survival, with inflammation also contributing to the development of acquired resistance by stimulating the priming and proliferation of cytotoxic T cells, which mediate the protective primary and memory responses against *Listeria* (Pamer, 2004; Qiu et al., 2018).

The propensity of *Listeria* to invade and damage the liver has long been documented in rodents; indeed, among the first names proposed for this bacterium were *Bacillus hepatis* (by Hülphers, in 1911) and *Listerella hepatolytica* (by Pirie, in 1927), based on observations of liver necrosis in rabbits and gerbils. In humans, however, while liver abscesses are described in neonatal listeriosis, clinical symptoms of liver injury due to *Listeria* are rarely reported during invasive listeriosis in adults. Moreover, there is a lack of knowledge on the fate of bacteria in organs, including the liver, during the asymptomatic incubation period, which can be very long in cases associated with pregnancy [up to seventy days (Goulet et al., 2013)]. In addition, asymptomatic carriage of *Listeria* exists in healthy humans (Slutsker and Schucha, 1999), as well as in many farm or wild mammal species (Gray and Killinger, 1966; Yoshida et al., 2000; Leclercq et al., 2014; Hurtado et al., 2017; Parsons et al., 2020), yet our understanding of this asymptomatic carriage, particularly in its hepatic stage, is severely lacking.

Recently, the notion has emerged that, in addition to the well-known phases of active replication and motility in the cytosol of host cells, *Listeria* may enter a quiescent phase in vacuolar compartments, ranging from slow growth to dormancy, which may play an important role in asymptomatic infections (Bierne et al., 2018). These vacuolar niches are particularly formed in liver cells, including hepatic macrophages (Birmingham et al., 2008) and hepatocytes

(Kortebi et al., 2017), by distinct mechanisms. In particular, we have shown that in the human hepatocyte cell line HepG2 and in primary human hepatocytes, *Listeria* enters a resting phase in acidic vacuoles, called "*Listeria*-containing vacuoles" (LisCVs) (Kortebi et al., 2017). LisCVs are generated late (i.e., after 2-3 days of infection), when the bacteria cease to express ActA and polymerize actin, and are partially degradative; therefore, a subpopulation of bacteria survives in a quiescent state, raising the possibility of long-term persistence of *Listeria* in the liver parenchyma. Stages of persistence in hepatocytes have been described for other pathogens, such as hepatic viruses and parasites. For instance, *Plasmodium vivax* can enter a quiescent state within a parasitophorous vacuole to go undetected for years (Prudencio et al., 2006).

The objective of this study was to characterize the transcriptional response of the hepatocyte to long-term *Listeria* infection in order to identify a gene expression signature associated with intracellular bacterial persistence. We report the development of three robust cellular models to obtain a homogeneous population of hepatocytes hosting *Listeria* in the late LisCV stage. By comparing the *Listeria*-induced transcriptional pattern in these three different models, we found a common signature associated with *Listeria* persistence in the hepatocyte. Our data suggest that down-regulation of key hepatic innate immunity genes involved in the acute phase response (APR) may contribute to silent carriage of *Listeria* in the liver.

MATERIALS AND METHODS

Bacterial Strains and Human Hepatocyte Cell Lines

We used *Listeria monocytogenes* laboratory strains EGDe and 10403S (Becavin et al., 2014) and a clinical isolate of the 4b serotype, CLIP80459 (Hain et al., 2012). Bacteria were grown on brain-heart infusion (BHI) medium at 37°C. The human hepatocellular carcinoma cell lines HepG2 (ATCC HB-8065) and Huh7 (CLS 300156) were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 2 mM L-glutamine (Sigma) and 10% fetal bovine serum (FBS, Sigma) at 37°C in a humidified 5% CO_2 atmosphere and placed at 10% CO_2 during infection assays.

Isolation and Culture of Primary Mouse Hepatocytes

Primary mouse hepatocytes (PMH) were isolated from 8- to 10-week-old female C57BL/6 mice, by collagenase perfusion of the liver, as previously described (Fortier et al., 2017). Briefly, mice were anesthetized, and a midline laparotomy was performed. The inferior vena cava was perfused with a 0.05% collagenase solution (collagenase from *Clostridium histolyticum*, Sigma C5138). The portal vein was sectioned, and the solution allowed to flow through the liver. Upon liver digestion, hepatic cells were removed by mechanical dissociation, filtered through a sterile 70 µm cell strainer (BD Falcon), washed twice by centrifugation at 300 x g for 4 min. After a filtration step through a sterile 40 µm cell strainer (BD Falcon), cells were resuspended in serum-containing culture medium [DMEM, 10% FBS, 1% penicillin–streptomycin (Sigma); 100 µg/mL fungizone antimycotic B (Gibco)]. Cell count and viability were assessed by trypan blue exclusion. Cells were seeded in 6-well collagen-coated plates for 6 h at 37°C in a 5% CO_2 atmosphere. After complete adhesion of the hepatocytes and washes to remove the dead cells, PMH were cultured at 37°C in a 5% CO_2 atmosphere for 4-6 days before infection in serum-free hepatocyte culture medium [William's E medium and GlutaMAX™ Supplement (Gibco); 100 U/ml penicillin/streptomycin, 0.5 µg/ml fungizone antimycotic B, 4 µg/ml insulin, 0.1% bovine serum albumin (BSA) and 25 nM dexamethasone (Sigma)] that was renewed daily.

Antibodies and Reagents

The primary antibodies used in this study were anti-*L. monocytogenes* polyclonal rabbit antibody (BD Difco, 223001) and anti-human LAMP1 monoclonal mouse antibody (BD Bioscience, 555801). Fluorescent secondary antibodies were Alexa Fluor 488-conjugated goat anti-rabbit (Life Technologies) and Alexa Fluor Cy3-conjugated goat anti-mouse (Jackson ImmunoResearch Laboratories). Alexa fluor 647-conjugated phalloidin (Life Technologies) and Hoechst (Thermo Fisher Scientific) were used to label F-actin and nuclei, respectively. All recombinant human proteins were obtained in lyophilized form from R&D Systems and reconstituted according to the manufacturer's instructions: IL-29/IFN-λ1 (1598-IL), IFN-β (8499-IF), interleukin IL-6 (206-IL) and IL-1β/IL-1F2 (201-LB).

Bacterial Infections and Cytokine Treatment

HepG2 and Huh7 cells were seeded in 12- or 6-well plates in order to reach approximately 90% confluency on the day of infection. HepG2 cells were grown on collagen-coated wells or coverslips (type I collagen from rat tail, Sigma). Cells were counted using a hemocytometer (Neubauer-improved, Hausser Scientific) before infection to determine the multiplicity of infection (MOI). Inoculums were prepared in serum-free culture medium using bacteria grown overnight to stationary-phase and washed twice in PBS. Cells were infected as described in (Bierne et al., 2021) and subsequently incubated for the indicated times with culture media containing 25 µg/ml gentamicin to kill extracellular bacteria. To determine the number of intracellular bacteria at a given time-point post-infection (p.i.), cells were washed in serum-free culture medium and lysed in cold distilled water. Serial dilutions of cell lysates were plated on BHI agar and the number of intracellular bacteria was determined by counting colony-forming units (CFU) after 48 h incubation at 37°C. In parallel, host cells were enumerated following trypsin detachment and trypan-blue staining. When HepG2 and Huh7 infected and non-infected cells were stimulated with recombinant human cytokines, experiments were performed in 6-well plates and the

specified concentration was obtained by adding 200 µl of culture medium containing 25 µg/ml gentamicin and the necessary volume of either the cytokine solution or a mock solution. PMH were infected using the same protocol as for the human cell lines, with the following differences: inoculum was prepared using William's E medium and replaced after 1 h with hepatocyte culture medium depleted of penicillin/streptomycin and supplemented with 25 µg/ml gentamicin. Medium was renewed daily throughout the infection time course. Infected and non-infected cells were handled identically, all experiments were performed in triplicate and reproduced at least three times.

Immunofluorescence Microscopy

Observation of intracellular *L. monocytogenes* by microscopy was performed as in (Bierne et al., 2021). Briefly, cells grown on 12 mm or 22 mm coverslips were rinsed in 1× phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde (PFA) in PBS for 30 minutes at room temperature. Cells were washed in PBS, permeabilized in 0.4% Triton X-100 in PBS, washed 3 times in PBS and incubated in blocking solution (2% bovine serum albumin (BSA) in PBS), before being processed for immunofluorescence, successively with the primary antibody solution, and secondary antibody solution containing Alexa fluor 647-conjugated phalloidin and Hoechst (in 2% BSA). Coverslips were mounted on glass slides using Fluoromount-G mounting medium (Interchim, Montlucon). Samples were analyzed with a Carl Zeiss AxioObserver.Z1 microscope equipped with 20× non-oil immersion or 40×, 63× and 100× oil immersion objectives connected to a CCD camera. Images were processed using ZEN (Carl Zeiss) or ImageJ.

RNA Extraction, RNA-Sequencing, and Functional Gene Analysis

HepG2 or Huh7 cells were infected with strain EGDe or remained non-infected, in independent biological triplicates, and PMH isolated from the livers of two mice (n=3 per mouse) were infected with strain 10403S or remained non-infected. All infection assays were performed in 6-well plates (HepG2 and Huh7: 3 infected, 3 non-infected; PMH, 6 infected, 6 non-infected). RNA was extracted using the RNeasy Mini Kit (Qiagen) and genomic DNA was removed using TURBO DNA-free™ kit (Ambion), according to the manufacturer's instructions. The integrity, purity and concentration of RNA samples was assessed on an RNA 6000 Nano chip using the Agilent 2100 electrophoresis Bioanalyzer. RNA Integrity Numbers (RIN) for all samples used for RNA sequencing (RNA-seq) library preparation was superior to 9.5. RNA-seq and data analysis procedures, as well functional gene analysis, and information on data repository, are detailed in the **Supplementary Material**. Each RNA-seq analysis was validated by RT-qPCR (see below), with *YWHAZ*, *PPIA*, or *Pdk1* used to normalize gene expression in infected relative to non-infected HepG2, HuH7 and PMH, respectively. Pearson correlation analysis was applied to the log2 FC of 14 significantly deregulated genes obtained by RNA-seq and RT-qPCR, as shown in the **Supplementary Material**, and indicates a very strong correlation.

Reverse Transcription-Quantitative PCR

RNA was extracted from cultured cells and depleted for DNA, as described above. RNA concentration and purity were assessed using a NanoDrop spectrophotometer (Thermo Scientific). One microgram of total RNA was used for reverse transcription with the LunaScript™ RT SuperMix Kit (NEB). Quantitative Real-Time PCR was performed on StepOne Plus Real-Time PCR Systems (Applied Biosystems) using Luna® Universal qPCR Master Mix (NEB) as specified by the supplier. Each 20 µl reaction was performed in triplicate. Target gene expression levels were normalized to an endogenous control gene whose expression stability was assessed using RefFinder (http://www.leonxie.com/referencegene.php) (Xie et al., 2012). PCR efficiencies were calculated to ensure equivalent amplification between target genes and endogenous control genes. Relative expression of target genes was calculated from cycle threshold (CT) values using the comparative CT (ΔΔCT) method with untreated or non-infected cells used as calibrator (reference) samples. Standard deviation of ΔCT values was determined according to the Applied Biosystems Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR (available at https://assets.thermofisher.com/TFS-Assets/LSG/manuals/cms_042380.pdf). A transcript was considered undetectable at CT ≥ 35; all negative controls in the absence of cDNA template generated CTs above this threshold. As IFN gene expression was absent in non-infected cells, this CT threshold was applied to all non-infected samples in order to allow the calculation of a relative expression value. Statistical significance of the difference in mean expression of genes from at least 3 experimental replicates was evaluated using the two-tailed paired-sample *t* test; a p value <0.05 was considered significant. Sequences of human and mouse gene specific primers are listed in **Supplementary Table S1**.

ELISA Assays

Sandwich enzyme-linked immunosorbent assay (ELISA) kits were used to determine the concentrations of IFN-λ1 (Invitrogen, 88-7296), IFN-β (R&D Systems DIFNB0), and C3 (Abcam, ab108823) in the supernatants of infected and non-infected HepG2 and Huh7 cells, according to the respective manufacturers' instructions. Conditioned media were collected at the indicated time-points p.i., centrifuged at 300 × g for 5 minutes at 4°C and the collected supernatants stored at -80°C. 50 µl of each sample supernatant and all standards were assayed in duplicate. For the C3 ELISA, conditioned media were diluted 100-fold in the diluent buffer according to the supplier's protocol. Signals were detected using a Tecan Infinite 200 device (Tecan Trading AG, Switzerland) by reading the absorbance at 450 nm, from which readings at 570 nm were subtracted for wavelength correction. Statistical analyses were performed using Student's two-tailed T-test on calculated concentrations.

Protein Extraction and LC-MS/MS Analysis of Culture Supernatants

HepG2 and Huh7 cells in 6-well plates either remained non-infected or were infected with *L. monocytogenes* EGDe (MOI~1-5). After 72 h, cells were rinsed twice in serum-free medium and

left for an additional 24 h in serum-free medium containing 25 µg/ml gentamicin. Conditioned media was then collected, supplemented with protease inhibitors mixture (Complete, EDTA-free, Roche), centrifuged (1000 × g, 5 min) and the supernatant concentrated ten times using an Amicon centrifugal filter unit (3K cut-off, Millipore). Cells were lysed in 500 ul of lysis buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 1% Igepal, 0.1% SDS, 5% glycerol, supplemented with protease inhibitors cocktail) and incubated on ice (30 min). The cell lysate was cleared by centrifugation (14000 × g, 10 min, 4°C) with the supernatant assayed for protein concentration using the Bradford assay (Biorad). Protein concentration of cell lysates was used to normalize the amount of conditioned medium to concentrate between experimental conditions and replicates. Sample preparation for proteome analysis, liquid chromatography and Mass Spectrometry, as well as data analyses are detailed in the **Supplementary Material**.

RESULTS

Establishment of Cellular Models of Long-Term *Listeria* Infection in Hepatocytes

To study the response of hepatocytes to long-term intracellular *Listeria* infection, we took a comparative approach using different cell models. However, prior to transcriptomic studies, it was essential to optimize protocols by testing different hepatocyte types, culture conditions, *Listeria* strains, and multiplicity of infection (MOI), in order to obtain populations of homogenously infected cells at 3 days post-infection (p.i.). We first selected the human hepatoblastoma cell line HepG2, widely exploited as an *in vitro* model of human hepatocytes and previously used to characterize the *Listeria* persistence stage (Kortebi et al., 2017). As these cells grow in clusters, leading to the formation of highly infected foci among predominantly uninfected islets, we seeded HepG2 cells on collagen-coated wells to allow the formation of a cell monolayer. Microscopic observation revealed that, for an infection at MOI~1-5 with the laboratory *Listeria* strain EGDe, collagen treatment was accompanied by a marked increase in bacterial intercellular spread during the 3-day course of infection (**Supplementary Figure S1A**), leading to LisCV formation at 72 h p.i. (**Supplementary Figure S1B**), as previously observed in conventionally grown HepG2 cells (Kortebi et al., 2017), but in a higher proportion of cells.

We also selected the hepatocellular carcinoma-derived cell line Huh7, which has been instrumental in exploring the mechanisms behind chronic hepatic viral infections (Hu et al., 2019; Todt et al., 2020). As opposed to HepG2, Huh7 naturally grow in monolayers and are highly permissive to *Listeria* cell-to-cell dissemination (**Figure 1A**). For the same MOI ~1-5 and *Listeria* strain (EGDe), the mean number of bacteria per infected cell at 72 h p.i. was on average ~10-fold higher in Huh7 cells than in HepG2 cells, as evaluated by Colony-Forming-Unit (CFU) and cell counts. Importantly, immunofluorescence experiments revealed that *Listeria* switched from polymerizing host actin at day 1 to the actin-free stage at day 3 (**Figure 1B**) accompanied by engulfment of bacteria into LAMP1-positive compartments (**Figure 1C**). Similar results were obtained with another commonly used laboratory strain, 10403S, as well as an epidemic clinical strain, CLIP80459 (**Supplementary Figure S2**). These results validate Huh7 as an additional model to study *Listeria* persistence in human hepatocytes.

As a third cell model, we chose primary hepatocytes, which could reveal cellular pathways potentially dysfunctional in carcinoma-derived cell lines. We previously showed that *Listeria* enters the persistence phase in primary human hepatocytes (PHH) (Kortebi et al., 2017), but we could not improve the infection protocol to use these cells for transcriptomic purposes, as they remained weakly infected with *Listeria*, regardless of the MOI or strain used. In particular, we found that thawing commercial PHH generated a significant viability issue, limiting the formation of intercellular junctions. We therefore used mouse hepatocytes, as they could be isolated fresh from animal livers immediately prior to infection. We found that freshly isolated primary mouse hepatocytes (PMH) were highly permissive to *Listeria* infection with strain 10403S at an MOI of 10 (**Figure 1A**). Furthermore, in PMH *Listeria* also shifted from the actin-dependent intercellular dissemination phase, at day 1, to the vacuolar persistence phase, at day 3 (**Figures 1B, D**). Overall, these results established that HepG2 and Huh7 cell lines and PMH are suitable models to study the hepatocyte response to persistent *Listeria* infection.

Transcriptional Responses of Hepatocytes After a 3-Day *Listeria* Infection

In order to identify host gene signatures specific to long-term *Listeria* infection in hepatocytes, we performed RNA-seq analysis on RNA extracted from HepG2, Huh7 or PMH infected for 72 h with *Listeria* strain EGDe (HepG2 and Huh7) or 10403S (PMH) and compared the gene expression profile to that of non-infected cells. The number of cells and intracellular bacteria were monitored in parallel to assess cell viability, revealing little cytotoxicity induced by infection (**Figure 2A**), and to compare intracellular bacterial loads between experiments and models (**Figure 2B**). Analysis of differentially expressed protein-coding genes between infected and non-infected cells (p<0.05 and log2 fold change (FC) >0.5 or <-0.5), herein termed "DEGs", identified 1134 DEGs in HepG2 cells (443 upregulated and 691 downregulated, **Supplementary Tables S2**, **S3**), 5981 DEGs in Huh7 cells (3198 upregulated and 2783 downregulated, **Supplementary Tables S4**, **S5**) and 2138 DEGs in PMH (379 upregulated and 1759 downregulated, **Supplementary Tables S6**, **S7**) (**Figure 2C**). While intracellular bacterial loads were of the same range in all three models (on the order of 10^5 CFU per well) (**Figure 2B**), we noted that the higher infectivity observed in Huh7 and PMH cells compared with HepG2 cells (7-fold and 4-fold, respectively) paralleled with a higher number of DEGs (5-fold and 2-fold respectively).

Functional analysis of upregulated DEGs revealed that interferon (IFN) responses were strongly activated in HepG2 and PMH cells, but not in Huh7 cells, after a 3-day infection with *Listeria* (see below). The 15 most highly upregulated DEGs in HepG2 cells included the interferon lambda 1 gene (*IFNL1*), as

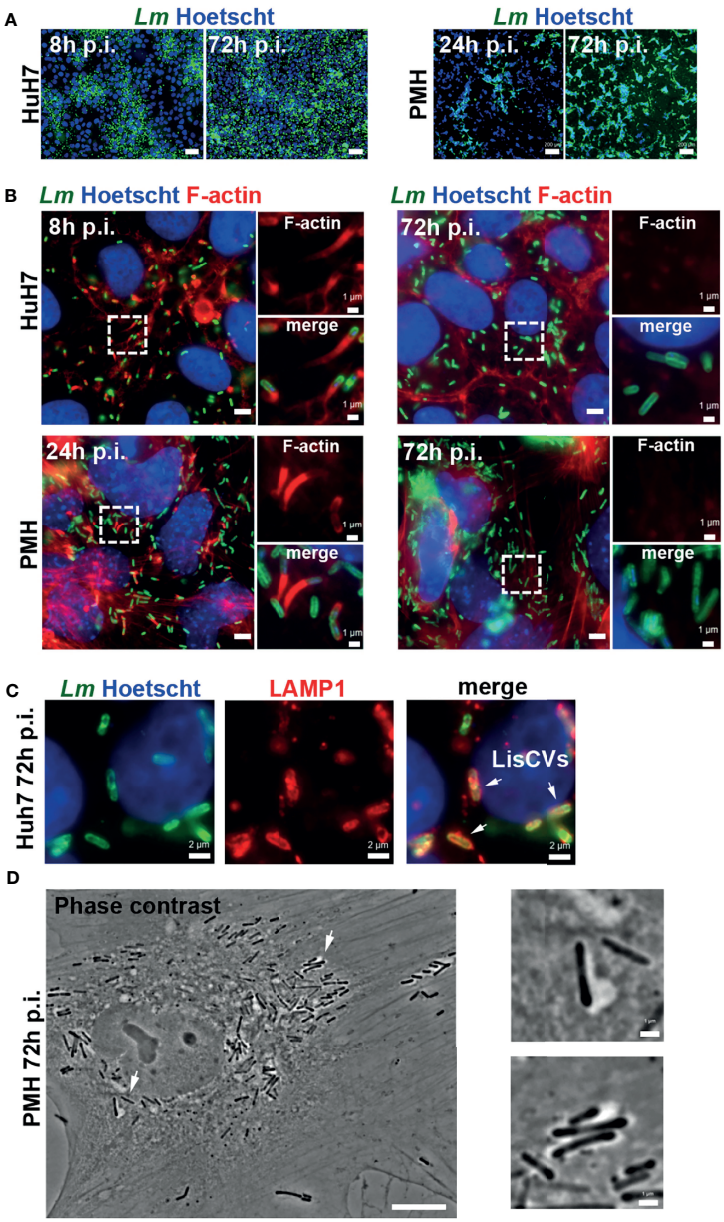

FIGURE 1 | Optimization of hepatocyte culture systems for modeling persistent *Listeria* infection. Different cell seeding conditions, MOI and *Listeria* strains (EGDe or 10403S) were tested to obtain optimal long-term *Listeria* infection of HepG2 (see **Supplementary Figure S1**), Huh7 or PMH. Infected cells were examined at day 1 (d1) and at day 3 (d3) by immunofluorescence microscopy: representative examples under optimized conditions are shown. **(A)** Low magnification micrographs of Huh7 cells infected with EGDe strain (MOI=1-5) or PMH infected with 10403S strain (MOI=10) for the indicated time. Images are overlays of *Listeria* (green) and Hoechst (blue) signals (bars: 50 µm, Huh7, or 200 µm, PMH). **(B)** High magnification micrographs of infected Huh7 or PMH showing *Listeria* (green), F-actin (red) and Hoechst (blue) signals. Bars: 5 µm. Boxed regions enlarged on the right show F-actin (top) or merged signals (bottom), highlighting actin-positive bacteria at d1 and actin-negative bacteria at d3 (bars: 1 µm). **(C)** Micrographs of an infected Huh7 cell at d3, showing *Listeria* (green), LAMP1 (red) and Hoechst (blue) signals. Arrows indicate 3 examples of LisCVs. **(D)** Phase contrast image of an infected PMH at d3 (bars: 10 µm). Arrows indicate 2 examples of bacteria within vacuoles, shown at a higher magnification on the right (bars: 1 µm).

well as Interferon Stimulated Genes (ISGs) (i.e., *IFI44, RSAD2, IFI44L, OASL, IFIT2, OAS2, CH25H, CCL5, EPSTI1, CMPK2, IFIT1, IFIT3*) (**Supplementary Table S2**), as in PMH (i.e., *Ccl5, Ifit3b, Ifr7, Isg15, Zbp1, Ifi44, Ifit1, Ifit3, Oas3, Apol9b, Oas2l, Oas1h, Tgtp1, Rsad2*) (**Supplementary Table S6**). Indeed, 166 (37%) and 126 (33%) of the upregulated DEGs in HepG2 and PMH, respectively, were mapped as ISGs in the interferome database (Rusinova et al., 2013), of which 56 were common to

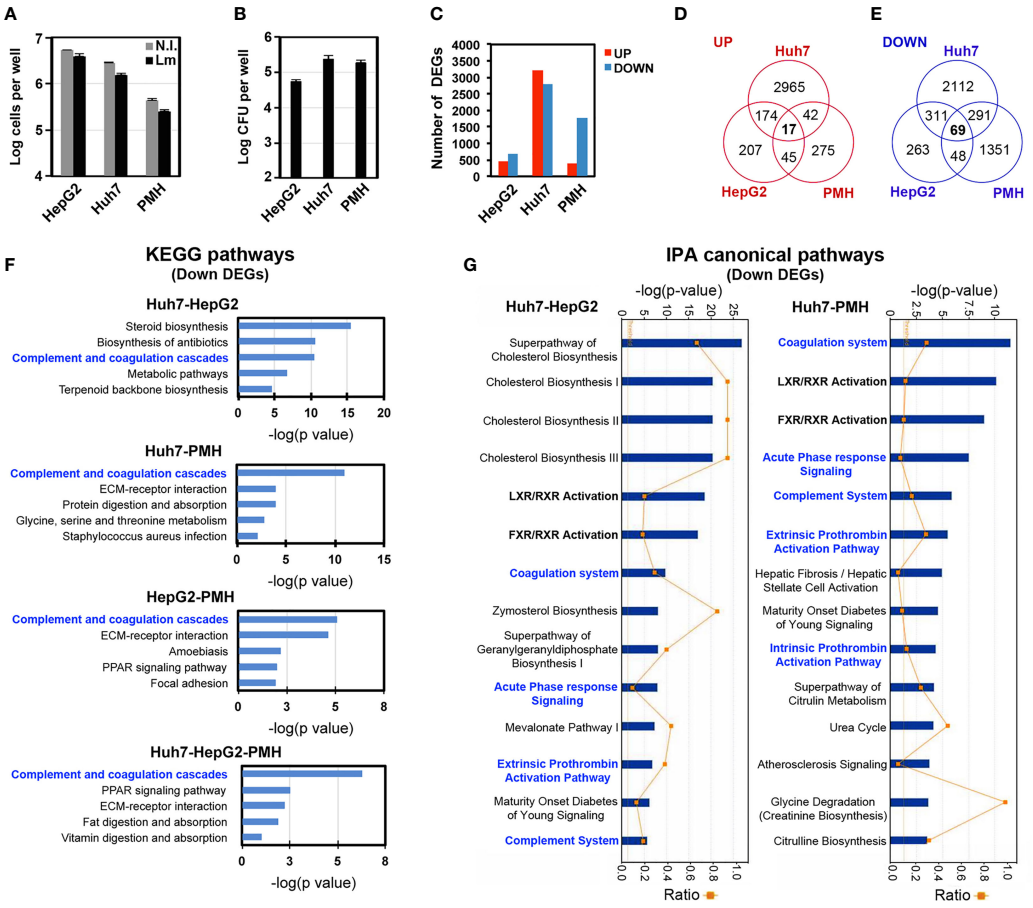

FIGURE 2 | Transcriptional responses to long-term *Listeria* infection in hepatocytes. HepG2, Huh7 and PMH were infected with *Listeria* for 72 h. **(A)** Cell counts (log cells per well, mean ±SD, n=3). **(B)** Intracellular bacteria load expressed as log CFU per well (mean ±SD, n=3). **(C)** Number of significant DEGs upregulated (red bars) or downregulated (blue bars) in infected hepatocytes compared to non-infected hepatocytes (adj $p<0.05$; $|\log2\ FC| > 0.5$). **(D, E)** Venn diagram showing the intersection of upregulated **(D)** and downregulated **(E)** DEGs between HepG2, Huh7 and PMH datasets. **(F)** The top 5 most significant KEGG pathways associated with downregulated DEGs in each of the 4 overlapping DEG datasets. **(G)** The top 14 most significant IPA canonical pathways associated with the Huh7–HepG2 (left) and the Huh7–PMH (right) overlapping downregulated DEGs.

both HepG2 and PMH datasets (**Supplementary Table S8**). In agreement with this, Gene Ontology of Biological Processes (GO-BP) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis showed that genes which were upregulated by infection in HepG2 and PMH cells significantly clustered in functions associated with viral infections and IFN responses (**Supplementary Table S9**). In contrast, upregulated DEGs in Huh7 cells were associated with entirely different biological processes and pathways, the most significant being "cell division" (GOBP) and "cell cycle" (KEGG) processes. This striking difference was also highlighted by the very limited overlap of upregulated DEGs between the three systems, with only 17 common genes (**Figure 2D**). 16 of these 17 genes were nonetheless categorized as ISGs (**Table 1** and **Supplementary Table S8**).

With respect to downregulated DEGs, only 69 were common to all three datasets (**Figure 2E**). However, intersecting DEGs from only two datasets yielded a significant number of genes commonly downregulated (i.e., 380 DEGs common to HepG2 and Huh7 and 360 common to Huh7 and PMH). Functional analysis of each individual or intersecting dataset (**Supplementary Table S10**) highlighted the "coagulation and complement cascades" as one of the top 3 KEGG pathways most significantly perturbed by infection (**Figure 2F**). Ingenuity Pathway Analysis (IPA) also identified the "coagulation system" and "complement system" among the top 15 statistically significant canonical pathways altered by long-term *Listeria* infection (**Figure 2G**). Consistent with the fact that most complement proteins and several coagulation factors are acute phase proteins (APPs), the "acute phase response (APR) signaling" pathway was also significantly deregulated. It should be noted that in these pathways, the C1S complement component and SERPINA1 are encoded by a single gene in the human genome and multiple paralogs in the mouse genome. We thus included these two genes to the overlapping DEG datasets, leading to 71 genes (Huh7-

TABLE 1 | Seventeen genes are upregulated by long-term *Listeria* infection in all hepatocyte models.

Gene Symbol	Gene Description	log2 FC		
		HepG2	Huh7	PMH
CCL5	C-C motif chemokine ligand 5	8.4	4.0	5.3
CXCL10	C-X-C motif chemokine ligand 10	5.7	1.2	2.1
DDX60	DExD/H-box helicase 60	8.3	1.2	2.6
DHX58	DExH-box helicase 58	1.5	2.3	2.8
IFI44	interferon induced protein 44	10.7	5.4	4.1
IFIT2	interferon induced protein with tetratricopeptide repeats 2	9.1	0.8	0.9
ISG15	ISG15 ubiquitin like modifier	3.7	0.6	4.3
ISG20	interferon stimulated exonuclease gene 20	1.3	0.6	0.7
OAS2	2'-5'-oligoadenylate synthetase 2	8.7	4.2	2.7
OAS3	2'-5'-oligoadenylate synthetase 3	3.2	1.5	3.7
PMAIP1	phorbol-12-myristate-13-acetate-induced protein 1	2.1	1.6	0.6
RSAD2	radical S-adenosyl methionine domain containing 2	10.5	4.3	3.6
SHLD3	shieldin complex subunit 3	0.7	1.0	0.5
STAT1	signal transducer and activator of transcription 1	2.2	0.8	1.8
TRIM5	tripartite motif containing 5	0.7	0.7	0.8
ZC3HAV1	zinc finger CCCH-type containing, antiviral 1	1.2	0.5	0.7
ZNFX1	zinc finger NFX1-type containing 1	1.2	0.9	0.9

Bolded gene symbols indicate genes that are classified as ISGs. Gray boxes indicate a log2 FC greater than 2 (i.e., 4-fold) in infected cells compared with non-infected cells.

HepG2-PMH, **Supplementary Table S11A**), 380 genes (Huh7-HepG2, **Supplementary Table S11B**) and 362 genes (Huh7-PMH, **Table S11C**), whose under-expression could be used as markers associated with *Listeria* persistent infection in hepatocytes (**Supplementary Table S11**).

In summary, the transcriptional signature of long-term *Listeria* infection is characterized by the robust activation of interferon response genes in HepG2 and PMH cells, and the inhibition of APR genes in all three hepatocyte models, with the downregulation of a large number of genes involved in the complement and coagulation cascades.

Different Interferon Responses to *Listeria* Infection in HepG2, Huh7, and PMH Cells

The *Listeria*-induced interferon responses in HepG2 and PMH, strikingly absent in Huh7, prompted us to further examine the expression of IFN genes. We first analyzed the RNA-seq data to have information about the absolute expression levels (fragments per kilobase of transcript per million mapped reads, FPKM) of IFN transcripts coding for either type I IFNs (IFN-I: IFN-α subtypes, IFN-β, -ϵ, -κ, -δ, -ζ, -ω), type II IFN (IFN-II: IFN-γ), or type III IFNs (IFN-III: IFN-λ1, -λ2, -λ3). A threshold FPKM value of 0.3 was applied to define detectable expression above background, as previously described (Ramskold et al., 2009). No IFN gene transcripts were detected in non-infected samples of any of the three models, nor in infected Huh7 samples (**Figure 3A**). In contrast, 3-day *Listeria* infection, triggered the expression of *IFNL1* (coding IFN-λ1*)* and, to a lesser extent, *IFNB1* (coding IFN-β) and *IFNL2* (coding IFN-λ2) transcripts in HepG2 cells, while low levels of *IFNB1* transcripts were detected in infected PMH.

Reverse transcription-quantitative PCR (RT-qPCR) was then used to compare IFN gene expression at 72 h p.i. with that of earlier infection time points, in order to determine the time course and amplitude of IFN gene expression in infected hepatocytes. In HepG2 hepatocytes, *Listeria* infection induced *IFNL1* and *IFNB1* expression at 24 h p.i., and more significantly at 72 h p.i. (10-fold more for *IFNL1* and 4-fold more for *IFNB1)*, but not at 8 h p.i. (**Figure 3B**). In addition, *IFNL1* expression was approximately 20-fold higher than that of *IFNB1*. In Huh7 cells, consistent with FKPM values, *Listeria* infection did not induce expression of any of these genes at any time point. ELISA assays confirmed the major production of IFN-λ1 and minor production of IFN-β proteins in response to *Listeria* infection in HepG2, with highest amounts at 72 h p.i., and the absence of both IFNs in Huh7 (**Figure 3C**). In PMH, quantification of *Ifnb1, Ifnl2*, and *Ifnl3* transcript levels [murine *Ifnl1* is a pseudogene (Hermant et al., 2014)] showed that infection elicited the expression of only *Ifnb1*, which was 40 times higher at 24 h than at 72 h p.i. (**Figure 3D**). The expression profile of *MX1*, an ISG known to be induced by both IFN-I and IFN-III, followed the expression profile of IFN genes in HepG2 and PMH and was not significantly induced in Huh7-infected cells (**Figure 3E**). We conclude that HepG2 and PMH both induce IFN genes when exposed to *Listeria*, but exhibit differences, with an early activation of IFN-I in PMH and a late activation of IFN-I/III – essentially *IFNL1* – in HepG2 cells. We also observed the Huh7 cell line to be defective in the signaling pathway leading to IFN production in the context of *Listeria* infection, as previously noted (Odendall et al., 2014).

However, Huh7 cells are known to have functional IFN receptors and can thus mount effective interferon secondary responses (Bolen et al., 2014; Odendall et al., 2014). We investigated whether the important bacterial burden observed in Huh7 cells could be the result of their defect in IFN signaling, by restoring functional responses using recombinant IFNs. Huh7 cells were infected with the EGDe strain for 24 h and treated daily with either IFN-β (3 ng/ml; ~800 IU/ml) or IFN-λ1 (100 ng/ml) for an additional 48 h. IFN treatment activated the expression of ISGs (*IFIT1, IFI6* and *MX1*), but did not result

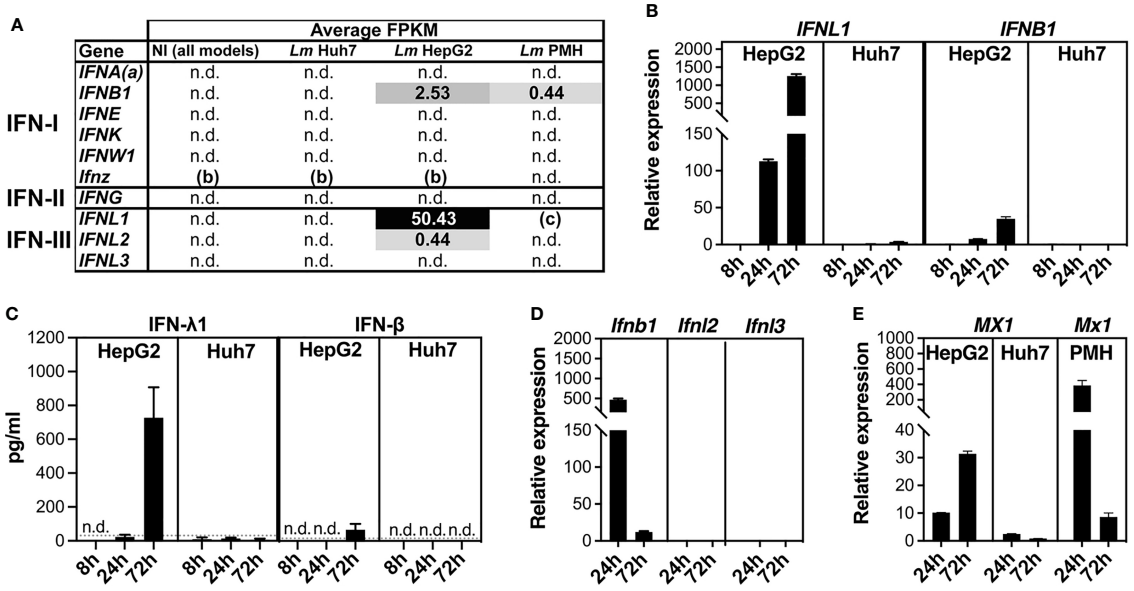

FIGURE 3 | *Listeria* infection triggers interferon responses in HepG2 and PMH, but not in Huh7 cells. **(A)** IFN gene expression in non-infected (NI) and infected (*Lm* EGDe or 10403S) HepG2, Huh7 and PMH cells at 72 h p.i., assessed by mean FPKM for each type I, II, or III IFN gene. (a) IFNA represents all genes coding for IFN-α subtypes; (b) *Ifnz* is present in the mouse but not the human genome; (c) *Ifnl1* is a pseudogene in the mouse genome. "n.d.", non detectable (FPKM < 0.3, the background level). **(B)** RT-qPCR analysis of *IFNL1* and *IFNB1* transcript levels in EGDe-infected HepG2 and Huh7 cells at 8 h, 24 h, and 72 h p.i., relative to NI cells, and normalized to *YWHAZ* and *PPIA* for HepG2 and Huh7, respectively. **(C)** ELISA quantification of IFN-λ1 (left) and IFN-β (right) protein secreted by EGDe-infected HepG2 or Huh7 cells. Conditioned media from EGDe-infected or NI cells was sampled at 8 h, 24 h, and 72 h p.i. Dotted grey lines: limit of quantification; "n.d.": below the limit of detection (LoD). All NI cell conditioned media sampled concurrently with infected samples were below the LoD (not shown). **(D)** RT-qPCR analysis of *Ifnb, Ifnl2* and *Ifnl3* transcript levels in 10403S-infected PMH at 24 h and 72 h p.i., expressed relative to NI cells, and normalized to *Pdk3* and *Pdk1* for 24 h and 72 h time points, respectively. **(E)** RT-qPCR analysis of *MX1* transcript levels in EGDe-infected HepG2 or Huh7 cells, and *Mx1* in 10403S-infected PMH, at 24 h and 72 h p.i., relative to NI cells, and normalized to *YWHAZ*, *PPIA*, and *Pdk3/Pdk1* for HepG2, Huh7, and PMH respectively. Values represent mean ± SD (n=3).

in a reduction in the number of intracellular bacteria at 72 h p.i. compared to untreated cells (**Supplementary Figure S3**).

Taken together, these results indicate that HepG2, Huh7 and PMH cells differ in their ability to mount interferon responses upon long-term *Listeria* infection, but interferon responses do not seem to affect the *Listeria* burden within hepatocytes.

Long-Term *Listeria* Infection in Hepatocytes Downregulates Genes of the Complement and Coagulation Cascades

Given the important roles of APP and other effectors of the complement and coagulation systems in immunity and host defense against pathogens (Merle et al., 2015; Antoniak, 2018; Ermert et al., 2019; Reis et al., 2019), dysregulation of these processes may have a strong impact on the outcome of *Listeria* infection. We thus explored further the nature of the downregulated DEGs associated with these innate immunity and hemostasis pathways. IPA and KEGG analysis, combined with a thorough literature search, identified 91, 44, and 43 APP genes which were downregulated by long-term *Listeria* infection in Huh7, HepG2, and PMH cells, respectively (**Figure 4A**), 19 of which were common to all three models. RT-qPCR was used to validate the downregulation of representative genes in HepG2, Huh7 cells (**Figure 4B**) and PMH (**Figure 4C**). The same downregulation was observed in Huh7 cells infected with *Listeria* strain 10403S or epidemic strain CLIP80459, compared to EGDe, demonstrating that the inhibitory effect was not strain-dependent (**Supplementary Figure S4**).

Downregulated APP genes unique to HepG2, Huh7 or PMH, or common to at least two models, were mapped onto the KEGG "complement and coagulation cascades" pathway (**Figure 5**). These complex cascades of tightly regulated proteolytic events act in crosstalk (Amara et al., 2008). The complement system can be activated by either the classical, lectin or the alternative pathway, which all converge to trigger the cleavage of a central component, the C3 protein and generate the same effector molecules. Strikingly, long-term infection with *Listeria* in Huh7 cells led to the downregulation of 30 genes, either involved in the complement cascade or acting as complement regulators (**Figure 4A**). Eight of these genes were similarly inhibited by *Listeria* infection in PMH: they encode (*i*) the activating enzyme C1S of the C1 complex, (*ii*) the central component C3, (*iii*) the membrane-attack proteins C6 and C8A, and (*iv*) four complement regulators (C1QTNF6, CFH, CFHR2, VTN) (**Figure 5**). Expression of genes encoding effectors of the blood coagulation and fibrinolysis systems were also altered by *Listeria* infection. The coagulation system includes the coagulation cascade and parallel regulatory

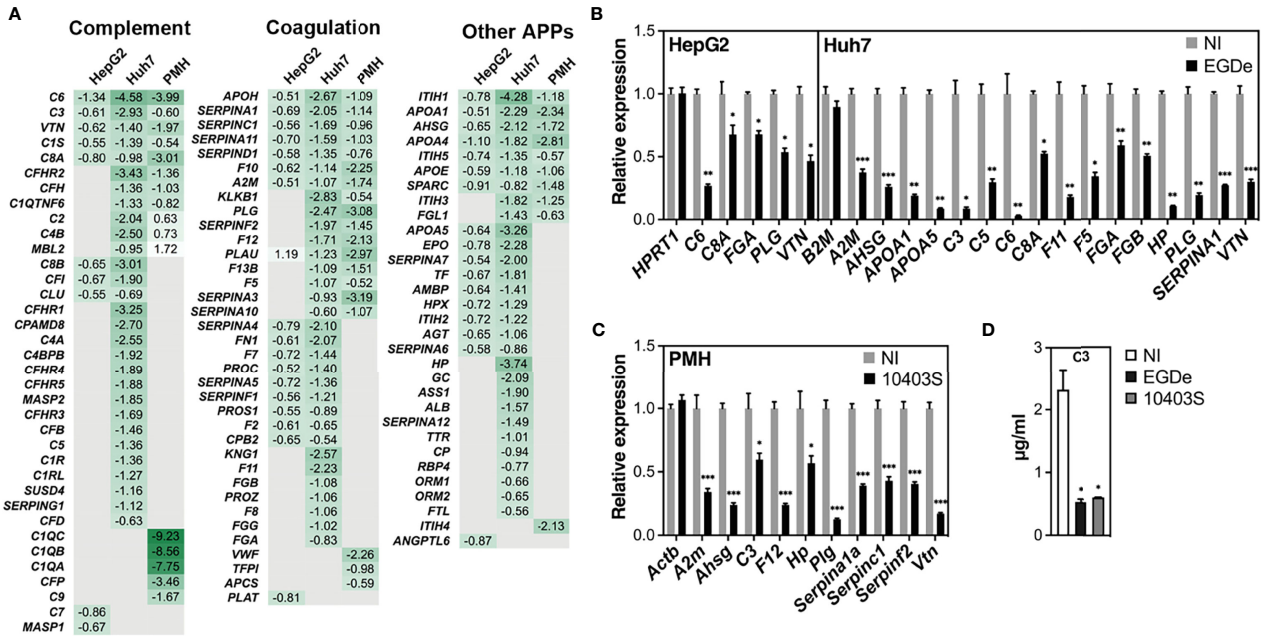

FIGURE 4 | Long-term *Listeria* infection downregulates expression of APP genes in hepatocytes. **(A)** Heatmap displaying log2 FC of APP genes significantly deregulated in infected cells compared to non-infected (NI) cells. APPs are classified according to their role in either the complement or coagulation systems or other functions. Gene symbols are indicated (corresponding gene names can be found in **Supplementary Tables S3, S5, S7**). Human *C1S* and *SERPINA1* have several murine orthologues; FC for murine *C1s1* and *Serpina1a* are shown. **(B, C)** RT-qPCR analysis of representative genes in **(B)** EGDe-infected HepG2 or Huh7 cells, and **(C)** 10403S-infected PMH, relative to NI cells. A non-differentially regulated control gene (*HPRT1* for Huh7, *B2M* for HepG2, and *Actb* for PMH) is included for each set. **(D)** C3 protein concentration in the conditioned media of NI or infected Huh7 cells at 72 h p.i. was measured by ELISA. All values represent mean ± SD (n=3). Statistical significance, Student's *t* test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

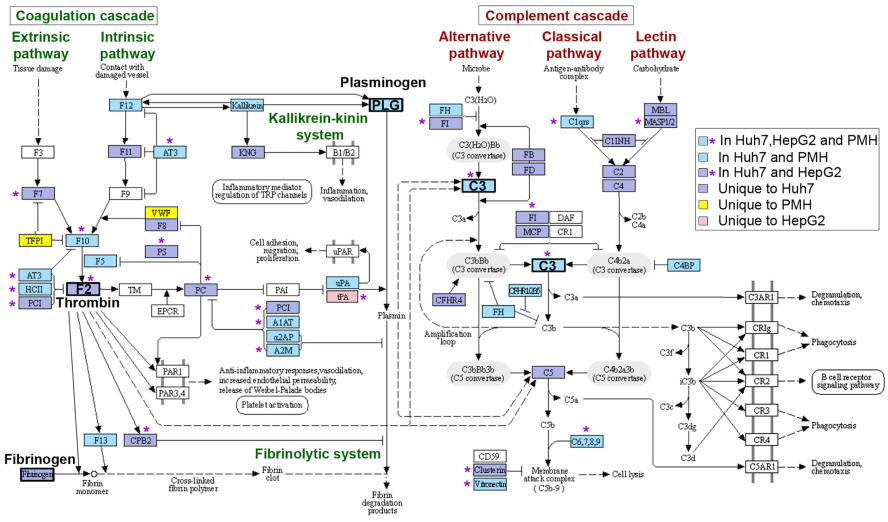

FIGURE 5 | Mapping of APP genes downregulated upon long-term *Listeria* infection on the "complement and coagulation cascade" KEGG pathway. Colored boxes and purple stars indicate components of the cascades specifically altered in three, two or one hepatocyte model (as shown by the legend on the right). The hsa04610 KEGG pathway was adapted to underline different pathways of the coagulation cascade and fibrinolytic system (left) or complement cascade (right). Key components are highlighted in bold: Thrombin (*F2*), Fibrinogen (*FGA, FGB, FGG*), Plasminogen (*PLG*) and complement C3 (*C3*).

systems that lead to a complex interplay of reactions resulting in the conversion of soluble fibrinogen to insoluble fibrin strands. The coagulation cascade is traditionally classified into the intrinsic and extrinsic pathways, both of which converge on Factor X activation, generation of thrombin, and subsequently fibrin to form fibrin clots. The fibrinolytic system (or plasminogen-plasmin system) is required for fibrin degradation and blood clot dissolution (**Figure 5**). Genes belonging either to extrinsic and intrinsic coagulation cascade, kallikrein-kinin system, fibrinolytic system and regulatory pathways, were widely downregulated upon long-term *Listeria* infection in Huh7 and PMH cells (32 and 19 DEGs, respectively), 16 of which were common to both hepatocyte models (**Figure 4A**). In particular, infection led to the repression of genes coding for (*i*) the coagulation factors F12, F10, F5, F13, and thrombin (F2), the enzyme responsible for the conversion of fibrinogen to fibrin, (*ii*) Kallikrein B1 (KLKB1) of the kallikrein-kinin system, a metabolic cascade resulting in the release of vasoactive kinins, (*iii*) plasminogen (PLG) and urokinase plasminogen activator (PLAU or u-PA) in the fibrinolytic system, (*iv*) a set of serpin proteases with regulatory functions (SERPINA1/α1-antitrypsin, SERPINA3/α1-antichymotrypsin, SERPINA10/Protein Z-dependent protease inhibitor, SERPINA11, SERPINC1/antithrombin, SERPIND1/heparin cofactor II, SERPINF2/α2-antiplasmin) and (*v*) other regulators (A2M/α2 macroglobulin, APOH/apolipoprotein H) (**Figure 5**). APP genes involved in other various biological processes were also downregulated in infected hepatocytes. In particular, common to Huh7 and PMH cells were 10 APPs contributing to the regulation of lipid metabolism (AHSG/Fetuin-A, APOA1, APOA4, APOA5, APOE, SPARC and ITIH5) or other functions (ITIH1, ITIH3, FGL1) (**Figure 4A**). The downregulation of complement and coagulation gene expression in infected hepatocytes compared to the basal level of expression in non-infected cells reflects the capacity of long-term *Listeria* infection to modify the hepatic innate immune landscape.

Long-Term *Listeria* Infection Reduces the Amount of Secreted APPs

We next examined whether the decrease in APP mRNA levels induced by long-term *Listeria* infection translated into a reduced abundance of secreted APPs. We first quantified the concentration of the C3 protein in cellular supernatants by ELISA. In agreement with mRNA expression data, a significant reduction in total C3 levels was observed in the conditioned media recovered from infected Huh7 and HepG2 cells, compared to non-infected cells (**Figure 4D**). We then performed a label-free MS quantitative proteomic analysis to explore the composition of the secretome of infected cells. The quantification of proteins in the conditioned media of long-term infected HepG2 or Huh7 cells compared to that of non-infected cells identified a significant decrease in the abundance of 42 and 153 proteins, respectively. Intersecting the secretome of each cell line with its corresponding transcriptome revealed a strong overlap, with 25 and 82 proteins that were downregulated at both the proteomic and transcriptomic level in HepG2 and Huh7, respectively (**Supplementary Table S12**), of which more than half were APPs (**Table 2**). These results suggest that long-term *Listeria* infection in hepatocytes alters APP secretion at a transcriptional level.

Long-Term *Listeria* Infection Impairs the Cytokine-Driven Expression of APP Genes

Given their important physiological functions, most APPs are produced at a basal constitutive level by hepatocytes. However, their amounts rapidly change in response to pro-inflammatory cytokines released by innate immune cells during the course of an infection, with interleukin-6 (IL-6) and interleukin-1 (IL-1)-type cytokines as the leading inducers (Zhou et al., 2016). As long-term *Listeria* infection reduced basal levels of APP gene expression, we sought to evaluate the effect of long-term *Listeria* infection on cytokine-induced APP gene expression. Since IL-6 and IL-1β produce selective gene induction, we first applied these cytokines to non-infected Huh7 cells, for 24 h, to identify representative IL-6- and IL-1β-specific APPs [also known as type I and type II APPs, respectively (Baumann and Gauldie, 1994)]. IL-6 induced the expression of *C5*, *C6*, *C8A*, *FGA*, *FGB* and *HP* genes, while IL-1β induced *C3*, *F11* and *HP* genes (**Figure 6A**). We subsequently examined the effect of *Listeria* infection on the cytokine-induced expression of these genes. Our results showed that upon inflammatory cytokine stimulation, the mRNA abundance of APPs was strongly reduced in Huh7-infected cells, when compared to non-infected cells, indicating that infection inhibits the inflammatory signaling pathway (**Figure 6B**). We confirmed this effect in HepG2 cells for the APPs *C6* and *FGA*, following stimulation with IL-6 (**Figure 6C**). These results reveal that long-term *Listeria* infection not only represses the constitutive expression of APP genes, but also counteracts the transcriptional stimulation induced by inflammatory stimuli.

Long-Term *Listeria* Infection Downregulates Genes of LXR-RXR, FXR-RXR, and Cholesterol Metabolism-Associated Pathways

IPA analysis of genes which were downregulated after 3-day *Listeria* infection, either in both human hepatocyte models, or both human Huh7 and PMH models, also identified pathways associated with ligand-dependent nuclear receptors liver X receptor/retinoid X receptor (LXR/RXR) and farnesoid X receptor/retinoid X receptor (FXR/RXR) (**Figure 2G**). LXR and FXR play a critical role in the regulation of metabolism, particularly cholesterol, fatty acid, bile acid and carbohydrate metabolism (Ding et al., 2014). Of note, several genes associated with these pathways encoded APPs (e.g., *AHSG, APOA1, APOA4, APOC1, APOC3, APOE, APOH* and *SERPINF2*), in line with the notion that LXR and FXR act in crosstalk with other transcription factors to control expression of a set of APP genes (Odom et al., 2004; Wollam and Antebi, 2011; Yin et al., 2011). This was confirmed at the protein level, as infection decreased the amount of secreted AHSG and apolipoproteins APOA1, APOE and APOH, in both HepG2 and Huh7 conditioned media, as well as of APOB, APOC3 and SERPINF2 in Huh7 (**Supplementary Table S12**). In addition, genes involved

TABLE 2 | Long-term *Listeria* infection decreases the abundance of APP in the secretome of HepG2 and Huh7 hepatocytes.

Uniprot accession	Gene symbol	Protein name	HepG2		Huh7 (a)	
			log2 Ratio	*p*-adj	log2 Ratio	*p*-adj
Complement						
P01024	C3	complement C3	-0.77	3.8E-19	-3.25	8.8E-59
P01031	C5	complement C5			-2.58	1.1E-16
P0C0L4	C4A	complement C4A			<-4.00	7.7E-15
P0C0L5	C4B	complement C4B			<-4.00	7.7E-15
P00751	CFB	complement factor B			-3.29	1.8E-09
P04004	VTN	vitronectin	-1.32	6.4E-03	-2.03	2.7E-06
P05155	SERPING1	C1-inhibitor			-4.09	1.6E-04
P06681	C2	complement C2			<-1.87	3.9E-04
P08603	CFH	complement factor H			-2.70	7.1E-18
Q03591	CFHR1	complement factor H related 1			<-2.74	1.3E-06
P10909	CLU	clusterin	-0.98	8.5E-03	-1.37	2.7E-04
P05156	CFI	complement factor I	-1.00	3.0E-03	<-1.87	3.9E-04
Coagulation						
P02751	FN1	fibronectin 1			-1.97	2.4E-28
P01023	A2M	alpha-2-macroglobulin	-0.90	1.7E-19	-2.08	8.8E-24
P00747	PLG	plasminogen			-4.55	1.9E-22
P01009	SERPINA1	alpha-1-antitryppsin	-0.80	4.1E-07	-2.06	5.2E-17
P01042	KNG1	kininogen 1			-3.91	5.5E-10
P02749	APOH	apolipoprotein H	-0.75	2.8E-03	-3.58	5.9E-10
P36955	SERPINF1	pigment epithelium-derived factor	-0.95	9.2E-06	-2.27	1.2E-07
P08697	SERPINF2	alpha-2-antiplasmin			-1.93	2.6E-06
P01008	SERPINC1	antithrombin			-1.97	6.2E-06
P05154	SERPINA5	plasminogen activator inhibitor-3	-0.98	9.3E-03	-2.37	8.8E-05
P05546	SERPIND1	heparin cofactor 2	-0.95	9.0E-03	-2.04	9.5E-05
P29622	SERPINA4	kallikrein inhibitor			<-1.58	1.5E-03
Q86U17	SERPINA11	serpin family A member 11	-1.23	2.4E-03	<-1.00	8.7E-03
P00748	F12	coagulation factor XII			-4.17	8.8E-05
P00734	F2	coagulation factor II, thrombin			-1.45	2.9E-04
P12259	F5	coagulation factor V			-3.91	5.0E-04
P02675	FGB	fibrinogen beta chain			-1.58	2.2E-03
Other APPs						
P02787	TF	transferrin	-0.65	7.0E-10	-1.43	1.6E-30
P02768	ALB	albumin			-1.22	2.4E-25
P02647	APOA1	apolipoprotein A1	-0.98	1.9E-06	-3.50	3.2E-22
P02760	AMBP	alpha-1-microglobulin/bikunin precursor	-0.89	2.4E-05	-2.12	1.4E-13
P02774	GC	GC, vitamin D binding protein			-5.09	4.8E-09
P19823	ITIH2	inter-alpha-trypsin inhibitor heavy chain 2	-0.86	4.4E-08	-1.15	1.4E-07
P01019	AGT	angiotensinogen			-1.76	2.2E-07
P02649	APOE	apolipoprotein E	-0.67	1.9E-03	-1.20	8.5E-07
P02765	AHSG	alpha 2-HS glycoprotein	-0.62	2.8E-03	-1.28	1.1E-05
P02753	RBP4	retinol binding protein 4			-1.24	8.0E-05
P00738	HP	haptoglobin			<-1.42	2.7E-03
P00450	CP	ceruloplasmin			-3.49	2.9E-11
Q08830	FGL1	fibrinogen like 1			-3.00	1.5E-03

A label-free quantitative proteomic approach was used to identify proteins differentially abundant in the conditioned medium of infected (I) compared to non-infected (NI) hepatocytes in 3 independent experiments. The relative abundance of individual proteins was assessed by significant differences in spectral counts (SC) between the two conditions (p-adj <0.01, see ***Supplementary Material****). Proteins with significant decreases, expressed as log2 (I/NI) SC ratio, were then selected on the basis of a decrease in RNA level of the corresponding gene in the transcriptome dataset, resulting in the identification of 25 and 82 proteins in HepG2 and HuH7, respectively (see* ***Supplementary Table S12****), of which 17 and 41 were APPs. (a) For eight proteins with SC=0 in the Huh7 infected condition, an arbitrary value of SC=1 was used to allow the calculation of a log2 ratio (in this case, the value is preceded by "<").*

in bile acid and cholesterol biosynthesis pathways, namely bile acid-CoA:amino acid N-acyltransferase (*BAAT*) and 3-hydroxy-3-methylglutaryl-CoA synthase 2 (*HMGCS2*), respectively, and several apolipoprotein genes (*APOA1, APOA4, APOC1, APOE, APOH*) were downregulated PMH as in Huh7 cells (**Supplementary Table S11**). It was particularly striking that cholesterol metabolism-related pathways were the most highly significantly altered pathways in HepG2 and Huh7 transcriptome datasets, as shown by KEGG (**Figure 3G**) and IPA enrichment analysis (**Figure 3F**). RT-qPCR validated the downregulation of representative genes (**Figure 7A**). As for APP genes, this inhibitory effect was not strain-dependent (**Supplementary Figure S4**). In both HepG2 and HuH7 hepatocytes, nearly all the genes of the cholesterol biosynthesis pathway were downregulated, including the genes encoding the rate limiting enzymes 3-hydroxy-3-methylglutaryl coenzyme A reductase (*HMGCR*), squalene epoxidase (*SQLE*), and lanosterol synthase (*LSS*) (**Figures 7B, C).** In Huh7, several genes of the classical bile acid (BA) synthesis

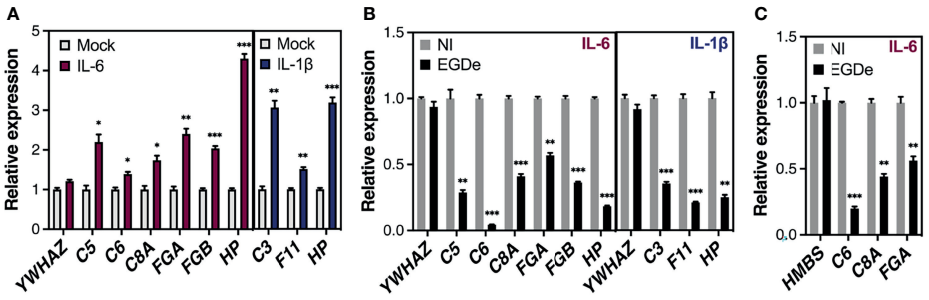

FIGURE 6 | Long-term *Listeria* infection reduces cytokine-driven expression of APP genes in human hepatocytes. **(A)** Huh7 cells were stimulated with IL-6 (50 ng/mL) or IL-1β (10 ng/mL) for 24 h. Gene expression was analyzed by RT-qPCR and is expressed as fold-induction relative to mock-stimulated cells; IL-6 induced the expression of *C5*, *C6*, *C8A, FGA*, *FGB* and *HP* genes, while IL-1β induced *C3*, *F11* and *HP* genes. **(B, C)** Effect of infection on cytokine-induced APP gene expression in Huh7 **(B)** and HepG2 cells **(C).** Cells were infected with strain EGDe (MOI~1-5) for 48 h or remained non infected (NI). IL-6 (50 ng/mL) or IL-1β (10 ng/mL) (Huh7) or IL-6 (25 ng/mL) (HepG2) was added to infected and NI cells and transcript levels relative to stimulated NI cells were quantified by RT-qPCR 24 h later (at 72 h p.i., i.e. 24 h post-stimulation). A control gene whose expression was affected neither by infection nor cytokine stimulation is included. **(B)** In Huh7 cells, the control gene was *YWHAZ* and gene expression was normalized to *PPIA*; **(C)** In HepG2 cells, the control gene was *HMBS* and gene expression was normalized to *YWHAZ*. All values represent mean ± SD (n=3). Statistical significance, Student's *t* test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

pathway were also downregulated, including the rate limiting enzyme cytochrome P450 family 7 subfamily A member 1 (*CYP7A1*). Together, these results suggest that long-term *Listeria* infection disturbs the expression of cholesterogenic-, lipogenic- and bile acid-associated genes.

Upstream Regulator Analysis of Gene Networks Perturbed by Long-Term *Listeria* Infection

To propose mechanistic hypotheses that could explain the inhibitory effect of *Listeria* infection on APP and lipid metabolism gene expression we used the Ingenuity Upstream Regulator Analysis (URA) analytical algorithm to predict host transcriptional regulators targeted by infection. This analysis identified several transcription factors (TFs), ligand-dependent nuclear receptors, as well as epigenetic factors, as possible regulators underlying infection-orchestrated transcriptional modulation (**Supplementary Table S13**). URA performed on the network of genes that were either deregulated by infection in Huh7 and PMH models, or in human Huh7 and HepG2 models, identified extensive overlap between the transcriptional regulators coordinating the observed patterns of gene dysregulation: the hepatocyte nuclear factors HNF1α and HNF4α, the ligand-dependent nuclear receptors PPARα, LXRβ, RXRα and RORC, and P53. The Huh7 and PMH models also revealed the possible involvement of CEBPA (CCAAT/enhancer binding protein alpha), a TF known to regulate APP expression with hepatocyte nuclear factors, as well as the epigenetic regulators EZH2, EP300, and SMARCB1. The sterol regulatory element-related transcription factors SREBF1 (or SREBP1) and SREBF2 (or SREBP2), on the other hand, were specific to Huh7 and HepG2 models, consistent with their function as master transcriptional regulators of cholesterol homeostasis (Shimano and Sato, 2017). Interestingly, the epigenetic regulator SIRT2, previously associated with *Listeria* infection (Eskandarian et al., 2013), was predicted to regulate the expression of ten infection-dysregulated genes involved in the cholesterol biosynthetic pathway (*ACLY, DHCR7, FDFT1, HMGCR, IDI1, LSS, MVD, MVK, SC5D, SQLE*) (**Supplementary Table S13**) (**Figure 7B**). Together, these analyses provide insights into transcriptional regulators that may orchestrate gene dysregulation mediated by long-term *Listeria* infection of hepatocytes and impact the intracellular persistence phase.

DISCUSSION

The ability of intracellular bacterial pathogens to hide long-term in host cells plays a key role in chronic or asymptomatic infections. However, the molecular mechanisms of this persistence are less well understood than those of virulence. Especially for *Listeria*, the concept of intracellular persistence is recent (Bierne et al., 2018) and its potential impact on host defenses remains unknown. Here, we established three cellular infection systems in human and murine hepatocytes to study how the host cell transcriptional landscape is remodeled once the bacteria enter a persistence stage in LisCVs. Convergent results indicate that this stage coincides with a profound deregulation of hepatic innate immune response genes, in particular a strong activation of the IFN response and repression of APR genes. We speculate that this immune deregulation may be propitious for asymptomatic *Listeria* carriage in the host (**Figure 8**).

Hepatocyte Models to Study *Listeria* Persistent Infection

A number of studies have analyzed the response of mammalian cells infected with *Listeria* using transcriptomic approaches (**Supplementary Table S14**). Some have used cultured cells of myeloid, endothelial, or epithelial origin, but during a short time of infection, when the bacteria are in the active phase of replication and/or dissemination (i.e., 24 h or less). Others have used organ biopsies after infection in mice, thus revealing a response arising from different cell types. In particular, in transcriptomic analyses of *Listeria* infection in the mouse liver (**Supplementary Table S14**),

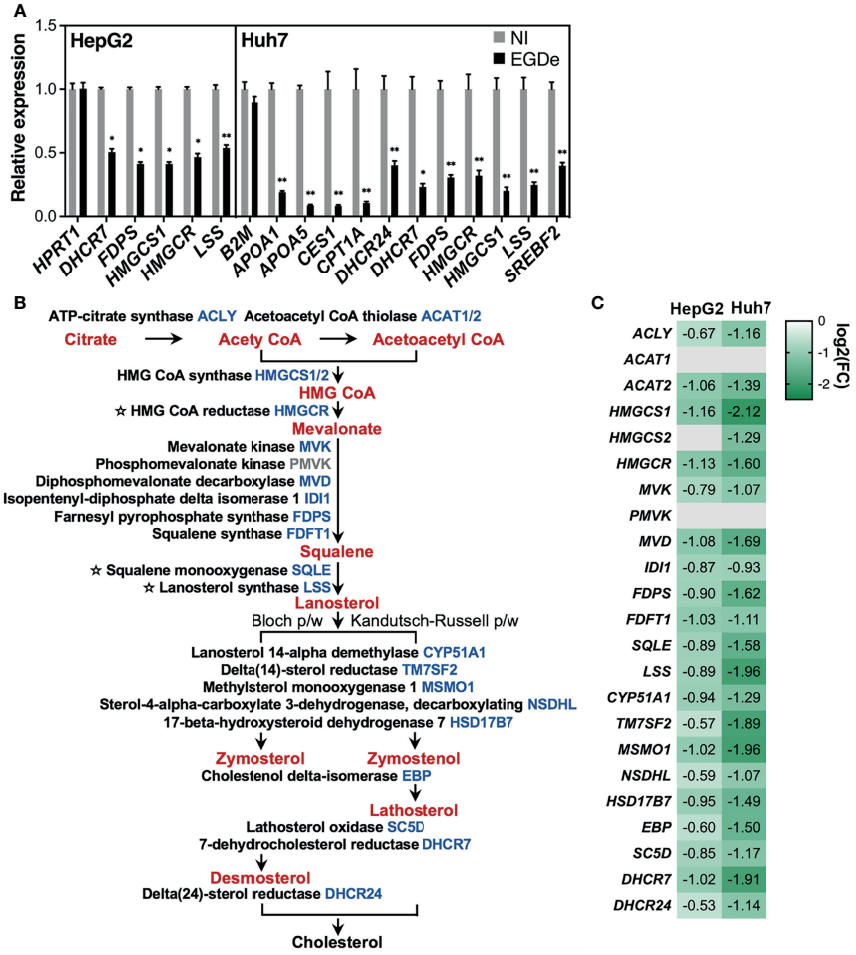

FIGURE 7 | Long-term *Listeria* infection downregulates almost all genes of the cholesterol biosynthesis pathway in both human HepG2 and Huh7 cells. **(A)** RT-qPCR analysis of transcript levels of representative genes involved in cholesterol metabolism and homeostasis in EGDe-infected HepG2 and Huh7 cells, relative to non-infected (NI) cells. Expression was normalized to *YWHAZ* or *PPIA* for HepG2 and Huh7 cells, respectively, and a non-differentially expressed control gene (*HPRT1* for HepG2, *B2M* for Huh7) is included. Values represent mean ± SD (n=3). Statistical significance, Student's *t* test (*$p < 0.05$, **$p < 0.01$). **(B)** Diagram illustrating the *de novo* cholesterol biosynthesis pathway. Enzyme names are in black and their corresponding gene symbols are blue when downregulated in infected cells, otherwise they are gray. Rate limiting enzymes are preceded by a star. Intermediary molecules are shown in red. Lanosterol can be transformed into cholesterol *via* two different routes: the Bloch pathway (left) and the Kandutsch-Russell pathway (right). **(C)** Heat map showing the log2 FC obtained by RNA-seq in HepG2 and Huh7 infected versus NI cells, for each of the cholesterol synthesis enzyme coding genes listed in **(B)**.

the specific contribution of hepatocytes versus immune cells was not determined. In addition, while these studies have proven extremely valuable in defining *Listeria* virulence and host immune responses during acute infection, they did not reveal strategies that might allow this pathogen to maintain long-term infection without causing symptomatic disease. A major challenge in studying persistent bacteria is due to the fact that slow-growing bacteria could be rare within tissues and thus difficult to detect and study *in vivo*. It is therefore crucial to develop efficient *in vitro* culture systems to model persistent infection. We show here that HepG2, Huh7 and PMH are good models to achieve this goal, as bacteria can efficiently replicate, disseminate, and finally be captured inside LisCVs, without causing major cytotoxicity. This latter observation is consistent with *in vivo* studies showing that *Listeria* can replicate extensively in hepatocytes without causing major cell death, when neutrophil recruitment to the sites of infection in the liver is inhibited (Conlan and North, 1991; Appelberg and Leal, 2000). We exploited these *in vitro* models in order to identify, for the first time, a "meta-signature" associated with long-term *Listeria* infection in hepatocytes.

The Intracellular Persistence Stage of *Listeria* in Hepatocytes Coincides With Inhibition of Complement and Coagulation Gene Expression

Hepatocytes play an important role in fighting bacterial infections, particularly as they are the primary source of many APPs, which they constitutively produce and overproduce in response to

infection (Zhou et al., 2016). For example, complement C3 and fibrinogen (FBN), the central compounds of the complement and coagulation systems, are APPs constitutively and almost exclusively produced by hepatocytes, and their serum levels are significantly increased during the APR (Zhou et al., 2016). Hepatocytes produce other complement and coagulation proteins that, like C3 and FBN, contribute to pathogen clearance, activation of innate immune cells, and enhancement of the adaptive immune response (Amara et al., 2008; Merle et al., 2015; Reis et al., 2019). It is therefore not surprising that pathogenic bacteria have evolved various mechanisms to evade the complement and coagulation systems (Hovingh et al., 2016; Antoniak, 2018; Ermert et al., 2019). However, while known mechanisms primarily involve bacterial protein effectors (e.g., capsules, proteases, inhibitors), mechanisms involving transcriptional repression are essentially undocumented. There is a plethora of data showing that systemic bacterial infections induce APP gene expression, and this knowledge has long been exploited for diagnostic purposes, as serum APP levels can be used as prognostic markers (Markanday, 2015). In contrast, to our knowledge, data regarding the impact of persistent bacterial infections on APP gene expression are scarce. In a recent study involving *Mycobacterium tuberculosis* (Mtb), a paradigm for asymptomatic persistent infections, the blood plasma proteome of healthy subjects was compared to those with active tuberculosis (TB) or latent tuberculosis (LTBI). This revealed that the amount of several APPs decreased in the plasma of LTBI cases compared to TB cases or even healthy controls (Teklu et al., 2020), suggesting that in asymptomatic Mtb carriers the expression of some APPs is not induced or is reduced relative to basal levels, and that APP levels could be used to predict the risk of TB reactivation. Interestingly, decreased APP expression has been associated with recurrent bacterial infections. For example, C3 levels in women with recurrent urinary tract infections have been shown to be lower than in healthy women (Syukri et al., 2015). In addition, mutations that cause complement deficiencies predispose individuals to recurrent infections (e.g., by *Streptococcus pneumoniae, Neisseria meningitidis and Haemophilus influenza*e) (Ram et al., 2010). Single-nucleotide polymorphisms (SNPs) in complement genes have also been linked, through genome-wide association studies (GWAS), to disease susceptibility and outcome of bacterial infections (van den Broek et al., 2020).

Here we show that long-term *Listeria* infection in hepatocytes can have a profound impact on both the constitutive and cytokine stimulated expression of APP genes. Several studies have demonstrated the importance of the complement system (Calame et al., 2016) and the coagulation system (Davies et al., 1981; Mullarky et al., 2005; Nishanth et al., 2013) in protecting the host from *Listeria*. The transcriptional inhibition observed here could therefore be a previously unappreciated effect induced by the pathogen to enhance its long-term survival. Among targeted APPs, the decrease in the amount of C3 protein in the secretome of *Listeria*-infected hepatocytes, compared to non-infected hepatocytes, is particularly striking, given the critical role of C3 in the response to this bacterial infection (Calame et al., 2016). For instance, blocking the functional activity of C3 through the use of an anti-CR3 antibody inhibits neutrophil recruitment to *Listeria* infection foci and reduces liver tissue damage (Conlan and North, 1991; Appelberg and Leal, 2000). In addition, through activation of its receptors CR3 and CRIg, C3 contributes to phagocytosis and elimination of *Listeria* by macrophages (Calame et al., 2016). C3 also plays a role in adaptive immunity, being required for efficient T cell activation during murine listeriosis (Nakayama et al., 2009; Verschoor et al., 2011; Tan et al., 2014). Beyond its role on immune cells, C3 could also counteract bacterial infection within hepatocytes. Indeed, an intracellular activity of complement proteins [known as "complosome" (Arbore et al., 2017)] was recently identified and, interestingly, a study showed that coating *Listeria* with C3 induces autophagy in epithelial cells (Sorbara et al., 2018). Since C3 is abundantly produced by hepatocytes, it is possible that the intracellular C3 form targets *Listeria* within this cell type. Blocking C3 gene expression, while sheltering in the LisCV niche, could be a strategy employed by *Listeria* to avoid autophagy and persist in the hepatocyte. Thus, inhibition of C3 production could promote bacterial persistence by several means, such as preservation of the hepatocyte niche and limitation of intracellular bacterial destruction. With respect to other APPs, it is worth mentioning the impaired innate response to *Listeria* infection in *ApoE*-deficient mice, as another example of how a decrease in APP expression could impact the host response to this pathogen (Roselaar and Daugherty, 1998).

Inhibiting a network of APP genes, rather than individual APPs, is an effective strategy to concomitantly target multiple host defenses. To our knowledge, this has never been described before for an intracellular bacterium. *Listeria* infection represses the constitutive expression of APP genes in hepatocytes and, importantly, this repression is maintained under conditions of inflammation, after stimulation with the pro-inflammatory cytokines IL-6 and IL-1β. These results suggests that long-term *Listeria* infection could impose a transcriptional block, desensitizing APP gene expression from inflammatory stimuli. However, it is known that acute infection with *Listeria* in mice stimulates the production by immune cells of these pro-inflammatory cytokines, which in turn stimulate APP expression in hepatocytes (Kopf et al., 1994). Notably, one study showed that after intravenous inoculation of mice with a sublethal dose of *Listeria*, a homogeneous infection of mouse livers was observed on day 1, followed by a peak in bacterial loads on day 3 and a highly significant reduction on day 9 p.i., representing the expected process of bacterial clearance (Kummer et al., 2016). At the same time, IL-6 and IL-1β mRNA levels increased in the infected livers on day 1 and remained elevated on day 3, followed by a sharp decrease on day 9 p.i. Consecutively to the induction of pro-inflammatory cytokines, the amount of many APPs increased on day 1, and especially on day 3, and then dropped to an nonsignificant level on day 9 p.i. in infected livers compared with uninfected controls (Kummer et al., 2016). We propose a model that combines these results with our own (**Figure 8**). On the one hand, acute infection triggers a major defensive inflammatory response in the host, including expression of APPs, leading to the elimination of the majority of bacteria and

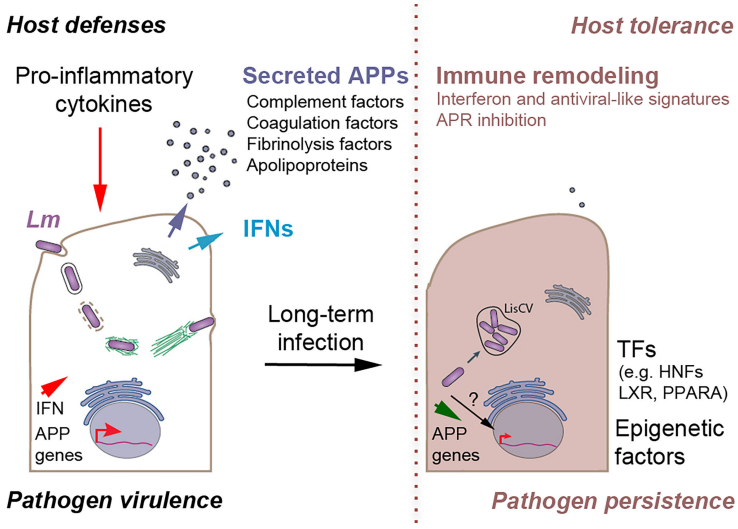

FIGURE 8 | Proposed model of *Listeria*-mediated immune remodeling to promote persistent infection in hepatocytes. *Acute infection* (left). *L. monocytogenes (Lm)* invades hepatocytes (through the InlA/B-mediated entry pathways or through cell-to-cell spread from infected macrophages, not represented). During the active phases of the infection process, *Lm* produces virulence factors that enable bacteria to enter into the cytosol and spread from cell-to-cell by the ActA-mediated actin-motility process. Hepatocytes detect intracellular *Lm* and secrete type I/III interferons (IFNs) and other cytokines (not represented), stimulating immune cells. In turn, immune cells secrete pro-inflammatory cytokines, such as IL-6 and IL-1β, which activate the expression of genes encoding acute phase proteins (APP) in hepatocytes. Secreted APPs, which include complement, coagulation and fibrinolytic factors and a set of apolipoproteins, have antimicrobial and/or immunomodulatory effects that contribute to the clearance of *Listeria* infection. *Persistent infection* (right). Long-term *Lm* infection exacerbates interferon and anti-viral like responses and imposes a block on APP gene expression in hepatocytes, by an unknown mechanism possibly involving the inhibition of transcription factors (e.g. hepatocyte nuclear factors (HNFs), LXR, PPARA) and epigenetic regulators, leading to the decrease in APP secretion and inhibition of the APR. Concomitantly, bacteria stop expressing ActA and are engulfed in membrane compartments to form *Listeria*-containing vacuoles (LisCV), where they enter a persistent non-replicative phase. The deregulation of the hepatocyte-specific innate immune responses prevents the complete elimination of *Listeria* in the liver, favoring the creation of a long-term reservoir of intracellular persistent bacteria. In addition, it could modulate adaptive immunity to favor the tolerance of cells harboring dormant pathogens.

infected hepatocytes. On the other hand, bacteria infecting hepatocytes control the expression, and thus the secretion, of APPs, which could counteract the massive activation of the APR and limit liver damage, while attenuating the immune response. We suggest that this process may prevent the complete elimination of *Listeria* in the liver, favoring the creation of a long-term reservoir of a small bacterial subpopulation that has entered a dormant state in LisCVs. Moreover, inhibition of APP gene expression could have profound implications for the development of effective adaptive immunity. Given the fact that the liver is a naturally immunotolerant organ (Zheng and Tian, 2019), downregulation of APPs could enhance host tolerance to *Listeria* persistent forms in asymptomatic carriers.

The mechanisms by which *Listeria* interferes with APP gene expression remain to be elucidated, but avenues of research exist based on the acquired knowledge of how this bacterium modulates host gene expression, e.g., by interfering with the signaling pathways that control TFs, impacting their stability or nuclear localization, or by acting on epigenetic regulators thus controlling the chromatin structure at target genes (Bierne and Hamon, 2020; Eldridge et al., 2020). Among possible hypotheses, *Listeria* could alter the functions of TFs essential for constitutive expression of APPs, such as hepatocyte nuclear factors (HNFs) HNF1α and HNF4α (Zhou et al., 2016). HNFs are particularly interesting candidates since they play a central role in the liver-specific transcriptional program. They coordinate the effect of various signaling pathways to fine-tune the expression of many liver genes (Odom et al., 2004), including those shown here to be downregulated by infection and involved in the complement and coagulation cascades or other facets of the APR (Zhou et al., 2016).

The Intracellular Persistence Stage of *Listeria* in Human Hepatocytes Coincides With Inhibition of Cholesterol Metabolism Genes

HNFs regulate hepatic gene expression in concert with other ligand-dependent nuclear receptors such as LXR, FXR and PPARA that regulate lipid and bile acid metabolism (Shih et al., 2001; Odom et al., 2004; Wollam and Antebi, 2011; Yin et al., 2011). HNF4α and LXR, in turn, regulate the activity of SREBPs (Shimano and Sato, 2017), and are thus directly implicated in the regulation of cholesterol homeostasis. In Huh7 and HepG2 human hepatocytes, the pathway the most significantly inhibited by infection was the cholesterol biosynthesis gene network, with almost all genes of the pathway downregulated in both human hepatocyte models (**Figure 7**). The role of cholesterol metabolic reprogramming in persistent *Listeria* infection of epithelial cells deserves further investigation, given the role of cholesterol in the establishment of

vacuolar niches for intracellular pathogens (Samanta et al., 2017) and of oxysterols in host immune functions (Cyster et al., 2014; Abrams et al., 2020). In addition, controlling cholesterol metabolism plays a key role in *Mtb* and *Helicobacter pylori* persistent infections (Pandey and Sassetti, 2008; Morey and Meyer, 2019). In PMH, long-term *Listeria* infection was not associated with the cholesterol biosynthesis pathway. Growth conditions, metabolic disparity between primary cell and carcinoma-derived cell lines and, importantly, species specificity, could account for the differences in the infection-associated transcriptional program between human hepatoma cell lines and PMH. However, it should be noted that some genes involved in cholesterol metabolism were also inhibited by infection in PMH, such as *Hmgcs2* and several target genes of LXR and FXR, including the cholesterol transporter gene *Abcg1*, the lipoprotein lipase gene *Lpl*, and several apolipoprotein genes, like in human hepatocytes.

Interferon and Antiviral-Like Signatures of Long-Term *Listeria* Infection in the Hepatic Transcriptome

IFN-I and/or IFN-III expression is a hallmark of the epithelial cell response to viral or bacterial invasion, including by *Listeria* (Bierne et al., 2012; Dussurget et al., 2014). Here we show that human HepG2 hepatocytes respond to intracellular *Listeria* infection with significant IFN-III production, in agreement with our previous results (Bierne et al., 2012). PMH cells, on the other hand, respond with IFN-I expression. These differences are consistent with the species-specific expression of IFN-I and IFN-III genes in the liver, as well as of their specific receptors. Human hepatocytes express high levels of IFN-III and respond strongly to stimulation by this IFN, whereas murine hepatocytes do not respond to IFN-III and preferentially express IFN-I (Nakagawa et al., 2013; Hermant et al., 2014; Broggi et al., 2020). In addition, we report differential expression dynamics of IFN-I and IFN-III genes, which may have significance in the specific roles of the two classes of IFNs. This emphasizes the need to address the role of IFN-III in human listeriosis. As for Huh7 cells, they do not produce any IFN in response to *Listeria*, as previously observed when these cells are challenged with other IFN-triggering stimuli, such as dsRNA (Lanford et al., 2003), poly(I-C) (Li et al., 2005) or hepatitis C virus (Israelow et al., 2014). A possible explanation for this lack of response is that Huh7 cells have a defective TLR3 signaling pathway (Li et al., 2005). This deficiency can be useful in identifying IFN-independent processes. In particular, the downregulation of cholesterol biosynthesis gene expression in response to *Listeria* infection occurs in Huh7 in the absence of IFN. In contrast, IFN is involved in the downregulation of sterol biosynthesis during viral infections (Blanc et al., 2011).

Are IFN-I and IFN-III pathways beneficial or detrimental to persistent *Listeria* infection? The answer to this question is complex, since IFNs can have opposing effects on bacterial pathogens (Boxx and Cheng, 2016; Cohen and Parker, 2016; Ng et al., 2016; Alphonse et al., 2021). In fact, in mouse models of listeriosis, IFN-I has been implicated in both the restriction and promotion of infection (Dussurget et al., 2014; Boxx and Cheng, 2016), likely resulting from the pleiotropic roles of IFN-I in distinct cell environments and at different stages in the infectious process. Our data suggest that IFN responses do not result in an antibacterial effect in hepatocytes. Indeed, restoring a functional IFN response in Huh7 cells, by exogenous supply of IFN-β or IFN-λ1, does not produce a reduction in bacterial load at 3-day p.i. However, it is likely that IFN secretion by hepatocytes modulates infection *in vivo* by acting on immune cells. Interestingly, IFN-I promotes some chronic bacterial infections (Boxx and Cheng, 2016) and can lead to immunosuppression in chronic viral infections by inhibiting DC and T cell activation (Ng et al., 2016). In murine listeriosis, the IFN-I response has inhibitory effects on T cells (Carrero et al., 2004; Archer et al., 2014). The role of IFN-III in listeriosis has not yet been directly studied, but it should be noted that this IFN limits inflammation and leukocyte responses that are detrimental to epithelial barrier integrity (Broggi et al., 2020). Based on this, we propose the hypothesis that excessive IFN signaling could promote cellular conditions that support bacterial persistence by contributing to immune suppression and tissue tolerance (**Figure 8**).

Proteins encoded by the genes activated downstream of IFN (ISGs) are an important family of innate immunity factors produced in response to microbial infections (Schneider et al., 2014), including listeriosis (Dussurget et al., 2014). Like APPs, ISGs have various functions and are activated in a network, allowing for the simultaneous induction of diverse responses. The striking result of our study is that long-term infection with *Listeria* in human (HepG2) and murine (PMH) hepatocytes leads to a prominent ISG signature, astonishingly mimetic of an antiviral response. In addition, we identified 16 ISGs whose expression is enhanced in all hepatocyte models at 3 days p.i. (**Table 1**). Since *Listeria* infection does not induce IFN expression in Huh7 cells, these 16 ISGs are, in this context, activated by alternative pathways to IFN signaling. Indeed, *CCL5, CXCL10*, *PMAIP1*, *IFIT2, RSAD2* and *ISG15* can be activated independently of IFNs (Varley et al., 2003; Werts et al., 2007; Knowlton et al., 2012; Ashley et al., 2019). In particular, *ISG15* expression is known to be induced independently of IFN-I/III signaling upon *Listeria* infection and its product, the ubiquitin-like modifier ISG15, inhibits *Listeria* infection in fibroblasts (Radoshevich et al., 2015). The role of ISG15 in hepatocytes has not yet been directly addressed, but the study of a mouse model of increased ISGylation suggests an important role of ISG15 in the liver, including metabolic processes and autophagy (Zhang et al., 2019). Interestingly, hyper-ISGylation is associated with larger infection foci in the liver, suggesting that uncontrolled ISGylation could promote *Listeria* survival in this organ.

Concluding Remarks

The inhibition of the APR and exacerbation of interferon responses in hepatocytes sheds new light on the mechanisms by which *Listeria* might escape the host immune system to create a niche of persistence in the liver. Our results open up new questions about the cell biology of *Listeria* infection, for which the cellular models presented here would prove useful. For example, what is the role of intracellular activities of complement proteins, ISG

products, and cholesterol metabolism, on the fate of cytosolic or vacuolar bacteria? What are the transcriptional and/or epigenetic mechanisms behind the deregulation of the identified genes? This study also raises the question of the role of APPs, IFN-I and IFN-III *in vivo*, especially in the physiology of the asymptomatic phase of *Listeria* infections. However, no relevant animal model to study a long-lasting silent *Listeria* infection is yet available. Exploiting the results of our study might guide the development of such models, through genetic modifications or the use of drugs targeting host genes involved in the APR and IFN signaling. In addition, as SNPs and other mutations in complement and IFN genes have been linked to immune deregulation and susceptibility to infection, GWAS in humans or animals with listeriosis would be valuable in the investigation of the potential roles of APR and IFN pathway-related genes in the susceptibility to *Listeria* infections.

DATA AVAILABILITY STATEMENT

The RNA-seq data presented in the study are deposited in the Gene Expression Omnibus (GEO) repository (https://www.ncbi.nlm.nih.gov/geo/), accession numbers GSE184697, GSE184729 and GSE184808. The MS proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository (https://www.ebi.ac.uk/pride/), accession number PXD027154.

ETHICS STATEMENT

Mice were housed under specific pathogen free conditions in the local animal facility of IERP (INRAE, Jouy-en-Josas) in agreement with animal welfare guidelines. The animal house was maintained on a 12-hour light/dark cycle. The animal study was reviewed and approved by the local ethics committee, the COMETHEA ("Comité d'Ethique en Expérimentation Animale du Centre INRAE de Jouy en Josas et AgroParisTech"), under the registration numbers 19-08 and by the French Ministry of Higher Education and Research (APAFIS #20380-2019060315249683 v1) and were performed in accordance with European directive 2010/63/EU.

AUTHOR CONTRIBUTIONS

Investigation and methodology: ND, AP, CH, KG, CA, AD-B, and HB. Formal analysis and data curation: ND, LJ, KG, CH, and HB. Visualization: ND and HB. Resources: LG, PS, and HB. Funding acquisition: HB, LG, and CA. Writing - original draft preparation: HB. Writing - review and editing: ND, AP, CA, PS, LG, and HB. Supervision: HB, AP, and CA. Conceptualization and administration: HB. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.761945/full#supplementary-material

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Supplementary Material

1 Supplementary Materials and Methods

1.1 Transcriptome analysis

1.1.1 RNA-sequencing

Directional RNA-seq libraries of infected and non-infected cell RNA were assembled using 500-1300 ng total RNA (as determined by Qubit assay (Invitrogen)) using the TruSeq® mRNA Stranded Library Prep kit (Illumina) which includes polyA-selection. The RNA-seq libraries were monitored for quality on the Bioanalyzer 2100 using an Agilent High Sensitivity DNA Kit. Libraries were pooled in equimolar proportions and sequenced in paired-end 50-35 bp runs on an Illumina NextSeq500 instrument, using NextSeq 500 High Output 75 cycles kits. Demultiplexing was performed (bcl2fastq2 V2.2.18.12) and adapters were trimmed with Cutadapt (v1.15); only reads longer than 10 bp were kept. TopHat (version 2.1.1) was used for alignment on the reference genomes: Ensembl-98 human genome (HepG2 and Huh7 samples) or Ensembl-100 GRCm38 mouse genome (PMH samples). Data were evaluated through principal component analysis and hierarchical clustering after transformation of the count data using RLOG function. Dendograms were built using Euclidian distance function and Ward criterion as linkage function. Normalization and differential analysis were carried out using the DESeq2 package, with a pre-filter step to remove genes with low counts (sum of all replicates counts<10, in all conditions compared). Results were considered statistically significant with adjusted p<0.05, with correction according to the Benjamini and Hochberg's procedure.

1.1.2 Functional gene analysis

Gene pathways analysis was performed on differentially expressed protein-coding genes using DAVID (Huang da et al. 2009) version 6.8 (http://david.abcc.ncifcrf.gov), KEGG (Kanehisa et al. 2000) (http://www.genome.jp/kegg/pathway.html) and QIAGEN's Ingenuity Pathway Analysis (IPA®, QIAGEN Redwood City). A p value <0.05 was considered to indicate a statistically significant difference. Analysis using the interferome database (Rusinova et al. 2013) was performed by selecting "hepatocytes" as the cell system, "all species", and a fold change value greater or equal to 2.

1.1.3 RT-qPCR validation

Each RNA-seq analysis was validated by RT-qPCR with *YWHAZ*, *PPIA*, or *Pdk1* used to normalize gene expression in infected relative to non-infected HepG2, HuH7 and PMH, respectively. Pearson correlation analysis was applied to the log2 FC of 14 significantly deregulated genes obtained by RNA-seq and RT-qPCR. Pearson's correlation coefficients (R^2) of 0.9365, 0.9582, and 0.9728 were obtained for HepG2, Huh7 and PMH respectively, indicating very strong correlation and thus the precision of both methods in the relative quantification of gene expression.

1.1.4 Data Availability

The RNA-seq data presented in the study are deposited in the Gene Expression Omnibus (GEO) repository (https://www.ncbi.nlm.nih.gov/geo/), accession numbers GSE184697 (*Lm* EGDe versus non-infected HepG2, 72 h), GSE184729 (*Lm* EGDe versus non-infected Huh7, 72 h) and GSE184808 (*Lm* 10403S versus non-infected PMH, 72 h).

1.2 Proteome analysis

1.2.1 Sample preparation

For the proteome analysis, 5 µg of protein extract were used for a short migration 1D gel electrophoresis (NuPAGE® 4-12 % Bis-Tris Gel, Novex). Proteins were visualized using Coomassie G-250 (SimplyBlue™ SafeStain, Invitrogen) and the whole-colored part of each lane was cut into small pieces. The gel pieces were destained using Solvent A (10 % v/v acetic acid, 40 % v/v ethanol) and Solvent B (50 % v/v 50 mM ammonium bicarbonate, 50 % v/v acetonitrile). The proteins contained in the gel were reduced by 10 mM dithiothreitol (Sigma) and alkylated by 55 mM iodoacetamide (Sigma). The proteins were digested with 200 ng of trypsin (Promega) and afterwards extracted using a solution of 0.5 % v/v trifluoroacetic acid and 50 % v/v acetonitrile. The peptides were dried completely using a concentrator (Savant™ SPD121D, Thermo Fisher Scientific) and taken up in 50 µl loading buffer (0.08 % v/v trifluoroacetic acid, 2 % v/v acetonitrile) for LC-MS/MS proteome analysis (4 µl = 400 ng peptides per injection).

1.2.2 Liquid Chromatography – Mass Spectrometry

Mass spectrometry was performed on the PAPPSO platform (MICALIS, INRAE, Jouy-en-Josas, France; http://pappso.inrae.fr). We used an Orbitrap Fusion™ Lumos™ Tribrid™ (Thermo Fisher Scientific) coupled to an UltiMate™ 3000 RSLCnano System (Thermo Fisher Scientific). A 4 µl sample was loaded at 20 µl/min on a precolumn (µ-Precolumn, 300 µm i.d x 5 mm, C18 PepMap100, 5 µm, 100 Å, Thermo Fisher) and washed with loading buffer. After 3 min, the precolumn cartridge was connected to the separating column (Acclaim PepMap®, 75 µm x 500 mm, C18, 3 µm, 100 Å, Thermo Fisher). Buffer A consisted of 0.1 % formic acid in 2 % acetonitrile and buffer B of 0.1 % formic acid in 80 % acetonitrile. The peptide separation analysis was achieved at 300 nl/min with a linear gradient from 0 to 30 % buffer B for 50 min and 30 % to 40 % for 5 min. One run took 66 min, including the regeneration step at 98 % buffer B. Positive ionization (1.6 kV ionization potential) and capillary transfer (270 °C) were performed with a liquid junction and a capillary probe (SilicaTip™ Emitter, 10 µm, New Objective). Peptide ions were analyzed using Data Dependent Acquisition (DDA) with HCD (Higher-energy Collisional Dissociation) mode and the machine settings were as follows: 1) full MS scan in Orbitrap (scan range [m/z] = 400–1600), and 2) MS/MS using HCD (30 % collision energy) in Orbitrap (AGC target = 5.0×10^4, max. injection time = 150 ms, data type = centroid). Analyzed charge states were set to 2-4, the dynamic exclusion to 100 s and the intensity threshold was fixed at 2.0×10^4.

1.2.3 Data Analyses

Identification. The *Homo sapiens* database (Uniprot, version 2021 taxonomy identifier = 9606, 20 396 entries) was searched by using X!TandemPipeline version 0.4.24 (Langella et al., 2017). The proteome identification was run with a precursor and a fragment mass tolerance of 10 ppm. Enzymatic cleavage rules were set to trypsin digestion ("after Arg and Lys, unless Pro follows directly after") and no semi-enzymatic cleavage rules were allowed. The fix modification was set to cysteine carbamidomethylation and methionine oxidation was considered as a potential modification. In a second pass, N-terminal acetylation was added as another potential modification, whereas all other previous settings were retained. The identified proteins were filtered as follows: 1) peptide E-value < 0.05 with a minimum of 2 peptides per protein and 2) a protein E-value of $< 10^{-4}$.

Quantification. Proteins were quantified by the spectral counting (SC) method. MassChroqR (version 0.5.2), an R package developed by PAPPSO platform (http://pappso.inrae.fr) was used to check the quality of data and practice statistical analysis in proteomic. The abundance in number of spectra was

modeled using the following generalized mixed model (GLM) with a Poisson distribution, as already described (Millan-Oropeza et al., 2017). Filters consist in removing proteins showing low numbers of spectra (Filter was at least 3 spectra per protein, for example, if cutoff = 3, all the proteins quantified with only 0, 1, 2 spectra in each of the injections will be removed) and the selection criteria is the ratio between the minimal and the maximal mean abundance values computed for a factor or a combination of factors of interest (here, cutoff =1.5). Protein abundance change were detected by analysis of variance (ANOVA) using a Chi-square test. The obtained *p*-values were adjusted for multiple testing by the Benjamini-Hochberg approach. Adjusted *p*-values obtained from ANOVA for the proteome was considered significant below a value of 0.01. For HepG2 samples, 486 proteins (sub groups) were detected and quantified. 214 proteins remained after filter application, and statistical analysis (adj *p*-value < 0.01) selected 42 proteins as less present in infected samples compared to uninfected samples. For Huh7 cells, 502 proteins (sub groups) were detected and quantified. 337 proteins remained after filter application and statistical analysis (adj *p*-value < 0.01), selected 153 proteins as less present in infected samples compared to uninfected samples. The data were then crossed with the transcriptome data to sort proteins whose corresponding gene was significantly downregulated in infected cells compared to uninfected cells.

1.2.4 Data Deposition

The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al. 2019) repository: **https://www.ebi.ac.uk/pride/archive/projects/PXD027154**

1.3 References

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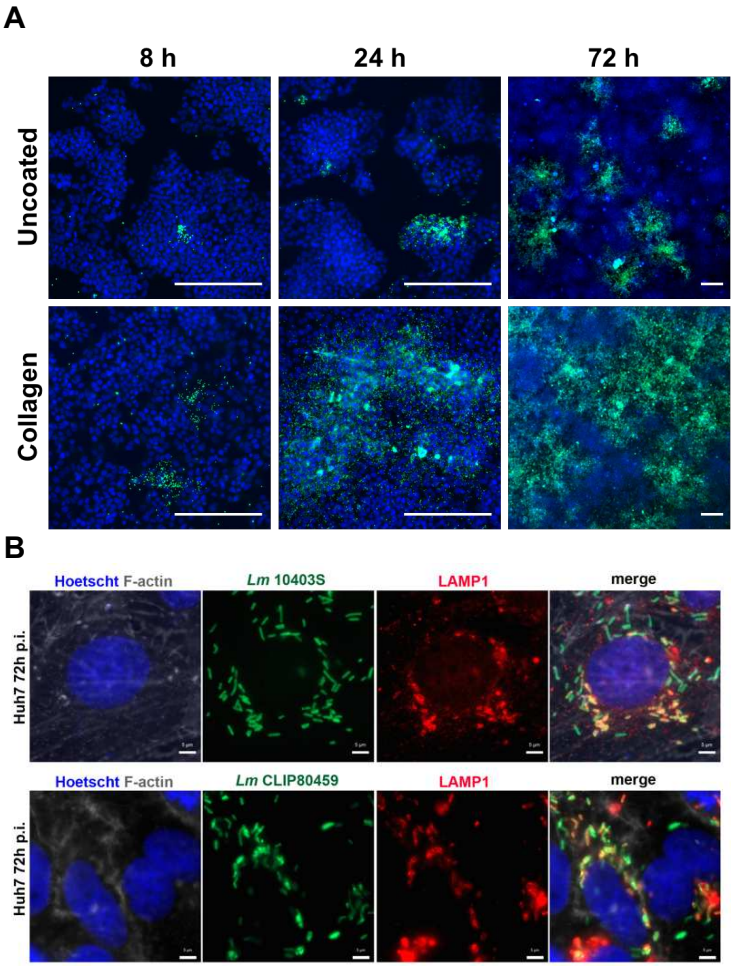
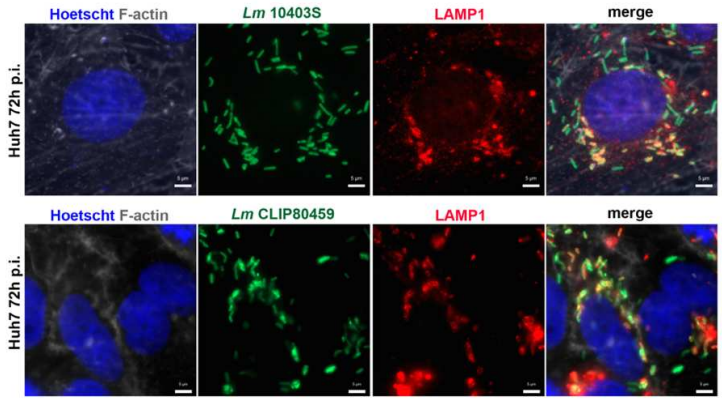
2 Supplementary Figures and Tables

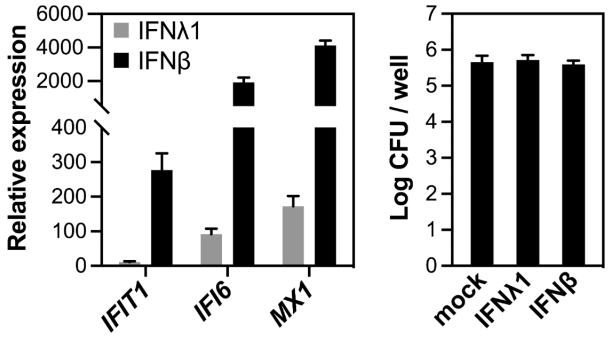
2.1 Supplementary Figures



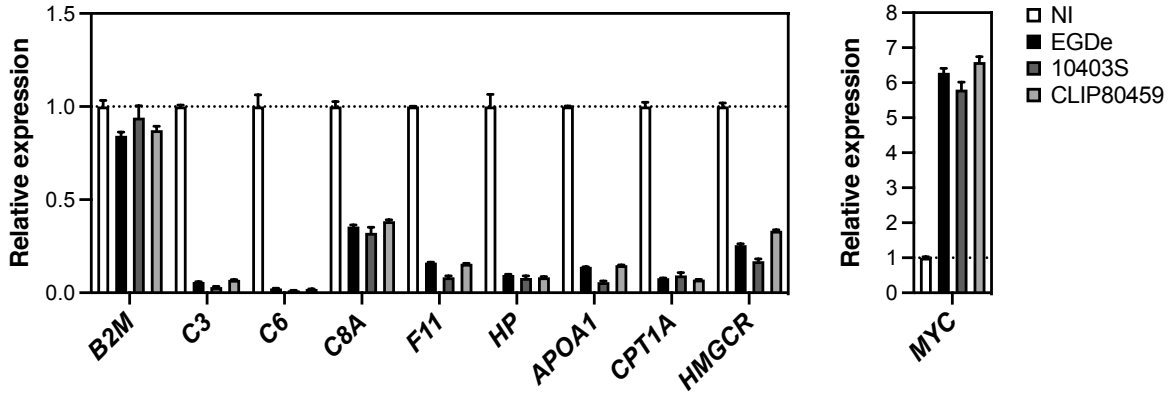
Supplementary Figure 1. Collagen coating increases bacterial spreading during 3-day *Listeria* infection in HepG2 cells. (A) HepG2 cells grown on uncoated or collagen-coated coverslips were infected with *Listeria* strain EGDe (MOI~1-5) and examined by immunofluorescence microscopy. **(A)** Low-magnification micrographs showing infected HepG2 cells at 8 h, 24 h and 72 h p.i., stained with Hoechst (to label nuclei, in blue) and *Listeria* antibodies (to label bacteria, in green). Monolayer growth of HepG2 on collagen decreases isolated clusters of cells, resulting in higher bacterial intercellular spreading at 8 h and 24 h p.i. and an increased number of infected cells at 72 h p.i. (bars: 200 µm). **(B)** High magnification micrographs showing cells at 72 h p.i. stained with Hoechst to label nuclei (blue), AF-647-phalloidin to label F-actin (white), *Listeria* antibody to label bacteria (green) and LAMP1 antibody to label LisCVs (red). Bacteria in LisCVs, which are LAMP1-positive, are observed both in HepG2 grown on uncoated (top) or collagen-coated coverslips (bottom).



Supplementary Figure 2. Formation of LisCVs in Huh7 cells is strain-independent. Huh7 cells were infected with laboratory strain 10403S or clinical strain CLIP80459 for 72 h and examined by immunofluorescence microscopy. High magnification micrographs show cells stained with Hoechst to label nuclei (blue), AF-647-phalloidin to label F-actin (white), *Listeria* antibody to label bacteria (green) and LAMP1 antibody to label LisCVs (red). LAMP1-positive bacteria are observed with both strains. Bars: 5 µm.



Supplementary Figure 3. Activation of IFN responses with recombinant IFN-β or IFN-λ1 does not change bacterial loads in Huh7 cells. Huh7 cells were infected with *Listeria* EGDe (MOI~1-5) for 24 h to let bacteria carry out the early phases of infection, and subsequently treated with either recombinant IFN-β (3 ng/ml; ~800 IU /ml) or IFN-λ1 (100 ng/ml) for an additional 48 h. IFN treatment activated the expression of ISGs as evaluated by RT-qPCR analysis of the transcript levels of *IFIT1, IFI6* and *MX1*, relative to mock-stimulated cells and normalized to *YWHAZ* **(A)**, but did not result in the reduction of the number of intracellular bacteria at 72 h p.i., compared to untreated cells **(B).** For all experiments, histograms represent means and standard deviations of triplicate experiments.



Supplementary Figure 4. Dysregulation of APP and lipid-metabolism gene expression in hepatocytes is strain-independent. RT-qPCR analysis of transcript levels of representative APP genes (C3, C6, C8A, F11, HP), or lipid metabolism-associated genes (*APOA1*, *CPT1A*, *HMGCR)*, in Huh7 cells infected with *Listeria* EGDe, 10403S, or CLIP80459 at 72h p.i., relative to non-infected cells (NI) cells. *PPIA* expression was used to normalize target gene expression and a control gene (*B2M*) whose expression was unaffected by infection, and an upregulated gene (*MYC*) are included. Histograms represent means and standard deviations of triplicate experiments.

2.2 Supplementary Tables

All supplementary Tables are attached as a single Supplementary Excel file, and are as follows:

Table S1. List of RT-qPCR primers.

Table S2. Protein-coding genes upregulated in HepG2 cells infected with *L. monocytogenes* EGDe for 72h, relative to non-infected HepG2 cells.

Table S3. Protein-coding genes downregulated in HepG2 cells infected with *L. monocytogenes* EGDe for 72h, relative to non-infected HepG2 cells.

Table S4. Protein-coding genes upregulated in Huh7 cells infected with *L. monocytogenes* EGDe for 72h, relative to non-infected Huh7 cells.

Table S5. Protein-coding genes downregulated in Huh7 cells infected with *L. monocytogenes* EGDe for 72h, relative to non-infected Huh7 cells.

Table S6. Protein-coding genes upregulated in PMH infected with *L. monocytogenes* 10403S for 72h, relative to non-infected PMH**.**

Table S7. Protein-coding genes downregulated in PMH infected with *L. monocytogenes* 10403S for 72h, relative to non-infected PMH.

Table S8. Genes upregulated in both HepG2 cells and PMH infected with *L. monocytogenes* for 72h.

Table S9. Gene Ontology of Biological Processes (GO-BP) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of genes upregulated in HepG2, Huh7 or PMH infected with *L. monocytogenes* for 72h.

Table S10. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of genes downregulated in HepG2, Huh7 or PMH infected with *L. monocytogenes* for 72h.

Table S11. Genes downregulated by 72h *L. monocytogenes* infection in all HepG2, Huh7 and PMH datasets (A) or intersecting Huh7 and HepG2 datasets (B), or Huh7 and PMH datasets (C).

Table S12. Proteins identified as significantly less abundant in the conditioned medium of 72h infected HepG2 (A) or Huh7 (B) cells, compared to non-infected cells.

Table S13. Ingenuity Upstream Regulator Analysis (URA) on downregulated genes common to both human Huh7 and murine PMH models (A) or both human Huh7 and HepG2 models (B).

Table S14. List of references providing information on host transcriptional responses to *L. monocytogenes* infection in cellular models *in vitro* (A) or in mouse models *in vivo* (B).

Supplementary Tables can be found online at:
https://www.frontiersin.org/articles/10.3389/fcimb.2021.761945/full#supplementary-material

1.2. Unpublished results

The results described below are complementary to Descoeudres et al., 2021, and address some of the questions raised by this study including:

(*i*) The time-dependent inhibition of the cholesterol biosynthesis and APP-coding gene downregulation observed during long-term infection.

(*ii*) The capacity of cultured hepatocytes to induce APP expression in an autocrine manner, by the secretion of IL-6, the major APR-inducing inflammatory cytokine.

(*iii*) The role of InlC, a *Lm* virulence factor known to inhibit NF-κB activation, in the downregulation of APP gene expression, regulated by NF-κB.

Transcriptional deregulation increases from 24 h to 72 h in Huh7 cells

We sought to address the question of whether the inhibitory effect of *Lm* infection on APP and cholesterol biosynthesis gene expression was specific to long-term infection, or whether this transcriptional repression could be observed at an earlier time point in infection. To answer this question, we employed the Huh7 cell line as the number of deregulated genes and the magnitude of gene deregulation was highest in this model (Descoeudres et al., 2021). RNA was extracted from infected and non-infected (NI) cells at 24 h p.i. in the same experiments from which RNA was obtained at 72 h p.i., providing a two-point infection time course to evaluate the evolution of the transcriptional host response. RT-qPCR was used to validate the RNA-seq results; a Pearson's correlation coefficient (R^2) of 0.9594 was obtained between fold change (FC) values calculated using the two methods, indicating very strong correlation. Huh7 cell monolayers are heavily and uniformly infected at both 24 h and 72 h, with an equally small proportion of NI cells at both timepoints (Figure 28A), leading to the assumption that eventual differences in the transcriptional signatures observed at the two time points would not be due to a larger proportion of NI cells at the earlier (24 h) timepoint. The number of cells and intracellular bacteria were monitored to assess cell viability and to compare intracellular bacterial loads over the two time points. There was less cytotoxicity at 24 h than at 72 h (Figure 28B) and the number of intracellular bacteria assessed by CFU counts was approximately one order of magnitude higher at 24 h than at 72 h p.i. (Figure 28C); a decrease of the same order was previously observed in HepG2 cells (Kortebi et al., 2017). It has been proposed that the degradation of a subpopulation of bacteria, and the entry of another subpopulation into a VBNC state, could explain this decrease (Kortebi et al., 2017).

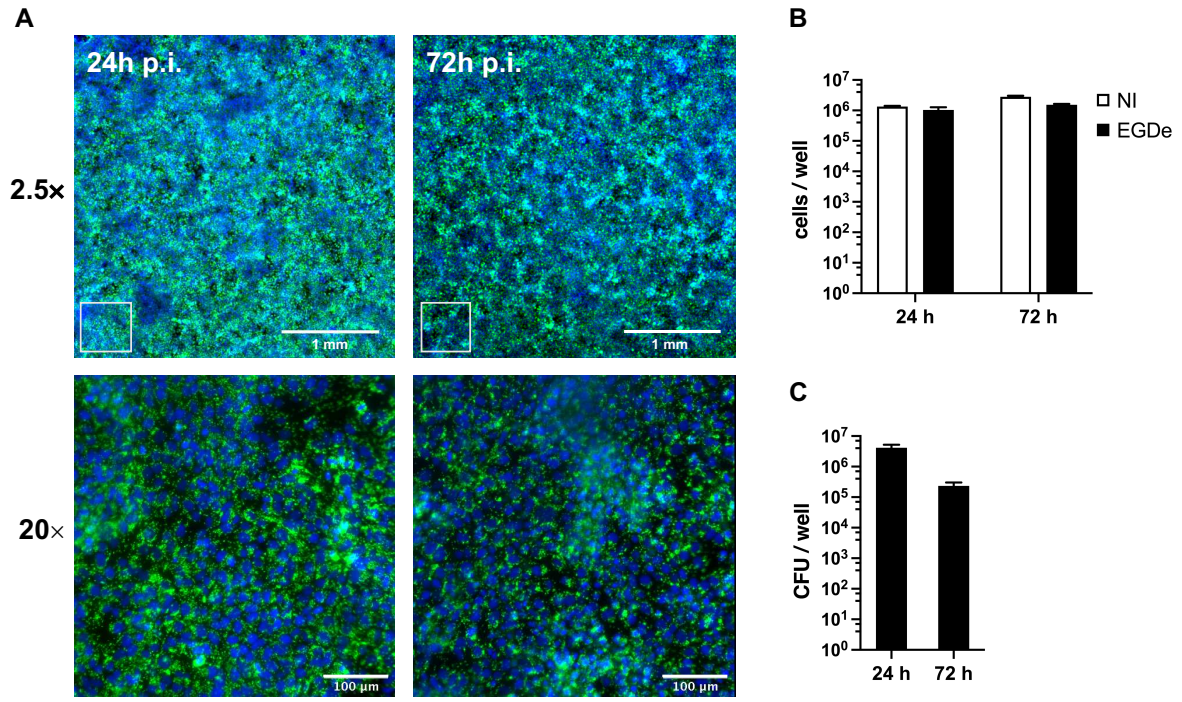


Figure 28 | Microscopy and intracellular bacterial quantification of *Lm* infected Huh7 cells at 24 h and 72 h p.i. reveal heavily infected cells at both timepoints.
(**A**) Very low (top) and low (bottom) magnification immunofluorescence microscopy images of *Lm* EGDe infected (MOI 1) Huh7 cells at 24 h (left) and 72 h p.i. (right) showing a heavily and uniformly infected cell monolayer at both time points; *Lm* (green), cell nuclei (blue). Top images: 2.5× magnification, scale bar 1 mm; bottom: 20× magnification, scale bar 100 µm. The squares in the bottom left corners of the 2.5× magnification micrographs represent the proportion of the images visualised at 20× magnification. (**B**) Cells per well (log scale, mean ± SD, n=3). (**C**) CFU per well (log scale, mean ± SD, n=3).

Using the same criteria to define significant differential gene expression between infected and NI cells at 72 h p.i. ($p<0.05$ and $|\log2(FC)| >0.5$) (Descoeudres et al., 2021), we identified 1856 differentially expressed protein-coding genes (DEGs) in Huh7 cells at 24 h p.i., of which 957 were upregulated and 899 downregulated (Figure 29A). This represents less than one third of the number of DEGs significantly deregulated at 72 h p.i. (i.e., 3198 and 2783 up- and downregulated DEGs, respectively (Descoeudres et al., 2021)). Differential expression was also somewhat more pronounced at 72 h than at 24 h, with, for upregulated DEGs a median log2(FC) of 0.77 at 24 h versus 0.88 at 72 h, and, for downregulated DEGs, -0.78 at 24 h versus -1.00 at 72 h (Figure 29B). These results indicate that the effect of infection on host cell transcription – both activation and repression – was significantly magnified over time despite the decrease in the number of culturable intracellular bacteria quantified at 24 h and 72 h p.i.

Time-dependent inhibition of APP and cholesterol gene expression in Huh7 cells

Most downregulated DEGs at 24 h p.i. (702/899) were also downregulated at 72 h p.i., whereas 2081 genes were specifically inhibited at 72 h p.i. (Figure 29A). Ingenuity Pathway Analysis (IPA) identified similar canonical pathways (CPs) inhibited at both timepoints, including those involved in cholesterol biosynthesis, APR signalling, the coagulation and complement systems, and FXR/RXR and LXR/RXR activation (Figures 29C and 30).

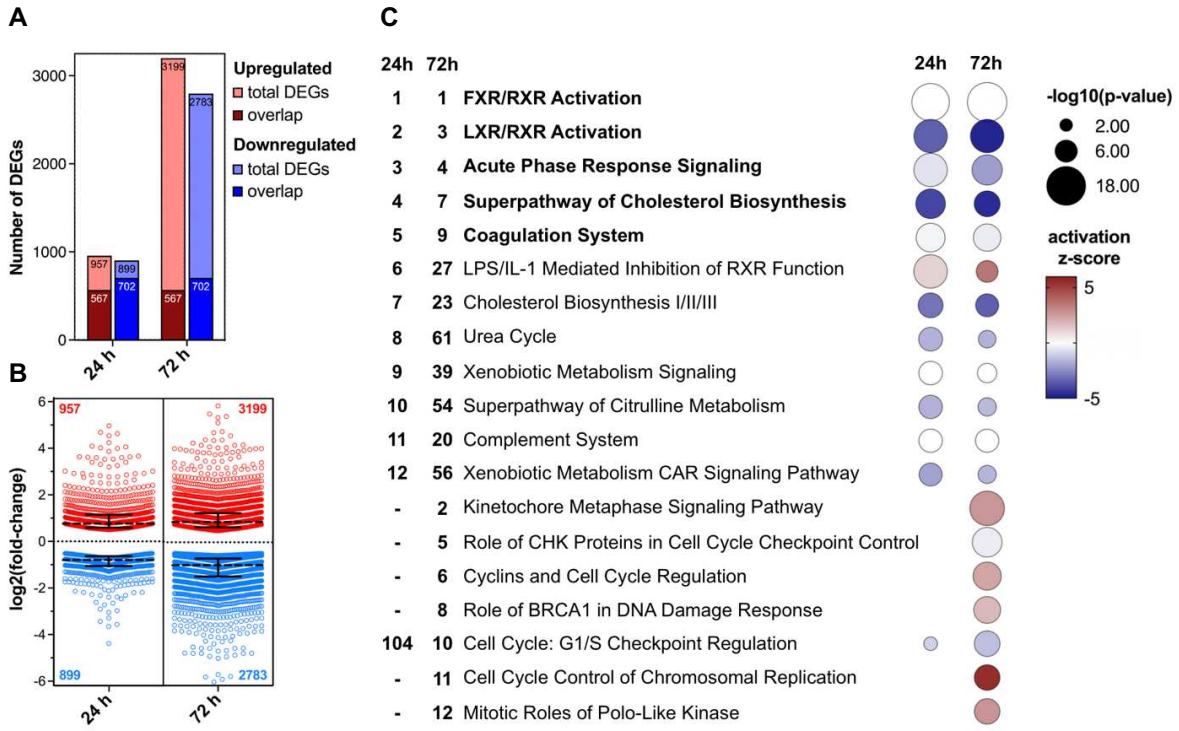


Figure 29 | Transcriptional response to *Lm* infection at 24 h and 72 h p.i. in Huh7 cells.
(**A**) Total DEGs upregulated (red) or downregulated (blue) at 24 h and 72 h p.i. timepoints, with DEGs common to both timepoints indicated within the bar. (**B**) Log2(FC) distribution of DEGs at 24 h and 72 h timepoints; medians and interquartile ranges are indicated by dashed and solid lines respectively. (**C**) IPA analysis was performed on total DEG sets obtained at 24 h and 72 h p.i. The top 12 deregulated pathways for each timepoint, ranked according to p-value, are shown. Rank is indicated on the left and *p*-value and activation *z*-score are indicated by bubble size and colour, respectively. The *z*-score indicates a predicted activation or inhibition of a CP: a negative *z*-score value connotates an overall pathway's inhibition, and a positive *z*-score value connotates an overall pathway's activation.

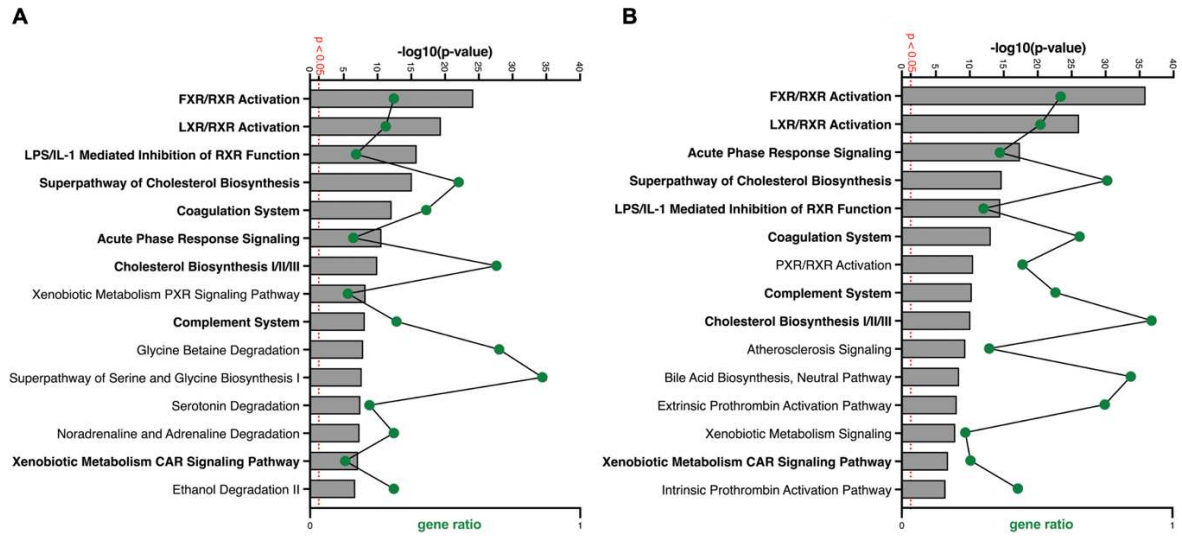


Figure 30 | Top 15 pathways downregulated at 24 h and 72 h p.i. in Huh7 cells.
IPA was performed on downregulated DEG sets obtained at 24 h (**A**) and 72 h p.i. (**B**) in Huh7 cells. Bars represent *p*-value, connected green circles represent gene ratio for each Canonical Pathway (CP). CPs common to both timepoints are indicated in bold.

We thus examined more closely the differential expression of genes encoding both cholesterol biosynthesis enzymes and APPs at 24 h and 72 h p.i. and found the number of downregulated genes as well as the magnitude of downregulation to be smaller (Figure 31). This result suggests that the repressive effect of intracellular *Lm* infection on APP and cholesterol synthesis gene expression increases during the infection time course. In the case of APP-coding genes, this could contribute to the establishment over time of an immunodeficient state in the liver that favours bacterial persistence in hepatocytes.

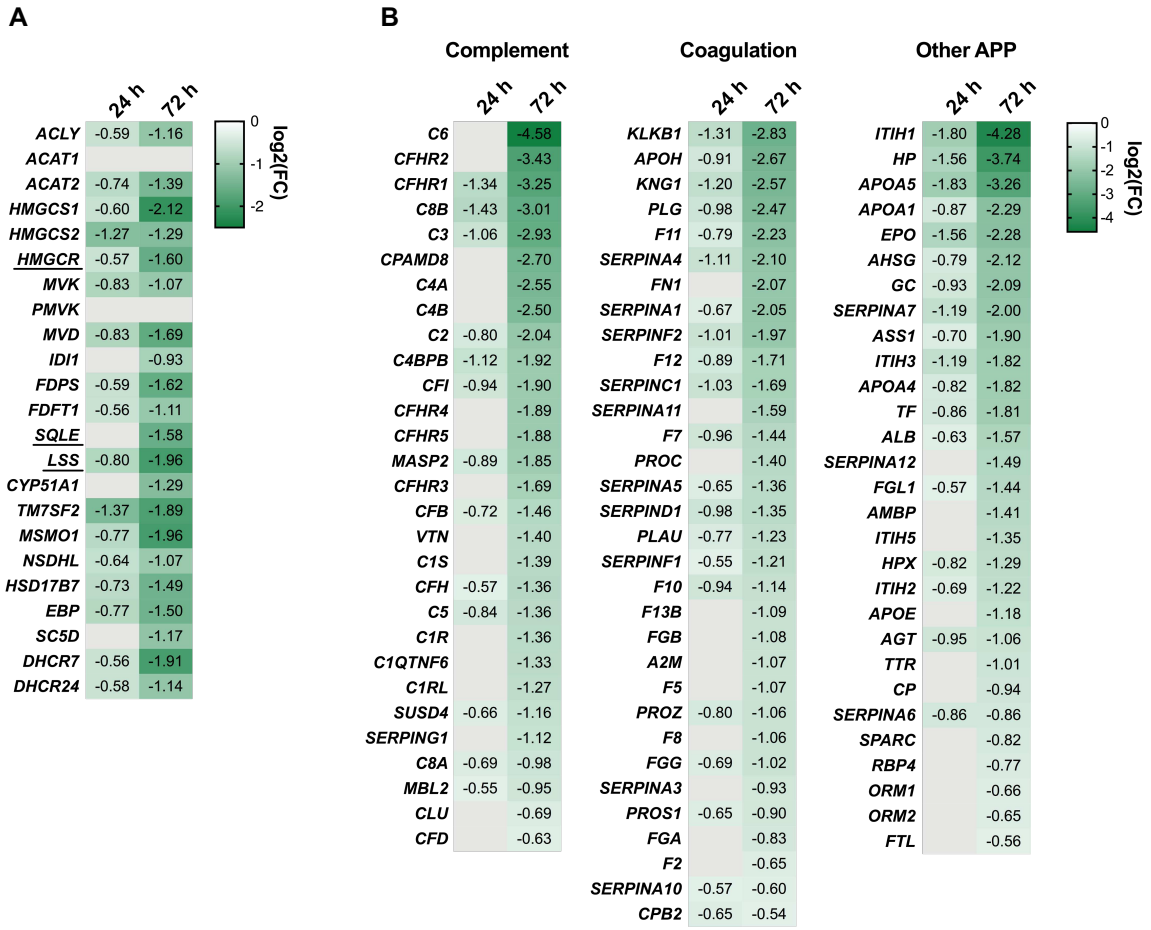


Figure 31 | The transcriptional downregulation of genes encoding cholesterol biosynthesis enzymes and APPs increases from 24 h to 72 h p.i. in Huh7 cells.
Heatmap displaying log2(fold change) of DEGs encoding cholesterol biosynthesis enzymes (**A**) and APP-coding genes significantly deregulated in infected compared to NI cells (**B**). APPs are classed according to their role in either the complement or coagulation systems or other functions in (B). DEGs encoding rate limiting enzymes are underlined in (A).

Lm infection does not elicit the expression of the APR-inducer IL-6 in hepatocytes

IL-6 is an important inducer of APP-coding gene expression during inflammation (Tanaka et al., 2014). It is mainly produced and secreted by innate immune cells. We cannot, however, exclude the production of IL-6 by hepatocytes in response to bacterial stimuli, as has been reported (Norris et al., 2014). To address this question, we examined whether *Lm* infection elicited the expression of this pro-inflammatory cytokine in hepatocytes using four different approaches. First, absolute *IL6* expression was estimated using the fragments per kilobase of transcript per million mapped reads (FPKM) values in the transcriptome datasets (Descoeudres et al., 2021). This analysis revealed that in all Huh7 and HepG2 samples *IL6* FPKM remained below the threshold value of 0.3, applied to define detectable expression above background as previously described (Ramsköld et al., 2009). Second, *IL6* transcripts in EGDe-infected and NI

Huh7 and HepG2 cells were quantified by RT-qPCR. Calculation of fold induction was possible, as despite low expression levels in infected cells, CT values obtained were below the threshold of 35 applied to define an absence of transcripts. While fold-change values suggest an induction of *IL6* expression in infected cells, they remain modest (Figure 32A). Third, to assess whether this low induction of expression translated into protein secretion, we carried out ELISA quantification of IL-6 in conditioned media recovered from infected and NI Huh7 and HepG2 cells. IL-6 was undetectable in all NI samples and detected in all infected samples but at very low levels (at the limit of quantification at around 1 pg/ml) (Figure 32B). Fourth, to assess whether these very low levels of secreted IL-6 could nonetheless induce APP gene expression, we treated uninfected Huh7 cells with conditioned media recovered from 3-day infected Huh7 cells and measured the expression of several representative APP genes at 24 h p.i. Conditioned medium from infected cells did not modulate the expression of complement *C3*, *C5*, *C6*, or of fibrinogen beta chain (*FGB*) genes in recipient cells in any way (Figure 32C). Taken together, these results strongly suggest that hepatocytes are not a source of IL-6, or other APR inducer, during *Lm* infection.

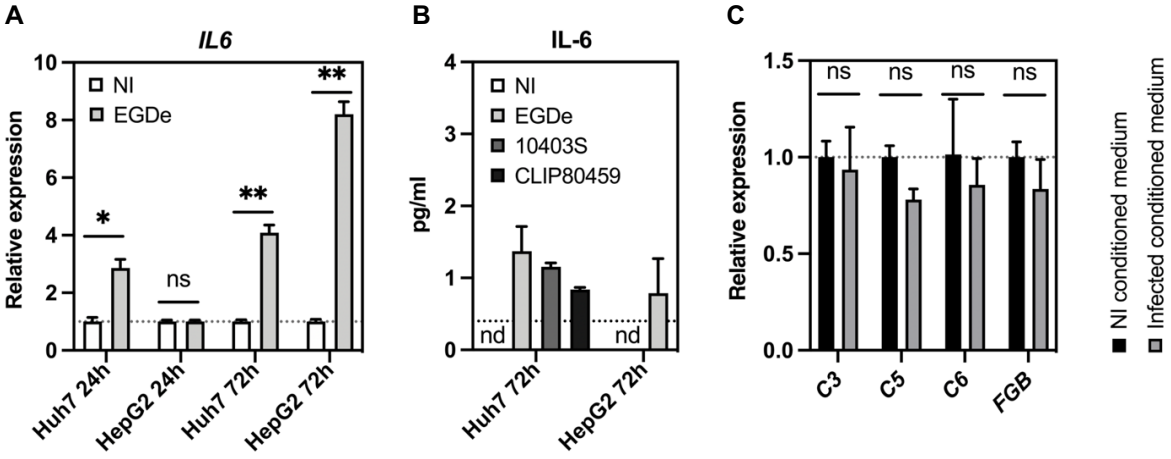


Figure 32 | Huh7 cells do not produce APR-stimulating cytokines during *Lm* infection.
(**A**) RT-qPCR quantification of *IL6* RNA transcript levels in Huh7 and HepG2 cells at 24 h and 72 h p.i. As *IL6* expression was below the detection level in NI samples, the CT threshold value (35) was applied to these samples in order to allow the calculation of a relative expression value. (**B**) ELISA quantification of IL-6 protein concentration in the conditioned media of NI or infected Huh7 or HepG2 cells at 72 h. Dotted line: limit of detection; nd: not detected. (**C**) Conditioned media recovered from 3-day *Lm* EGDe-infected ("infected conditioned medium") or non-infected ("NI conditioned medium") Huh7 cells was used to stimulate uninfected Huh7 cells for 24 h. *C3*, *C5*, *C6*, and *FGB* expression was then assessed by RT-qPCR and is expressed as fold-change in cells treated with infected conditioned medium relative to expression in cells treated with NI conditioned media. All values represent mean ± SD (n=3). Statistical significance was determined by Student's *t* test (* $p<0.05$, ** $p<0.01$, ns: not significant).

InlC contributes to the downregulation of type I but not type II APP-coding genes

As described in section C.4.3, the *Lm* virulence factor InlC interferes with the degradation of IκB, inhibiting the activation of NF-κB (Gouin et al., 2010). As NF-κB signalling is involved in the transcriptional regulation of APP-coding gene expression (Asselin and Blais, 2011) and notably type I APPs induced by IL-1-type cytokines (Bode et al., 2012), we investigated whether InlC plays a role in the *Lm* infection-induced downregulation of APP expression. To do this, we compared the transcript levels of representative APPs of both type I (*HP*, *C3*) and type II (*FGB*, *PLG*) in Huh7 cells following infection with EGDe (WT), an isogenic *inlC* mutant strain (Δ*inlC*) and a complemented strain (Δ*inlC*+*inlC*). We first assessed the ability of the Δ*inlC* and the Δ*inlC*+*inlC* bacteria to infect Huh7 cells and found intracellular bacterial levels to be similar to those observed for the WT strain at three observed timepoints (Figure 33A). At 72 h p.i., we extracted RNA from Huh7 cells infected by each of these strains and quantified APP gene expression levels. We found that in the absence of InlC, the inhibition of type I APP expression was less efficient than in the presence of InlC. In contrast, InlC did not appear to play a role in the downregulation of type II APPs (Figure 33B). These preliminary results suggest that InlC could play a role in repressing even basal levels of type I APP expression. Future work is needed to address whether this effect is NF-κB-dependent, however. Furthermore, a role for InlC in the context of inflammation remains to be examined. For this, a similar experiment will have to be performed in which hepatocytes are stimulated by the pro-inflammatory cytokines IL-6 or IL-1β concomitant to infection, to mimic physiologic conditions in which pro-inflammatory cytokines are secreted by innate immune cells.

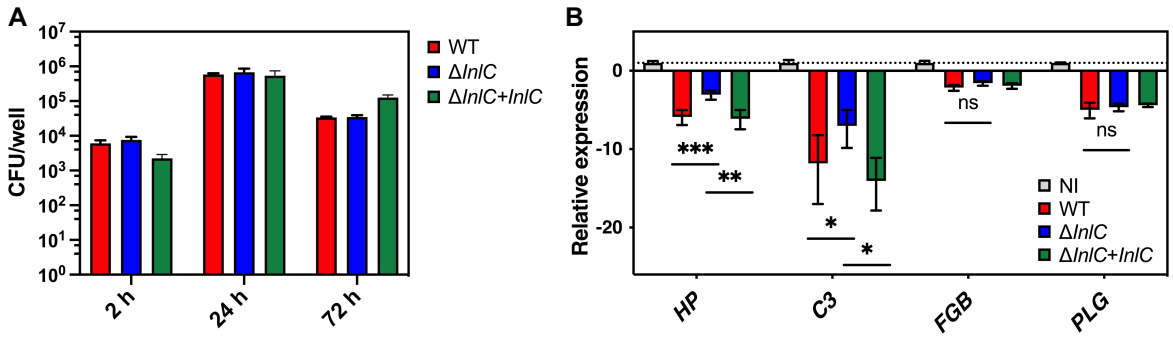


Figure 33 | InlC contributes to the downregulation of type I but not type II APP downregulation. (**A**) Huh7 cells were infected with EGDe (WT), *inlC* deletion mutant (Δ*InlC*), and the complemented strain (Δ*inlC*+*inlC*) for 72 h and intracellular bacterial loads were quantified at 2 h, 24 h and 72 h p.i. to verify similar bacterial entry, intracellular spreading and survival between the strains. (**B**) RT-qPCR quantification of two type I APP (*HP*, *C3*) and two type II APP (*FGB*, *PLG*) transcripts in WT-, Δ*InlC*-, and Δ*InlC*+*InlC*-infected Huh7 cells at 72 h p.i., expressed as fold induction relative to NI cells, and normalised to *PPIA*. All values represent mean ± SD (n=3); statistical significance was determined by Student's *t* test (* p<0.05, ** p <0.01, *** p<0.001, ns; non-significant).

Material and Methods

Bacterial strains and cell lines

Lm wild type EGDe (Bécavin et al., 2014) and mutant strains EGDe-Δ*inlC* and EGDe-Δ*inlC*+*inlC* (a kind gift from P. Cossart) were used in this study. EGDe-Δ*inlC* was generated by allelic exchange, as described previously (Arnaud et al., 2004) and used to construct EGDe-Δ*inlC*+*inlC*, as described in (Travier et al., 2013). The resulting in-frame deletion mutant lacking the InlC protein-coding region was verified by DNA sequence analysis of the chromosomal deletion. Strains and human hepatocellular/hepatoblastoma cell lines Huh7 (CLS 300156) and HepG2 (ATCC HB-8065) were grown as described in (Descoeudres et al., 2021).

Bacterial infection

Huh7 and HepG2 cells were seeded in 12- or 6-well plates in order to reach approximately 90% confluency on the day of infection. HepG2 cells were grown on collagen-coated wells or coverslips (type I collagen from rat tail, Sigma). Cells were counted using a haemocytometer (Neubauer-improved, Hausser Scientific) before infection to determine the inoculum necessary to obtain a multiplicity of infection (MOI) of 1. Inoculums were prepared in serum-free culture medium using bacteria grown overnight to stationary-phase and washed twice in PBS. Cells were infected as described in (Descoeudres et al., 2021).

RNA extraction, RNA-seq, and RT-qPCR

RNA extraction, RNA sequencing and RT-qPCR were performed as described in (Descoeudres et al., 2021). For RT-qPCR, Pearson correlation analysis was applied to the log2(FC) of 9 significantly deregulated genes obtained by RNA-seq and RT-qPCR (Figure 34).

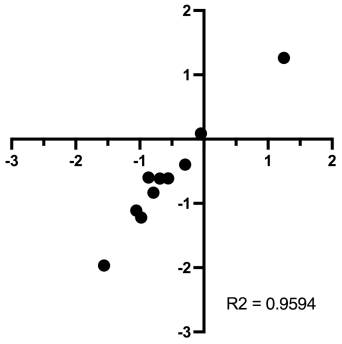


Figure 34 | RT-qPCR validation of Huh7 24 h p.i. RNA-seq results.
Pearson correlation analysis was applied to the log2(FC) of 9 DEGs and one non-regulated gene obtained by RNA-seq and RT-qPCR. A Pearson's correlation coefficient (R^2) of 0.9594 was obtained, indicating very strong correlation and thus the precision of both methods to measure gene expression.

A transcript was considered undetectable at CT ≥ 35; all negative controls (no cDNA template controls) generated CTs above this threshold. As *IL6* gene expression was below the detection level as determined by this CT threshold in NI samples, the CT threshold value was applied to these samples in order to allow the calculation of a relative expression value. Statistical significance was determined using Students paired *t* test on biological triplicates; a *p* value <0.05 was considered significant. Primer sequences are listed below:

Gene symbol	Gene name	Forward sequence	Reverse sequence
PPIA	peptidylprolyl isomerase A	AGACAAGGTCCCAAAGACAGC	ACCACCCTGACACATAAACCC
IL6	interleukin 6	CCACTCACCTCTTCAGAACG	CATCTTTGGAAGGTTCAGGTTG
HP	haptoglobin	CACAGAAGGAGATGGAGTGTAC	TGGGTTTGCCGGATTCTTG
C3	complement C3	AGTCTTTGTACGTGTCTGCC	ACTTGGGTGTCTTGGTGAAG
PLG	plasminogen	GAAGACCCCAGAAAACTACCC	TTTCAGGTTGCAGTACTCCC
APOA1	apolipoprotein A1	AGGATGAAAGCTGCGGTG	TCTTTGAGCACATCCACGTAC
F11	coagulation factor XI	TGGGTGTGCTTCAGTAGACAA	CAGTTGCCAAGAGTGCTCAAG
C8A	complement C8 alpha chain	GCAGCCAGTATGAACCAATTC	CCCTCCATTCCCCATTGTATAC
DHCR7	24-dehydrocholesterol reductase	CCAAGTTCACCCACGAGTCC	GGCCCTCTCGGTTTGTCTTC
SREBF2	sterol regulatory element binding transcription factor 2	TTCCTGTGCCTCTCCTTTAAC	TCATCCAGTCAAACCAGCC
B2M	beta-2-microglobulin	CACCCCCACTGAAAAAGATG	ATATTAAAAAGCAAGCAAGCAGAA
MYC	MYC proto-oncogene, bHLH transcription factor	CGTCTCCACACATCAGCACAA	CACTGTCCAACTTGACCCTCTTG

IL-6 ELISA assay

The human IL-6 uncoated enzyme-linked immunosorbent assay (ELISA) (Invitrogen, 88-7066) was used to determine the concentration of IL-6 in the conditioned media of infected and NI Huh7 and HepG2 cells, according to the manufacturers' instructions with minor modifications to increase the sensitivity of the assay (9-point standard curve from 0.4–100 pg/ml and overnight incubation of samples). Conditioned media was collected at the indicated time points, centrifuged at 300 × g for 5 minutes at 4°C and the collected supernatants stored at -80°C. 100 µl of each conditioned media sample and all standards were assayed in duplicate. The plate was read at 450 nm using a Tecan Infinite 200 device (Tecan Trading AG, Switzerland), from which readings at 570 nm were subtracted for wavelength correction.

Acknowledgments

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Part 2. The hepatocyte response to *Lm* infection in mixed versus pure populations of infected cells: contribution of bystander cells and role of epifactors BAHD1 and MIER1/3

Presentation

As described in Part 1 of the Results section, the *Lm* infection process in classically cultured human HepG2 cells is not homogeneous: foci of densely infected cells are observed among islets of uninfected cells. Transcriptional effects of long-term infection could therefore be masked by the presence of uninfected cells. To acquire the results presented in Part 1, HepG2 cells were grown on collagen to increase the intercellular spread of bacteria in order to obtain a higher proportion of infected cells. In this second part of the Results section, we report the use of a different approach: the sorting of infected cells from a mixed population of infected and NI cells. Fluorescence-activated cell sorting (FACS) of HepG2 cells infected with GFP-expressing *Lm* and microarray profiling were used to obtain the transcriptome of homogeneous populations of infected cells that was compared to the transcriptome of sorted NI controls. The transcriptome of heterogeneous populations of infected and uninfected cells was evaluated in parallel. The data from this microarray profiling were unpublished results obtained by Bierne and collaborators before my arrival in the laboratory and were the basis of my thesis project, as follows:

(*i*) Functional clustering of genes deregulated by long-term *Lm* infection indicated a downregulation of genes involved in complement activation and cholesterol biosynthesis pathways, a phenomenon that we have rigorously confirmed, as described in Part 1 of the Results section.

(*ii*) Transcriptomic data obtained from the homogeneously infected cells suggested an attenuation of the IFN response when compared to that of the heterogeneous population of unsorted infected and uninfected cells.

(*iii*) Microarray profiling indicated that the expression of genes encoding epigenetic factors BAHD1 (Bierne et al., 2009) and MIER1 and MIER3 (Derwish et al., 2017; Ding et al., 2003) were upregulated uniquely in homogeneously infected (sorted) cells.

As described in the Introduction (section C.5.1) of this thesis, BAHD1 and MIER proteins are hypothesised to be scaffolding proteins of a chromatin-repressive complex containing HDACs, histone methyltransferases (KMT), and heterochromatin markers (e.g., HP1, MBD1, CDYL, KAP1), and involved in gene silencing via heterochromatin formation (Lakisic et al., 2016). BAHD1 has been shown to repress the expression of a subset of ISGs during *Lm* infection in colon epithelial cells and to play a role in the pathogenicity of *Lm* infection in the murine model of listeriosis (Lebreton et al., 2011). In addition, phenotyping results of *Bahd1* KO mice indicate that BAHD1 plays a role in gene regulation in the placenta and the brain, two organs targeted by *Lm* (Lakisic et al., 2016; Pourpre et al., 2020). Finally, data from *Bahd1* KO mice also suggest that the BAHD1-MIER complex regulates glucose and cholesterol metabolism (Lakisic et al., 2016). Glucose metabolism was specifically affected in the liver as *Bahd1* KO mice had lower hepatic glycogen levels, while muscle glycogen levels remained similar to WT (Bierne and collaborators, unpublished data). Based on this data, we speculated that the BAHD1-MIER complex could play a role in the transcriptional response of hepatocytes to long-term *Lm* infection. To explore this hypothesis, my thesis project was constructed around a collaboration between my thesis director, H. Bierne, and L. Gillespie, head of a team at the Memorial University of Newfoundland, Canada, as part of the France-Canada Recherche Fund program. While H. Bierne and collaborators were at the origin of the discovery and characterisation of BAHD1 (Bierne et al., 2009; Lakisic et al., 2016; Lebreton et al., 2011; 2014; Pourpre et al., 2020), L. Gillespie and her team have long been conducting pioneering work on MIER proteins, and in particular MIER1, in the framework of their research on cancer signalling pathways (Blackmore et al., 2008; Derwish et al., 2017; Ding et al., 2003; 2004; McCarthy et al., 2008; Paterno et al., 1997; 1998).

Results

FACS procedure to isolate pure populations of long-term *Lm* infected cells for transcriptomic analysis and the comparison to heterogeneously infected cell populations

To obtain a homogeneous population of infected cells, a GFP-expressing *Lm* EGDe strain (*Lm*-GFP) was used to infect HepG2 cells to enable FACS. Approximately 10–15% of GFP-positive cells were isolated by FACS from 72 h-infected cultures, using stringent sorting gates (Figure 35). Microscopic examination confirmed that GFP-positive sorted cells were a homogeneous population of highly infected cells as described previously (Kortebi et al., 2017). The GFP-negative cell population of the infected cell cultures, however, was a heterogeneous population

of uninfected cells or cells containing a small number of bacteria; these cells were not exploited. Cultures of NI cells that underwent the same FACS procedure as infected cells were used as controls.

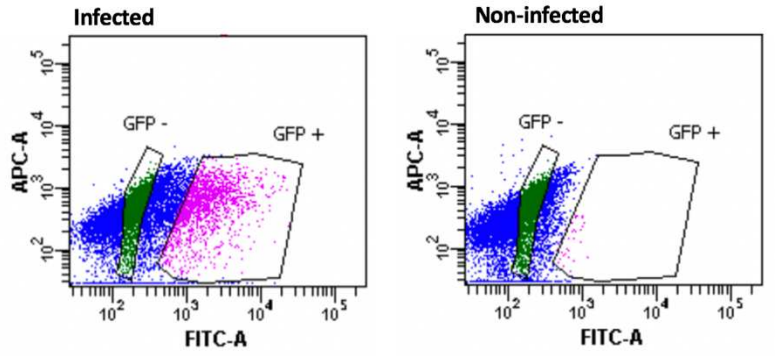


Figure 35 | FACS sorting of *Lm* EGDe-GFP infected and non-infected cells.
At 72 h p.i. *Lm*-GFP infected cells were selected by gating the GFP/FITC-positive population that was absent in NI cells.

Total RNA was isolated from FACS-purified infected and NI cells ("sorted" cells) in three independent biological replicates. In parallel, RNA was also extracted from infected and uninfected cells before FACS isolation ("unsorted" cells) (Figure 36A). Transcriptomic profiling was performed using Affymetrix Human Genome microarrays. Differentially expressed genes (DEGs) in long-term *Lm* infected cells relative to NI cells were defined as protein-coding genes that obtained a Benjamini-Hochberg adjusted *p*-value <0.05 and |fold-change| >1.4.

This analysis revealed 576 DEGs upon long-term infection in the heterogeneous unsorted cell population, and a significantly higher number of DEGs (1841) in the sorted cell population (i.e., about 3-fold more) (Figure 36B). These results showed that the enrichment of infected cells by FACS increases the power of identification of infection associated DEGs.

Downregulation of genes involved in complement activation and cholesterol biosynthesis and upregulation of genes involved in IFN responses upon long-term *Lm* infection

Functional annotation of up- or downregulated DEG sets that were common or unique to the sorted or unsorted HepG2 transcriptomes using Gene Ontology of Biological Processes (GOBP) terms was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang et al., 2009). With this approach, we aimed to reveal infection-specific pathways as well as eventual pathways to which uninfected cells in the mixed population contributed.

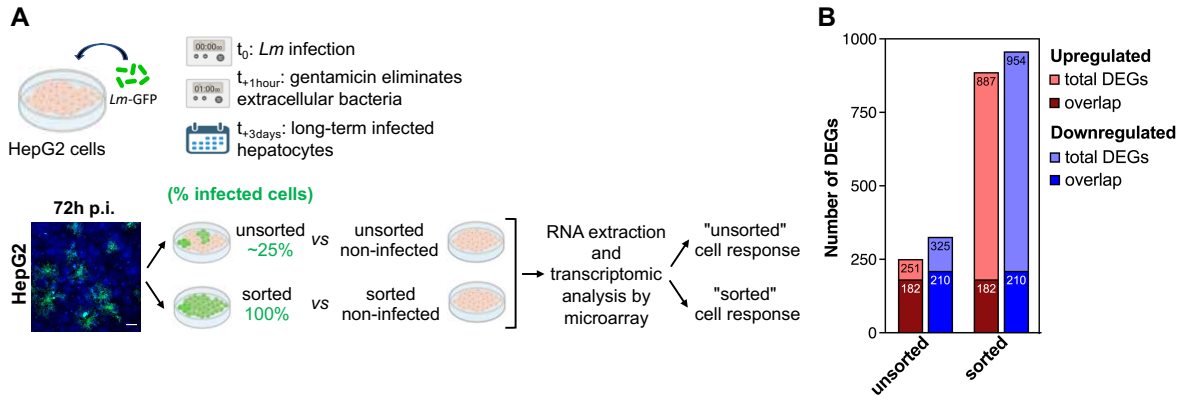
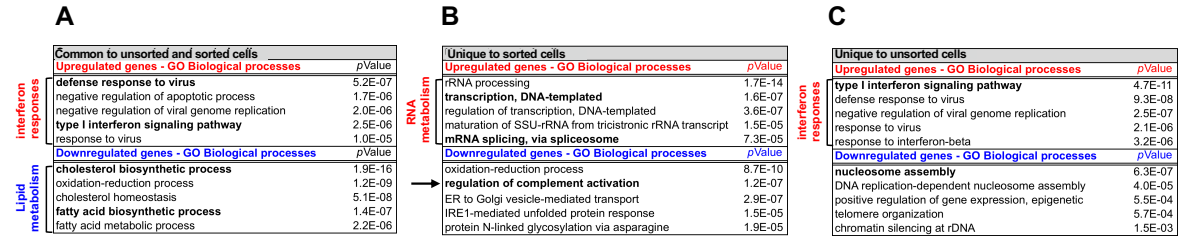


Figure 36 | Transcriptional analysis of HepG2 cells infected with *Lm* for 72 h with or without sorting procedures.
(**A**) Overview of the experimental procedure employed to obtain pure ("sorted") or mixed ("unsorted") populations of long-term *Lm*-infected cells. (**B**) Histograms represent the number of DEGs that are upregulated or downregulated in infected cells compared to NI controls (p-value <0.05 and |fold-change| >1.4), in either unsorted or sorted cell populations. The number of DEGs common to both datasets, or specific to each dataset, are indicated.



A — Common to unsorted and sorted cells

	Upregulated genes - GO Biological processes	pValue
interferon responses	**defense response to virus**	5.2E-07
	negative regulation of apoptotic process	1.7E-06
	negative regulation of viral genome replication	2.0E-06
	type I interferon signaling pathway	2.5E-06
	response to virus	1.0E-05
	Downregulated genes - GO Biological processes	pValue
Lipid metabolism	**cholesterol biosynthetic process**	1.9E-16
	oxidation-reduction process	1.2E-09
	cholesterol homeostasis	5.1E-08
	fatty acid biosynthetic process	1.4E-07
	fatty acid metabolic process	2.2E-06

B — Unique to sorted cells

	Upregulated genes - GO Biological processes	pValue
RNA metabolism	rRNA processing	1.7E-14
	transcription, DNA-templated	1.6E-07
	regulation of transcription, DNA-templated	3.6E-07
	maturation of SSU-rRNA from tricistronic rRNA transcript	1.5E-05
	mRNA splicing, via spliceosome	7.3E-05
	Downregulated genes - GO Biological processes	pValue
	oxidation-reduction process	8.7E-10
	regulation of complement activation	1.2E-07
	ER to Golgi vesicle-mediated transport	2.9E-07
	IRE1-mediated unfolded protein response	1.5E-05
	protein N-linked glycosylation via asparagine	1.9E-05

C — Unique to unsorted cells

	Upregulated genes - GO Biological processes	pValue
interferon responses	**type I interferon signaling pathway**	4.7E-11
	defense response to virus	9.3E-08
	negative regulation of viral genome replication	2.5E-07
	response to virus	2.1E-06
	response to interferon-beta	3.2E-06
	Downregulated genes - GO Biological processes	pValue
	nucleosome assembly	6.3E-07
	DNA replication-dependent nucleosome assembly	4.0E-05
	positive regulation of gene expression, epigenetic	5.5E-04
	telomere organization	5.7E-04
	chromatin silencing at rDNA	1.5E-03

Figure 37 | Functional annotation of DEGs identified upon long-term *Lm* infection in sorted and unsorted HepG2 cells.
The DAVID functional annotation tool was employed to identify enriched GOBPs in up- or downregulated gene sets common to sorted and unsorted long-term infected HepG2 cells (**A**), unique to sorted cells (i.e., a pure population of infected cells) (**B**), or unique to unsorted cells (i.e., a mixed population of infected and NI cells) (**C**). The 5 most significantly enriched biological processes, classed according to *p*-value, are shown.

Functional gene analysis showed that the responses common to sorted and unsorted infected cells included the upregulation of gene networks involved in IFN responses ("defense response to virus" and "type I IFN signaling pathway") and the downregulation of genes involved in lipid metabolism (cholesterol and fatty acid-associated metabolism) (Figure 37A). Analysis of the specific responses of sorted infected cells revealed an upregulation of RNA metabolism pathways and a downregulation of gene networks involved in the "regulation of complement activation" (Figure 37B). These results suggested a transcriptional-mediated inhibition of the complement system specifically in *Lm*-infected hepatocytes which we further established in additional hepatocyte models, as detailed in Part 1 of the Results section.

Finally, the analysis of the pathways specifically deregulated in unsorted infected cells highlighted upregulated GOBPs exclusively related to the interferon response and host defence responses to viruses (Figure 37C). These later results suggested that in a mixed population of infected and uninfected cells, there were responses specific to uninfected "bystander" cells that are important to consider, as presented below.

The contribution of uninfected bystander cells to interferon responses during long-term *Lm* infection of hepatocytes

With respect to upregulated genes, we noted that the IFN response was a signalling pathway significantly enriched in unsorted cells (Figure 37C) compared to sorted cells (Figure 37B). We therefore further compared the IFN response in the sorted and unsorted cell population, by identifying ISGs common or unique to the transcriptome datasets, using the Interferome database (Rusinova et al., 2013). Of the 182 DEGs which were common to sorted and unsorted HepG2 datasets, 13 (7%) encoded ISGs, indicating that IFN responses were activated in both the sorted pure population of infected cells and the unsorted heterogeneous mixed population of infected and uninfected cells. However, the proportion of upregulated ISGs was much higher in unsorted cells: ISGs accounted for 13% of the upregulated DEGs in unsorted cells, compared to only 1.5% in sorted cells. Moreover, of the 69 DEGs which were specifically induced in the unsorted cell population, 29% (20) were ISGs, whereas not a single ISG was identified in the 705 upregulated DEGs that were specific to sorted cells (Figure 38A).

In addition, the expression levels of ISGs upregulated in both unsorted and sorted cells were higher in unsorted than in sorted cells (Figure 38B, left). The expression levels of the 13 ISGs identified through the Interferome database are shown, as well as *CCL5*, an ISG induced during *Lm* infection, whose expression is regulated by both NF-κB and IRFs (Casola et al., 2001; Lebreton et al., 2011). Of note, upregulated ISGs unique to unsorted HepG2 cells include *IFNL1*, encoding the type III interferon IFNλ1, genes encoding proteins involved in IFN-I/III signalling (*IRF9*, *STAT1*), as well as *DDX58* that encodes RIG-I, an important intracellular PRR in epithelial cells (Figure 38B, right).

Overall, these results suggest that in heterogeneous populations of infected and uninfected cells, IFN secondary responses, consisting in the induction of ISGs and the *IFNL1* gene (which is itself an ISG), occur primarily in uninfected bystander cells during long-term *Lm* infection. They also suggest that IFN responses are specifically attenuated by an active mechanism of repression in infected cells.

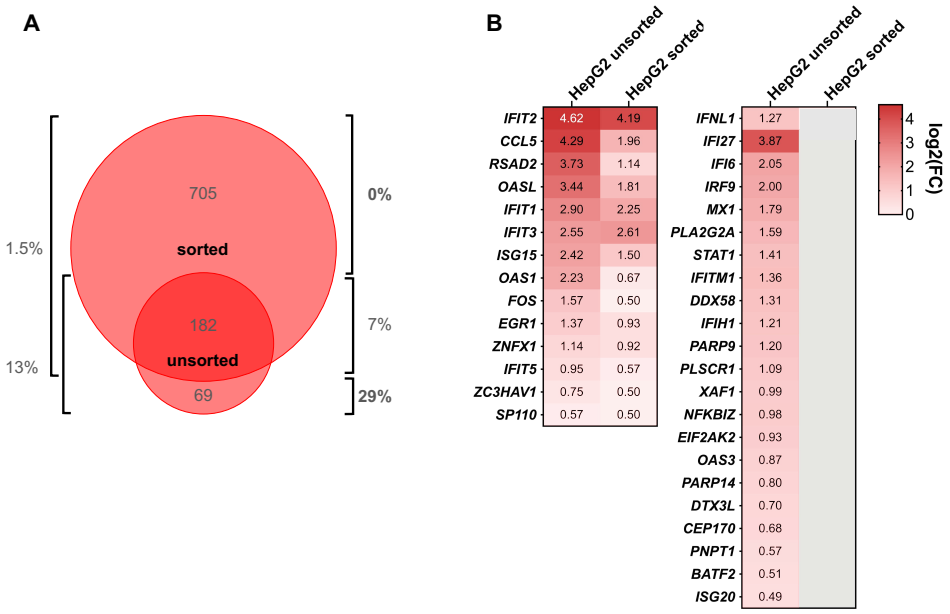


Figure 38 | Uninfected bystander cells appear to be the major contributors to the interferon response during long-term *Lm* infection.
(**A**) Proportional Venn diagram showing the intersection of upregulated DEGs in unsorted and sorted HepG2 cells infected for 72 h with *Lm*. The number exterior to the circles indicates the percentage of ISGs in each DEG set. (**B**) Heatmap displaying log2(FC) of *IFNL1* and ISGs significantly upregulated in infected unsorted and sorted HepG2 cells, relative to their corresponding NI controls.

The BAHD1-MIER complex contributes to the infection-dependent repression of *IFNL1* and ISGs in HepG2 cells

Interestingly, *BAHD1*, *MIER1*, and *MIER3* were among the DEGs that were specifically upregulated in sorted infected HepG2 cells, suggesting that intracellular *Lm* infection impacts the expression of these genes and that their products may play a role during hepatocyte infection. As BAHD1 is known to repress ISGs during *Lm* infection of LoVo cells (Lebreton et al., 2011), we looked for a similar effect in HepG2 cells, and also investigated the involvement of MIER1 and MIER3. With this aim, we sought to deplete HepG2 cells of components of the BAHD1-MIER complex, by an approach using siRNAs. A pool of siRNAs targeting both *BAHD1*, *MIER1*, and *MIER3*, was applied 2 days before infection and during the 3-day infection, resulting in a decrease in *BAHD1*, *MIER1*, and *MIER3* transcript levels by approximately 70–85% (Figure 39A). At the same time, we examined the expression of *IFNL1* and seven representative ISGs. Combined siRNA KD of BAHD1-MIER1/3 in long-term infected HepG2 cells resulted in an approximately 4-fold increase in *IFNL1* expression, as well as a 2- to 5-fold increase in the expression of representative ISGs (*CCL5*, *RSAD2, IFIT1*, *IFI27*, *IFI6*, *MX1*, *STAT1, OAS3*), relative to infected cells treated with control siRNA (Figure 39B).

These results, while requiring confirmation through complementary approaches, provide the first evidence for a role of the BAHD1-MIER chromatin-repressive complex in attenuating IFN responses in hepatocytes hosting intracellular *Lm*.

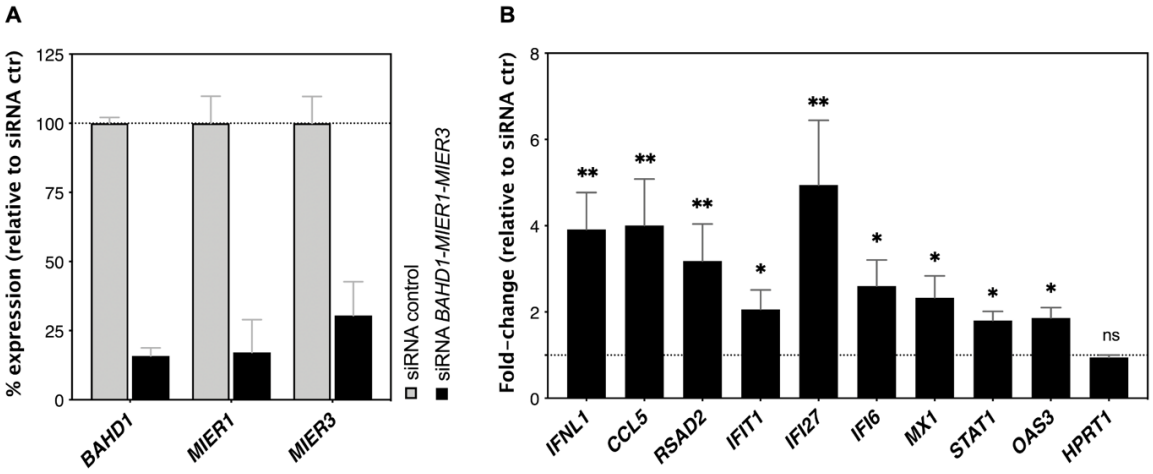


Figure 39 | BAHD1, MIER1 and MIER3 contribute to the repression of ISG expression in HepG2 cells during long-term *Lm* infection.
(**A**) RT-qPCR validation of *BAHD1*, *MIER1*, and *MIER3* combined KD in long-term infected HepG2 cells pre- and post-treated with siRNA targeting *BAHD1*, *MIER1*, and *MIER3*. (**B**) RT-qPCR quantification of *IFNL1* transcripts and representative ISGs (*CCL5*, *RSAD2, IFIT1*, *IFI27*, *IFI6*, *MX1*, *STAT1, OAS3*) in long-term infected HepG2 cells pre- and post-treated with siRNA targeting *BAHD1*, *MIER1*, and *MIER3*. *HPRT* is included as an unregulated control gene. All values represent mean ± SD (n=4). Statistical significance was determined by ratio paired *t* test (* $p<0.05$, ** $p<0.01$, ns: non-significant).

Discussion

The transcriptomics data obtained by microarray analysis of the response in "unsorted" versus "sorted" cells that is presented here is unique in that it provides an insight into the role of uninfected "bystander" cells in the host response to long-term *Lm* infection. In addition, it allowed the identification of genes and pathways that are unique to long-term LisCV stage *Lm*-infected cells, such as genes involved in the complement system and genes involved in chromatin-remodelling (i.e., *BAHD1*, *MIER1*, *MIER3*). It thus highlighted the need for a sufficiently homogeneous population of infected cells in order to accurately profile the specific host cell response to infection. This led us to optimise cell seeding and infection protocols in order to achieve this objective without the use of FACS, as this procedure could not be employed in my host laboratory due to the absence of flow cytometry facilities in level 2 biosafety conditions.

Surprisingly, while the number of deregulated genes was about 3-fold higher in homogeneously infected "sorted" cells, compared to unsorted cells, only 13 upregulated genes were annotated as ISGs in the Interferome database, compared to 33 in unsorted cells. The magnitude of induction was also lower in the pure infected cell population. This suggests that the transcriptional response in uninfected "bystander" cells during bacterial infection could contribute significantly to the global host response. These observations also suggest the existence of paracrine or juxtacrine signalling between infected cells and adjacent or neighbouring uninfected cells. This form of signalling termed "bystander activation" has been observed in both viral and bacterial infections and is a particular form of innate immunity adaptation. Bystander activation during *Lm* infection has been shown to occur through cell-contact dependent mechanisms in epithelial cells, resulting in chemokine secretion (Kasper et al., 2010). More surprisingly, in macrophages, IFN-I expression was induced through paracrine signalling in the form of secreted extracellular vesicles containing bacterial DNA (Nandakumar et al., 2019). Bystander activation is particularly important when infection represses the activation of innate signalling in infected cells, as we observed for IFN expression in hepatocytes. Exploring if infected hepatocytes can signal to uninfected hepatocytes to induce IFN-III expression or if bystander expression is merely secondary IFN signalling induced from a first wave produced by infected cells merits attention.

The BAHD1-mediated repression of ISGs and *IFNL2* in a subset of epithelial cells has previously been reported in colon epithelial cells (Lebreton et al., 2011). We have expanded

on this result and report the BAHD1-mediated repression of a subset of ISGs and *IFNL1* in hepatocytes. Moreover, the upregulation of BAHD1-MIER proteins has never before been observed upon microbial stimulus, to our knowledge. The specificity of this upregulation to the pure infected cell population is testimony to the utility of FACS to obtain homogeneously infected cells. The infection-specific transcriptional upregulation of BAHD1-MIER components, combined with the infection-specific repression of *IFNL1* and ISGs and the role of the BAHD1-MIER complex in their repression suggests that BAHD1-MIER complex-mediated repression is induced uniquely by the intracellular presence of *Lm*, and cannot be induced through paracrine or juxtacrine signalling.

The mechanism by which *Lm* infection triggers BAHD1 recruitment to ISGs is not yet established and would be an interesting perspective to pursue. Interestingly, the protozoan *Toxoplasma gondii* – an intracellular pathogen that, like *Lm*, targets the liver and the brain – was found to partially abrogate the expression of the majority of IFN-γ induced genes during infection through HDAC-mediated repression at IFN-γ-responsive promoters (Lang et al., 2012). Work by Olias *et al*. showed that the *T. gondii* secretory protein "*Toxoplasma* Inhibitor of STAT1-dependent Transcription" (TgIST), translocates to the host cell nucleus where it recruits NuRD to STAT1-dependent promoters to block ISG expression in IFN-γ-activated macrophages (Olias et al., 2016). As described in the Introduction of this thesis (section C.5.1), BAHD1 is a chromatin-remodelling complex that shares structural and functional similarities with NuRD. *Lm* thus shows convergence with *T. gondii* through the common modulation of IFN responses via the targeting of functionally related chromatin-remodelling complexes, BAHD1 and NuRD. Lebreton et al. (Lebreton et al., 2012) proposed a model linking the BAHD1 complex to STAT1/2, via its components HP1 and KAP1, which are known to interact with STAT transcription factors (Lebreton et al., 2011). In the case of *Lm*, however, the virulence factor LntA has been identified and found to alleviate BAHD1-mediated repression of ISG expression; it is not known whether a distinct virulence factor acts in a divergent manner to activate BAHD1 repression (as in the case of *T. gondii*-mediated recruitment of NuRD), or whether the recruitment of BAHD1 to ISG promotors and subsequent ISG repression is a cell autonomous response to *Lm* infection.

Material and Methods

Bacterial strains and cell lines

Lm wild type strain EGDe (BUG1600) (Bécavin et al., 2014) and the mutant strain EGDe-GFP (BUG2538) (Balestrino et al., 2010) were used in experiments presented in this Results section. Strains were grown on brain-heart infusion (BHI)-agar plates or in BHI at 37°C under shaking. The human hepatocellular carcinoma cell line HepG2 (ATCC HB-8065) was grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 2 mM L-glutamine (Sigma) and 10% foetal bovine serum (FBS, Sigma) at 37°C in a humidified 5% CO2

Bacterial infection

HepG2 cells were infected as described (Kortebi et al., 2017) at an MOI of 1–5. In order to ensure maximum invasion and infection, cells were centrifuged with the inoculum at 300 × g for 2 minutes and incubated for 1 h, before the addition of gentamicin 100 µg/ml for 10 minutes, followed by the addition of gentamicin 25 µg/ml for the entire duration of the infection to eliminate extracellular bacteria. Intracellular bacterial counts and immunofluorescence were performed at 24 h and 72 h p.i. to verify infection progression.

Cell sorting

HepG2 cells were grown in T25 cell flasks and infected with EGDe-GFP at an MOI of 1–5 or remained NI. At 72 h p.i., a batch of flasks was used to determine bacterial loads by CFU counts after cell lysis in water and remaining flasks were processed for RNA extraction (unsorted cells) or washed in PBS, trypsinised, resuspended in DMEM and subjected to cell sorting using a FACSAria II Cell Sorter (BD Bioscience) (sorted cells). Infected cells were selected based on GFP fluorescence; ~ 5×10^5 pure GFP-positive cells were collected from each of 3 independent experiments. Similar numbers of control NI cells from parallel experiments were also sorted. Sorted cells were immediately placed in the RNeasy Mini Kit RLT buffer (Qiagen) containing 10 µl β-mercaptoethanol to preserve RNA from degradation. A flask of infected cells was used for microscopic examination of GFP-positive and GFP-negative cells.

RNA extraction and microarray analysis

Following the infection and sorting protocols, total RNA was extracted from cells infected or NI, sorted or unsorted, using the RNeasy Mini Kit (Qiagen) and genomic DNA was removed using TURBO DNA-free™ kit (Ambion), according to the manufacturer's instructions. RNA

concentration and purity were assessed using Experion^{TM} automated electrophoresis system and reagents (BioRad). For gene expression analysis, 50 ng total RNA was reverse transcribed and amplified using the Ovation Pico WTA System v2 (Nugen Technologies #3302-12), fragmented and biotin labelled using the Encore Biotin Module (Nugen Technologies #4200-12) following the protocol provided by the manufacturer. Gene expression was determined by hybridization of the labelled template to HuGene 1.0 ST microarrays (Affymetrix). Hybridization cocktail and post-hybridization processing was performed according to the "Target Preparation for Affymetrix GeneChip® Eukaryotic Array Analysis" protocol found in the appendix of the Nugen protocol of the fragmentation kit. Arrays were hybridized for 18 hours and washed using fluidics protocol FS450_0007 on a GeneChip Fluidic Station 450 (Affymetrix) and scanned with an Affymetrix Genechip Scanner 3000, generating CEL files for each array. Quality assessment and normalization of the arrays was performed with the tools available in Expression Console v1.1 (Affymetrix) and Bioconductor packages in R. Gene-level expression values were derived from the CEL file probe-level hybridization intensities using the model-based Robust Multichip Average algorithm (RMA) (Bolstad et al., 2003). RMA performs normalization, background correction and data summarization. Local-Pooled-Error test *(LPE test)* was performed to statistically evaluate the significance of each gene's differential expression (Jain et al., 2003), and differentially expressed genes were selected based on a *p*-value threshold of $p < 0.05$. P-values were corrected for multiple comparisons using Benjamini & Hochberg's method to control the false discovery rate (Benjamini and Hochberg, 1995). The microarray data generated can be found in the Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE53285.

Functional gene analysis and ISG identification

Over-representation analysis was performed on upregulated or downregulated DEGs common or unique to the infected sorted or unsorted HepG2 transcriptomes using DAVID (http://david.abcc.ncifcrf.gov, version 6.8) (Huang et al., 2009). ISGs were determined using the interferome database (Rusinova et al., 2013), using the following criteria: "liver", "hepatocytes", and "*Homo sapiens*" were selected to determine the organ, cell system, and species, respectively. The database was probed uniquely for ISGs with a fold change value greater or equal to 4.

siRNA treatment

HepG2 cells were seeded on collagen coated plates one day prior to forward siRNA transfection using an equimolar mix of Dharmacon ON-TARGET plus siRNAs targeting *BAHD1* (J-020357-09), *MIER1* (J-014201-08), and *MIER3* (J-015618-08), or control non-targeting siRNA (D-001810-10-05) (Thermo Scientific) at a final total concentration of 9 nM. Cells were infected 48 h later and forward transfected for a second time 6 h p.i. using the same protocol.

RT-qPCR

RNA extraction and RT-qPCR were performed as described in Part 1 of the Results section. Briefly, RNA was extracted using the RNeasy Mini Kit (Qiagen) and genomic DNA was removed using TURBO DNA-free™ kit (Ambion), according to the manufacturer's instructions. 1 µg of total RNA was used for reverse transcription with the LunaScript™ RT SuperMix Kit (NEB). Quantitative Real-Time PCR was performed on StepOne Plus Real-Time PCR Systems (Applied Biosystems) using Luna® Universal qPCR Master Mix (NEB) as specified by the supplier. Relative expression of target genes was calculated from cycle threshold (CT) values using the comparative CT (ΔΔCT) method with untreated or NI cells used as calibrator (reference) samples. Target gene expression levels were normalized to *B2M*. Statistical significance was determined using a two-tailed ratio paired *t* test performed on ΔCT values obtained from biological quadruplicate infection experiments; a p value <0.05 was considered significant. Primer sequences are as follows:

Gene symbol	Gene name	Forward sequence	Reverse sequence
B2M	beta-2-microglobulin	CACCCCCACTGAAAAAGATG	ATATTAAAAAGCAAGCAAGCAGAA
BAHD1	bromo adjacent homology domain containing 1	AGTGTTGCCCCTGTCTGTTA	GAGAGGAAATTCCAACTGGC
CCL5	C-C motif chemokine ligand 5	CCCAGCAGTCGTCTTTGTCA	TCCCGAACCCATTTCTTCTCT
HPRT1	hypoxanthine phosphoribosyltransferase 1	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT
IFI27	interferon alpha inducible protein 27	TCTGCCGTAGTTTTGCCC	ATCATCTTGGCTGCTATGGAG
IFI6	interferon alpha inducible protein 6	CCCAAGTAGGATTACAAGCATG	GGTTGTTGGGGAGAGTGATAG
IFIT1	interferon induced protein with tetratricopeptide repeats 1	CTTGGGTTCGTCTACAAATTGG	AAAGTGGCTGATATCTGGGTG
IFNL1	interferon lambda 1	GAAGCAGTTGCGATTTAGCC	GAAGCTCGCTAGCTCCTGTG
MIER1	MIER1 transcriptional regulator	CAGTGGCTGTAGTGGGGAAAA	GGCATCTTGAGAAGCAACAGA
MIER3	MIER family member 3	CTGCATGGACGGAAGAAGAA	ATGCTACACACTCAGCAACT
MX1	MX dynamin like GTPase 1 (interferon-induced GTP-binding protein Mx1)	GAAGATAAGTGGAGAGGCAAGG	CTCCAGGGTGATTAGCTCATG
OAS3	2'-5'-oligoadenylate synthetase 3	GTGTGGACTTTGATGTGCTG	CCCGTTGTAGCTCTGTGAAG
RSAD2	radical S-adenosyl methionine domain containing 2	AGAATACCTGGGCAAGTTGG	GTCACAGGAGATAGCGAGAATG
STAT1	signal transducer and activator of transcription 1-alpha/beta	ACCGCACCTTCAGTCTTTTC	GCAATTTCACCAACAGTCTCAAC

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DISCUSSION AND PERSPECTIVES

Lm has been a paradigm for the study of host-pathogen interactions since the early 1980s and the use of this model bacterium in cellular microbiology has not only been at the forefront of the elucidation of the host response to intracellular infection, but also at the origin of the discovery of previously unidentified host factors and novel mechanisms of mammalian transcriptional regulation. In addition, significant knowledge concerning the immune response has been gained by dissecting the transcriptomic response to *Lm* in phagocytic cells, notably macrophages, during the early and active phase of *Lm* infection. The response in non-immune cells, however, is lacking.

An intravacuolar persistence phase in the life cycle of *Lm* upon long-term infection of hepatocytes was recently identified that raises many questions concerning the eventual reprogramming of host cells to allow the creation and maintenance of this intracellular bacterial niche that could allow *Lm* to persist long term in the hepatic parenchyma in LisCVs. The results of this doctoral thesis contribute to a better understanding of how the hepatocyte transcriptional landscape is remodelled by *Lm* once the bacteria enter this persistence stage and how this response could contribute to long-term intracellular parasitism through the downregulation of the innate immune response. This work is not only unique in addressing the novel question of host adaptation to intracellular long-term parasitism by *Lm*, it is also the first study to address the question of the transcriptional response to *Lm* in hepatocytes. Although the liver is a key target organ of *Lm* during *in vivo* infection, and hepatocytes the principal hepatic replicative niche, little is known of the liver phase of human listeriosis, nor of the role of the liver in asymptomatic carriage or during the lengthy incubation periods documented for invasive listeriosis. We established three cellular infection systems in human and murine hepatocytes that allowed us to establish a common signature of long-term infection that was cell line- and species-independent. These long-term infection models lay the foundation for further *in vitro* characterisation of the host response that we suggest could promote long-term asymptomatic carriage in the liver. We show that long-term infection coincides with a profound reprogramming of hepatic innate immune response genes, with an activation of IFN responses and the repression of crucial hepatocyte-specific innate immune proteins that appears to increase over the time course of infection, resulting in significantly decreased secretion after three days of infection. These results suggest an immune deregulation that could be propitious for long term survival of vacuolar *Lm* in hepatocytes, favouring persistent colonisation of the host.

In vitro models to study *Lm* persistent infection in hepatocytes

In this study, we sought to quantify and dissect the global transcriptional responses to persistent *Lm* infection in human adult hepatocytes and as such strove to establish relevant *in vitro* cellular models. As established hepatocyte cell lines do not always accurately portray the metabolic or immune responses observed *in vivo* or in primary hepatocytes (Brownell et al., 2013; Franko et al., 2019) we used, in parallel, two hepatocyte cell lines of different origin as well as primary mouse hepatocytes (PMH). As we did not choose to employ a single-cell transcriptomics approach, the global transcriptional response obtained would be the averaged gene expression from a bulk population of cells. It was therefore crucial to obtain sufficiently homogeneous cell populations of either viable NI cells or LisCV stage *Lm*-infected cells. To achieve this objective, two strategies were employed: FACS sorting of *Lm* EGDe-GFP infected HepG2 cells was used to obtain a population of 100% infected cells, which could be compared to NI cells, as well as to an unsorted mixed population. The results of the microarray profiling of these cells were part of the preliminary results obtained before my arrival and around which my thesis project was constructed. While this first strategy has several advantages, notably the reproducible acquisition of purely infected cells and the possibility of evaluating the contribution of uninfected bystander cells by comparing sorted and unsorted cell populations, it could also induce cellular stress that impacts transcription. Moreover, it is a complex procedure that requires a FACS facility under biosafety level 2 conditions, and the lack of such facilities in my host laboratory prevented us from employing this strategy.

The second strategy, and the one I implemented, was the optimisation of infection and cell culture protocols to allow sufficient bacterial entry and spreading in order to obtain a homogenously infected cellular monolayer. Three models were generated, each with advantages and disadvantages (Figure 40). HepG2 cells were grown on collagen to allow the formation of a cell monolayer and increased bacterial spreading, although maximal infection was not obtained. The second hepatocyte cell line, Huh7, had the advantage of growing naturally in a monolayer and being very permissive to infection so that at an MOI of 1, almost all cells were infected by 24 h p.i. However, IFN responses were absent in this cell line which excluded their use in any investigation related to this important facet of the host response to *Lm* infection. In regard to primary cells, primary human hepatocytes would have been our first choice, but infection efficiency is low, and the prohibitive costs of these commercially available cryopreserved cells prevented us from optimising the infection protocol to obtain a

homogeneous population of infected cells without the use of FACS. In contrast, PMH could be obtained fresh, at little cost, and we were able to optimise the cell culture and infection protocols to obtain good cellular viability and infection efficiency. On the other hand, using murine hepatocytes also added the parameter of possible species-specific effects. In addition, it is important to note that although primary cells are more physiologically relevant compared to their carcinoma transformed counterparts, residual contamination with non-parenchymal cells during the extraction process is inevitable (Brownell et al., 2013; Jilg et al., 2014). Our RNA-seq data indicated that the expression of a number of genes known to be expressed specifically in innate immune cells was not null in uninfected PMH samples, suggesting that the removal of non-parenchymal cells was, indeed, not complete during the PMH isolation procedure. The expression of cytokines by residual immune cells in primary hepatocyte cultures could also influence hepatocyte gene expression (Crispe, 2016). Finally, the complex isolation procedure and the necessity of freshly isolated PMH also makes their use impractical in exploratory experiments.

To overcome the singularities of any one model, we intersected transcriptome data (DEG sets and functional pathways), allowing us to model a faithful robust transcriptomic signature in LisCV stage *Lm*-infected hepatocytes. Each of the cell lines found its utility in different experimental settings and these models will provide the framework for the continued study of the host response to long-term *Lm* infection. Huh7 cells are unique in the almost pure population of infected cells that they yield and provide an interesting background to study IFN-independent processes. The HepG2 cell line is fundamental to examining the role of IFNs in *Lm* infection, particularly IFN-III, and that of host factors controlling IFN-responses, such as BAHD1. Finally, FACS sorting of infected cells gave a first insight into the role of uninfected "bystander" cells in the host response to infection.

The importance of transcriptional regulation in the host response to long-term *Lm* infection

The different cellular models and the strategies employed resulted in a more or less homologous population of infected cells from which we were able to obtain a global transcriptomic signature (Figure 40A). Infection profoundly modified the transcriptional landscape, with the number of DEGs identified roughly correlating to the proportion of infected cells (Figure 40B). This is consistent with other transcriptional studies; in liver samples from

infected mice, for example, a high correlation (r=0.95) was observed between the bacterial load and the number of deregulated genes (Dieterich et al., 2008).

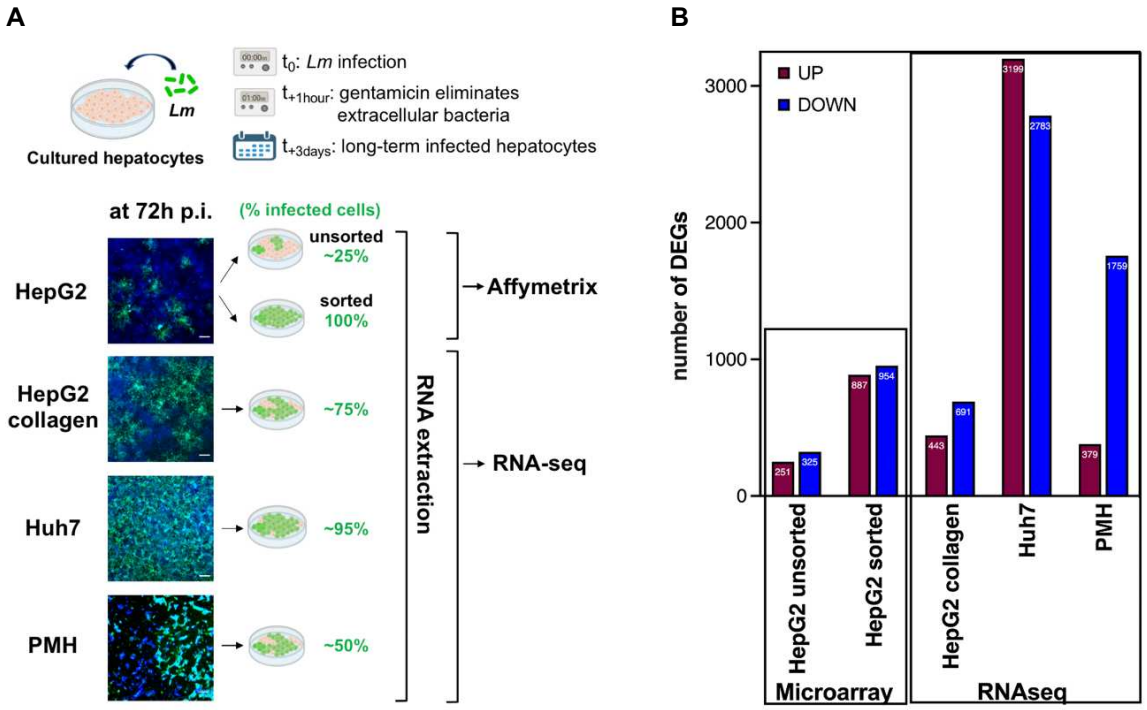

Figure 40 | Overview of the hepatocyte *Lm* infection models and the number of DEGs identified using each cellular model and strategy.
(**A**) Overview of the *in vitro* long-term *Lm* infection models. Very low magnification (2.5×) immunofluorescence microscopy images showing *Lm* (green) and cell nuclei (blue) allow an estimation of the proportion of infected cells (indicated to the right); scale bar: 200 µm. (**B**) Histograms represent the number of upregulated and downregulated DEGs identified in each of the hepatocyte models and strategies employed.

In the Huh7 cell model in which we observed the most effective *Lm* infection, the number of significant DEGs (both up- and downregulated, adj. $pV < 0.05$, $|\log2(FC)| > 0.5$) amounted to a total of 5982 genes, representing 30% of the 19,936 protein-coding genes of the current Ensembl annotation for *Homo sapiens*. In line with this profound reprogramming of gene expression, an incredibly large proportion (38%) of upregulated genes encode nuclear proteins (compared to 13% of downregulated genes), of which 17% are known epigenetic factors, suggesting the importance of transcriptional and possibly epigenetic regulation in the hepatocyte host response to *Lm*.

Most of the studies examining the host response to *Lm* over an infection time course *in vitro* have identified an increasing number of genes as the infection progresses, although none of these studies proceeded beyond the 24 h timepoint (Besic et al., 2020; McCaffrey et al., 2004;

Pontiroli et al., 2012; Popov et al., 2006). We addressed the kinetics of the transcriptional response in the Huh7 cell line by examining the global transcriptional response at an earlier timepoint (24 h) and observed both an important increase in the number of deregulated genes (3-fold) as well as the magnitude of gene deregulation from day 1 to day 3.

Inhibition of complement and coagulation components and APP expression in long-term infected hepatocytes

Hepatocytes play a major role in the immune response as the major source of circulating APPs, crucial innate immunity molecules of diverse function, including pathogen detection and clearance, haemostasis, as well as the regulation of the inflammatory and adaptive immune responses during infection (Zhou et al., 2016). Opsonins, complement cascade components, fibrinogen and other APPs are expressed constitutively by hepatocytes but their expression is also induced by pro-inflammatory cytokine stimulation during listeriosis (Khafaga et al., 2021; Kopf et al., 1994; Kummer et al., 2016). Bacteria have developed multiple and highly diverse mechanisms to resist complement activation, and inhibit fibrin formation or haemostasis, illustrating the importance of the complement and coagulation systems in innate defence (Ermert et al., 2019; Figueroa and Densen, 1991; Nelson et al., 2011). The *Streptococcus pyrogenes* virulence factor SpeB, for example, targets for degradation several APP and complement factors, including C3, fibrinogen, plasminogen, vitronectin, fibronectin, and alpha-2-macroglobulin (Nelson et al., 2011).

In this work, by intersecting results from all three hepatocyte models, we show that long-term *Lm* infection profoundly impacts both the constitutive and cytokine stimulated expression of hepatocyte-specific APP genes at both the transcript and protein levels. This result is novel in that the transcriptional downregulation of complement, coagulation or other innate immunity related APPs has not, to our knowledge, been previously reported in response to intracellular bacterial infection of hepatocytes.

Pathogen hepatotropism is generally considered the domain of hepatic viruses such as HCV and hepatitis B virus (HBV) as well as parasites such as *Plasmodium* spp. in their sporozoite form (Protzer et al., 2012). Hepatocyte cell lines are thus frequently used to study the host response to these pathogens. On the other hand, the hepatocyte response to facultative intracellular bacterial pathogens with liver stages (i.e., *Lm, Salmonella enterica* serovar Typhimurium*, Francisella tularensis, Brucella* spp.*, Burkholderia pseudomallei, Klebsiella*

pneumoniae) (Conlan and North, 1992; Talwani et al., 2011; Wisplinghoff and Appleton, 2008) has not been extensively investigated. The down regulation of complement protein expression at the transcriptional level has been observed in HCV-infected hepatocytes and in liver biopsies of patients chronically infected with HCV. Transcriptional deregulation results in reduced protein levels in the serum and liver biopsy samples of chronically infected patients, indicating that infection can negatively affect complement levels even in a physiological context involving paracrine or endocrine stimulation of hepatocytes by immune cells (Banerjee et al., 2011; Mazumdar et al., 2012; Patra et al., 2019).

The data we found providing information on APP serum or liver levels in listeriosis attest to APR activation in murine or ovine listeriosis. Several studies observed increased levels of serum amyloid A-1 (SAA1), SAA2, alpha-1-acid glycoprotein 1 (ORM1), ORM2, amyloid P-component, serum (APCS), and haptoglobin (HP) (Khafaga et al., 2021; Kopf et al., 1994; Kummer et al., 2016). With the exception of HP, however, none of these APPs were highly downregulated in any of our models (SAA1/2 were not downregulated in any model, and ORM1/2 were modestly downregulated uniquely in Huh7). The impact of *Lm* infection on the circulating or hepatic levels of the APPs for which we observed important transcriptional downregulation, for example, C3, vitronectin, or various apolipoproteins, remains unclear.

The physiological consequences of a reduction in both cellular and circulating complement components on the outcome of listeriosis *in vivo* or on intracellular bacterial degradation *in vitro*, for example, could be multiple and deserve further attention. C3, in particular, was downregulated in all our hepatocyte models, and is a central component of the complement cascade, with pleiotropic functions. A decrease in serum C3 levels could negatively affect the adaptive immune response to *Lm* infection as optimal CD8 and CD4 T cell expansion and contraction requires C3 (Nakayama et al., 2009; Tan et al., 2014). While innate clearance and killing of *Lm* is not impaired in the absence of opsonisation – indeed, intravascular *Lm* is more efficiently cleared in the absence of C3 – opsonisation favours *Lm* uptake by the spleen (Broadley et al., 2016; Verschoor et al., 2011). C3 opsonisation is necessary for the formation of *Lm*-platelet complexes that promote bacterial targeting to CD8a+ DCs in the spleen, whereas free *Lm* are cleared by KCs in the liver through scavenger receptor (SR) binding (Figure 41). Uptake of *Lm* by splenic DCs is necessary for efficient T cell priming (van Lookeren Campagne and Verschoor, 2018; Verschoor et al., 2016), which is necessary for sterilising immunity to *Lm* (Pamer, 2004). On a cellular level, C3 opsonisation has also recently been shown to favour

autophagy of *Lm* by epithelial cells and to favour listerial clearance in the intestine (Sorbara et al., 2018).

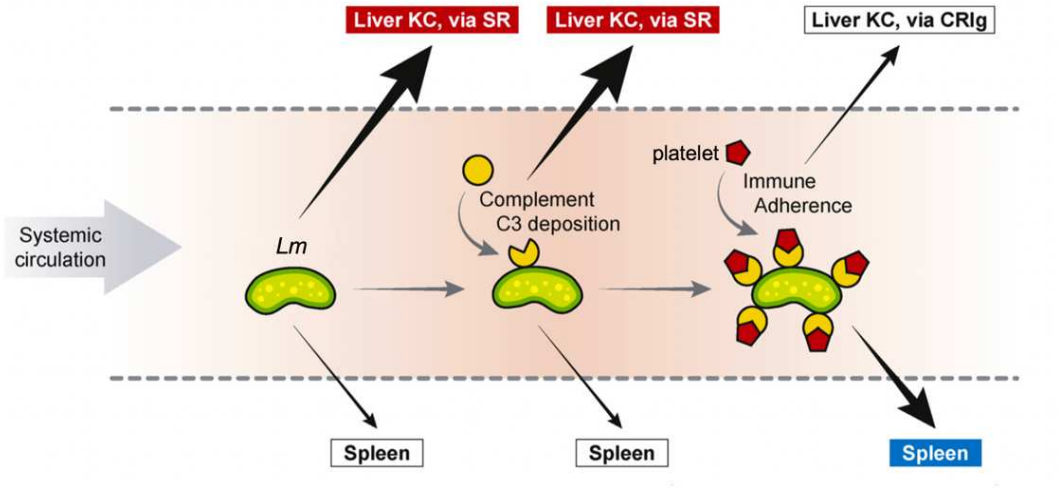

Figure 41 | The role of C3 opsonisation in the dual clearance dynamics of intravascular *Lm*.
In the absence of C3 opsonisation, *Lm* uptake is rapidly mediated by KCs in the liver through SR binding. C3 opsonisation, on the other hand, results in immune adherence or the formation of *Lm*-platelet complexes which favours a slower uptake by DCs in the spleen, promoting efficient T-cell priming. (Adapted from van Lookeren Campagne and Verschoor, 2018)

The deregulation of coagulation cascade proteins could also impact the outcome of listeriosis: fibrin, polymerised fibrinogen, serves as a scaffold for the adherence and migration of cells, and is particularly important in the liver where it plays a protective role during listeriosis to limit bacterial spread (Antoniak, 2018; Mullarky et al., 2005).

Regarding other APPs, the impaired innate immune response to *Lm* infection resulting in reduced survival of *ApoE* deficient mice can be cited as another example of how a decrease in APP expression could impact the host response to this pathogen (Roselaar and Daugherty, 1998). In this regard, it is interesting to note that the expression of several apolipoproteins (i.e., APOA1, APOA4, APOC1, APOC3, APOE, APOH), whose functions are at the crossroads of lipid metabolism and innate immunity, are inhibited by long-term *Lm* infection of hepatocytes.

Downregulation of cholesterol metabolism genes in long-term infected human hepatocytes

The association between cholesterol homeostasis and infection or inflammation has long been acknowledged: decreased plasma cholesterol levels during infection was first described in 1917 by W. Denis and correlate with infection severity (Denis, 1917; Feingold et al., 2000; Shimizu et al., 2018). Moreover, it has been suggested that low total cholesterol and low HDL (high

density lipoprotein) levels increase the chance of developing an infection and that, inversely, high cholesterol levels protect against bacterial infections (Feingold and Grunfeld, 2012). Hepatocytes are the major source of endogenous cholesterol *de novo* biosynthesis, which is esterified and stored or secreted into the blood as constituents of lipoproteins (Luo et al., 2019). In human Huh7 and HepG2 hepatocytes, the cholesterol biosynthesis pathway was the most significantly affected pathway identified by functional annotation using both IPA and DAVID. Almost all genes of the pathway were downregulated at 72 h in Huh7 and HepG2 (Figure 7, Descoeudres et al., 2021), and in Huh7 cells, all but four genes downregulated at 72 h were already downregulated at 24 h (Figure 31). As mentioned above, a large number of apolipoproteins, the protein fraction of lipoproteins and highly expressed by hepatocytes, were downregulated in all three models. In contrast, cholesterol biosynthesis pathway genes were not significantly perturbed in PMH on the whole. This difference with human cell lines may result from either the metabolic disparity between primary cells and carcinoma-derived cell lines or a species-specific response to infection. Indeed, in humans and other primates, infection decreases cholesterol serum levels while in non-primates such as rodents, rabbits and dogs, endotoxins and pro-inflammatory cytokines increase serum cholesterol (Hardardóttir et al., 1995).

Many pathogens have evolved mechanisms to increase cholesterol metabolism in host cells in order to establish infection: in hepatocytes *Plasmodium* spp. – auxotrophic for certain lipid species and metabolites, such as cholesterol – induces increased cellular cholesterol biosynthesis (Zuzarte-Luis and Mota, 2020). *Leishmania donovani* infection activates sterol regulatory element-binding protein 2 (SREBP2) – the master regulator of cholesterol and fatty acid metabolism (Luo et al., 2019) – to upregulate cholesterol biosynthesis, and modulates membrane cholesterol in order to facilitate entry and survival in macrophages (Mukherjee et al., 2014). Cholesterol is necessary to support viral replication, and cellular cholesterol uptake and synthesis by the host cell is increased during viral infection by members of the *Flaviviridae* family (i.e., Dengue, Zika, Yellow Fever, West Nile viruses (Osuna-Ramos et al., 2018), and HCV (Feingold and Grunfeld, 2012)). Host cholesterol trafficking pathways are also manipulated by both obligate and facultative intracellular bacteria in order to access nutrient-rich vesicles or to acquire membrane components for the bacteria or the bacteria-containing vacuole (Lai et al., 2013; Samanta et al., 2017).

The effects of *Lm* infection-mediated cholesterol metabolism gene reprogramming in hepatocytes deserves further investigation given the recently elucidated mechanism of

antibacterial defence that involves infected cells limiting plasma membrane cholesterol to reduce *Lm* entry and cell-to-cell spread (Abrams et al., 2020; Zhou et al., 2020), the role of cholesterol in inflammasome activation in macrophages (Dang et al., 2017), as well as the importance of cholesterol in the establishment of vacuolar niches and persistent infection described for a variety of intracellular bacteria (Morey and Meyer, 2019; Pandey and Sassetti, 2008; Samanta et al., 2017).

Mechanisms of regulation behind the transcriptional downregulation of APP and cholesterol genes

Implication of host transcription factors

How *Lm* infection interferes with the expression of APP-coding genes and cholesterol biosynthesis-associated genes, and whether this is a *Lm*-driven mechanism or a general response to cell invasion remains unclear. Ingenuity Upstream Regulator Analysis (URA) of the common gene sets revealed multiple TFs that are known to regulate APP and/or cholesterol homeostasis related genes (Table S13, Descoeudres et al., 2021). As APP downregulation was observed in the absence of cytokine stimulation, and as both cholesterol metabolism and constitutive APP production are part of the basal transcription program of hepatocytes, it seems reasonable to postulate the disruption of the functions of TFs essential for constitutive gene expression in the liver. HNFs are particularly interesting candidates: HNF4α, a nuclear receptor, is a constitutively active TF, and one of the most abundant liver-enriched TFs, contributing to the regulation of almost half of actively transcribed hepatic genes (Odom et al., 2004; Soccio, 2020). HNF4α expression is regulated by the lesser expressed HNF1α as well as HNF6 (ONECUT1) (Lau et al., 2018). Both HNF4α and HNF1α were predicted by URA analysis to be strongly inhibited in long-term *Lm* infection, and are known to play a central role in the transcriptional regulation of all the pathways we observed to be downregulated, including those involved with apolipoprotein synthesis, blood coagulation, and complement protein synthesis and activation (Pontoglio et al., 2001).

HNF4α regulates the activity and expression of other nuclear receptors that regulate lipid and bile acid metabolism, as well as inflammation, notably LXR and FXR (Lu, 2016; Wollam and Antebi, 2011), and PPARA (Soccio, 2020). HNF4α and LXR, in turn, directly regulate SREBP2 activity (Shimano and Sato, 2017), thus directly linking the regulation of cholesterol biosynthesis genes to HNF4α. The lipid-activated nuclear receptors LXR and PPARα are

involved in the regulation of C3 expression in human and rat hepatocytes (Jamali et al., 2010; Li et al., 2005a; Mazumdar et al., 2012; Mogilenko et al., 2013) and both were shown to play a crucial role in the host response to *Lm* as transcriptional regulators in myeloid cells (Abdullah et al., 2012a; Joseph et al., 2004). We performed experiments in Huh7 cells to examine the role of one of these nuclear receptors, PPARα, in APP-coding gene downregulation during *Lm* infection. We found that PPARα activation had no effect on APP gene expression, arguing against a role of this nuclear receptor in the mechanism at play (data not shown).

The mechanisms of APP gene induction, as part of the APR, is better comprehended than that of APP constitutive expression or repression (Asselin and Blais, 2011; Volanakis, 1995). It is well established that cytokine stimulation activates NF-κB and STAT3, as well as other TFs such as AP-1, which, in turn, stimulate APP expression in hepatocytes (Bode et al., 2012; Zhou et al., 2016). These TFs are not thought to play a role in constitutive expression, however: mice deficient in both STAT3 and RELA/p65 had an almost completely abrogated APR to bacterial pneumonia, demonstrating the transcriptional dependence of the APR on these two TFs alone, but no effect on gene expression was observed in the NI double KO mice (Quinton et al., 2012).

Implication of Lm *effectors*

As the secreted bacterial factor InlC inhibits the NF-κB pathway (Gouin et al., 2010), we searched for a role of this effector in the *Lm*-mediated inhibition of APP gene expression. We observed that the downregulation of type I APP genes was partially alleviated during infection with a *Lm*Δ*inlC* mutant, in comparison with the WT strain, suggesting that InlC amplifies the *Lm*-mediated inhibitory effect on some APP genes. However, these experiments were performed in the absence of cytokine stimulation. Thus, the role of InlC could be more important in an inflammatory context, and this hypothesis deserves to be tested.

The question of the contribution of other *Lm* virulence factors in the transcriptional deregulation we observed merits further investigation, through the use of mutant *Lm* strains, as *Lm* could employ mechanisms to actively inhibit APP expression, as observed for HCV. Exogenous expression of HCV proteins *in vitro* is sufficient to reduce both the basal and cytokine-induced expression of C3 and C4 in Huh7 cells and immortalised human hepatocytes, as well as *in vivo* (Banerjee et al., 2011; Mazumdar et al., 2012). The proposed model for the downregulation of C3 involves the HCV-mediated downregulation of the expression of the upstream TF C/EBPβ that binds to the IL-6 response element (Mazumdar et al., 2012). Expression levels of these TFs (C/EBPα, C/EBPβ, and C/EBPγ) was observed to increase

during the APR (Ramji and Foka, 2002). We observed the genes encoding all three isoforms (*CEBPA*, *CEBPB*, *CEBPG*) to be highly expressed in human and murine hepatocytes, and their expression was significantly downregulated in Huh7 cells. Both C/EBPs and AP-1 are involved in the induction of APP gene expression following cytokine induced MAPK pathway activation (Asselin and Blais, 2011). Their role in constitutive APP gene expression, however, is less well characterised. The correlation between reduced *CEBPB* mRNA and phosphorylated C/EBPβ levels and the expression levels of C3 in the absence of cytokine stimulation and independently of MAPK activation (Mazumdar et al., 2012), suggests a role for this TF in basal C3 expression.

Implication of host epifactors

Transcriptional repression of APP-coding genes in the absence of stimulation is known to depend on co-repressor complexes, such as nuclear receptor co-repressor (NCoR) 1 and silencing mediator for retinoid or thyroid-hormone receptors (SMRT or NCoR2) that are involved in chromatin silencing through the recruitment of HDACs and work in cooperation with nuclear receptors (Mottis et al., 2013). Our observation of the inhibition of an entire network of APP genes in response to *Lm* infection could suggest the involvement of other epigenetic mechanisms and epifactors in the form of chromatin-repressive machineries, capable of silencing entire gene networks over large genomic regions, as opposed to specific individual gene repression. One potential candidate epifactor is bromodomain containing 4 (BRD4), a member of the bromodomain and extraterminal (BET) protein family. BRD4's bromodomain binds acetylated lysine residues on histones and its C-terminal domain recruits the positive transcription elongation factor (P-TEFb); BRD4 thus directly stimulates RNA polymerase II-dependent transcription (Morgado-Pascual et al., 2019). BRD4 also contributes to NF-κB activation and regulates the expression of many immunity-associated genes and pathways (Wang et al., 2021). BRD4 inhibition in primary human hepatocytes results in a transcriptomic signature that greatly resembles the one induced by *Lm*: pathways involved in the APR, complement and coagulation cascades, and cholesterol and fatty-acyl biosynthesis are negatively regulated; reduced circulating levels of complement are also observed in patients treated with a BRD4 inhibitor (Gilham et al., 2016; Wasiak et al., 2017). The transcriptional repression of APR genes mediated by BRD4 inhibition is maintained upon IL-6 or IL-1β stimulation (Wasiak et al., 2017; 2020). Interestingly, BRD4 activity is regulated by phosphorylation (Wu et al., 2013), and could thus be regulated by infection-induced cell signalling pathways, in a similar manner to the epifactor SIRT2, the nuclear location of which depends on its infection-induced dephosphorylation (Pereira et al., 2018). The hypothesis of

BRD4 involvement in transcriptional regulation during *Lm* infection could be tested, for example, by determining phospho-BRD4 kinetics over the expression time course.

Both excessive and insufficient APR induction or complement expression and activity is detrimental in sepsis and systemic bacterial and viral infection, and in many different cardiometabolic diseases such as atherosclerosis, diabetes, and nonalcoholic fatty liver disease (Hertle et al., 2014; Ricklin et al., 2016). Deciphering the mechanisms behind the transcriptional downregulation of these proteins has far reaching implications that extend beyond infectious disease.

Downregulation of APP-coding and cholesterol synthesis genes is IFN-independent

Clinical studies have shown since the 1980s that hypocholesterolaemia is a common side effect of IFN treatment (Robertson and Ghazal, 2016). Moreover, viral infection has since been shown to downregulate sterol synthesis in an IFN-dependent manner (Blanc et al., 2011). The IFN induced expression of the ISG cholesterol 25-hydroxylase (CH25H) has been identified as one of the mechanisms behind this metabolic deregulation (Abrams et al., 2020; Blanc et al., 2013; Dang et al., 2017; Shibata et al., 2013). CH25H catalyses the production of 25-hydroxycholesterol, an oxysterol that inhibits cholesterol synthesis (Cardoso and Perucha, 2021). In contrast, our results show with no ambiguity that the transcriptional repression of cholesterol metabolism genes observed in response to *Lm* infection in hepatocytes is not associated with the IFN-I/III response nor the expression of CH25H. CH25H was one of the top 10 most highly upregulated genes in HepG2 but was not differentially regulated in Huh7 cells that exhibited no IFN response. Both cell lines downregulated cholesterol synthesis in response to long-term infection in a highly similar manner. Furthermore, IFN-λ1 and IFN-β treatment of HepG2 and Huh7 cells did not result in the downregulation of representative cholesterol synthesis genes (data not shown).

The IFN and ISG signature of long-term *Lm* infection

The expression of IFN-I and/or IFN-III is considered one of the hallmarks of the epithelial cell response to *Lm* (Bierne et al., 2012b; Dussurget et al., 2014). However, this conclusion is derived from the quantification of IFN expression almost exclusively at the transcriptional level. In this work, we clearly establish that human HepG2 hepatocytes respond to intracellular *Lm* infection with a major, but rather delayed, production of IFN-III (IFN-λ1) and a minor

production of IFN-I (IFN-β). This result is important due to the species specificity of the IFN response: IFN-λ1 is a pseudogene in mice, and murine hepatocytes predominantly express IFN-I and do not respond to IFN-III (Hermant et al., 2014; Nakagawa et al., 2013). This is supported by our observation that PMH respond to *Lm* with early IFN-β but no IFN-III expression. The magnitude and kinetics of ISG induction by IFN-I and IFN-III differ: while IFN-I induces rapid but transient ISG expression, IFN-III induction of ISG expression is slow but sustained (Stanifer et al., 2020). These differences in IFN type and expression kinetics may have important physiological impacts and highlight the need to study the role of IFN-III in human listeriosis. As for Huh7 cells, they do not produce any IFN in response to *Lm*, as previously observed when these cells are confronted with other IFN-triggering stimuli, such as double stranded RNA (dsRNA) (Lanford et al., 2003), poly I-C (a synthetic dsRNA) (Li et al., 2005b) or HCV (Israelow et al., 2014). A possible explanation for this unresponsiveness is that Huh7 cells have a defective TLR3 signalling pathway (Li et al., 2005b). This deficiency allowed us to identify the IFN-independence of certain processes, such as the downregulation of cholesterol biosynthesis gene expression observed in response to *Lm* infection, as discussed above.

A remarkable result of our transcriptomic study is the observation of very potent IFN secondary responses in *Lm*-exposed hepatocytes (at least in the models where IFN is produced, i.e., HepG2 and PMH). The exceptional number of activated ISGs after long-term infection is notably mimetic of an antiviral response. In human HepG2, we observed both IFN and downstream ISG expression to be triggered much later than in viral infections, however, with maximum expression observed at 3 days p.i. as opposed to < 24 h p.i. in virus-infected human epithelial cells, for example (Voigt and Yin, 2015).

The role of IFN signalling in persistent *Lm* infection merits further investigation. IFNs can have opposing effects and, in the murine listeriosis model, IFN-I has been implicated in both the restriction and promotion of infection, likely resulting from the pleiotropic roles of IFN-I in distinct cell environments and at different stages in the infectious process (Alphonse et al., 2021; Peignier and Parker, 2021). The role of IFN-III in listeriosis has not yet been directly studied, but it should be noted that this IFN type has immunomodulatory properties and could be less detrimental to epithelial barriers than IFN-I (Broggi et al., 2020b). Several recent reports have, however, provided evidence that IFN-λ contributes to disease pathology by exacerbating the innate immune response during chronic infection or autoimmune disease (Goel et al., 2021;

Read et al., 2021), and IFN-III signalling has been observed to be detrimental in murine models of bacterial respiratory infections (Kotenko et al., 2019).

Our data suggest that IFN responses do not result in a cell-autonomous antibacterial effect in hepatocytes in the timeframe observed (up to 72 h p.i.) but do not rule out an effect in the longer-term. However, it is likely that IFN secretion by hepatocytes modulates infection *in vivo* by acting on immune cells, impacting both innate and adaptive immunity. If prolonged, this IFN expression could contribute to innate immune dysfunction, as observed in chronic viral infections (Dagenais-Lussier et al., 2017). We propose the hypothesis that excessive IFN signalling during long-term *Lm* infection could promote cellular conditions that support bacterial persistence by contributing to immune suppression and tissue tolerance.

Contribution of bystander cells to the IFN response in hepatocytes

A key question that our work opens is that of the nature of the cell subpopulations engaged in these IFN responses within a tissue. In 1963, in one of the first observations of IFN production by cultured cells, Henle described an inverse relationship between the number of virus-infected cells and the IFN titers obtained (Henle, 1963). With the advent of single-cell techniques, the notion of cytokine production by "bystander" cells has gained attention, and several studies have highlighted the contribution to IFN and cytokine expression of uninfected cells within virus- or bacteria-infected cell cultures (Holmgren et al., 2017; Recum-Knepper et al., 2015). Bystander activation in *Lm* infection has been shown to occur through both cell-contact dependent mechanisms and paracrine signalling in the form of secreted extracellular vehicles (Kasper et al., 2010; Nandakumar et al., 2019). Through the use of FACS to select GFP-*Lm* long-term infected HepG2 cells, we could compare the extent of gene deregulation in a homogeneous as opposed to a heterogeneous population of infected cells and found that while gene deregulation was in general higher in the homogeneously infected cells, IFN and ISG expression was amplified in the heterogeneously infected HepG2 cell population.

Exploring how infected cells signal to uninfected cells is an interesting avenue of research that has not been thoroughly explored in *Lm* infection of epithelial cells. HepG2 cells could provide a suitable model as bystander activation is particularly important when infection represses the activation of innate signalling in infected cells, as we observed for IFN expression. A recent transcriptomic study of the *Lm* response to infection, for example, reported no difference

between cytokine expression levels between *Lm*-infected and uninfected cells in heterogeneously infected primary trophoblast monolayers (Johnson et al., 2021).

The role of the BAHD1-MIER chromatin-repressive complex in modulating gene expression during *Lm* infection of hepatocytes

The BAHD1-mediated repression of ISG expression in response to *Lm* infection was previously observed in colon epithelial cells (Lebreton et al., 2011) and we report a similar repression in long-term *Lm* infected hepatocytes. The mechanism by which *Lm* infection triggers BAHD1 recruitment to ISGs is not yet established, however, and is an interesting perspective to pursue. It has been proposed that BAHD1 could be recruited to ISG promoters through the interaction of STAT1/2 with BAHD1-MIER complex components HDAC1 and/or KAP1 (Lebreton et al., 2012) (Figure 42A).

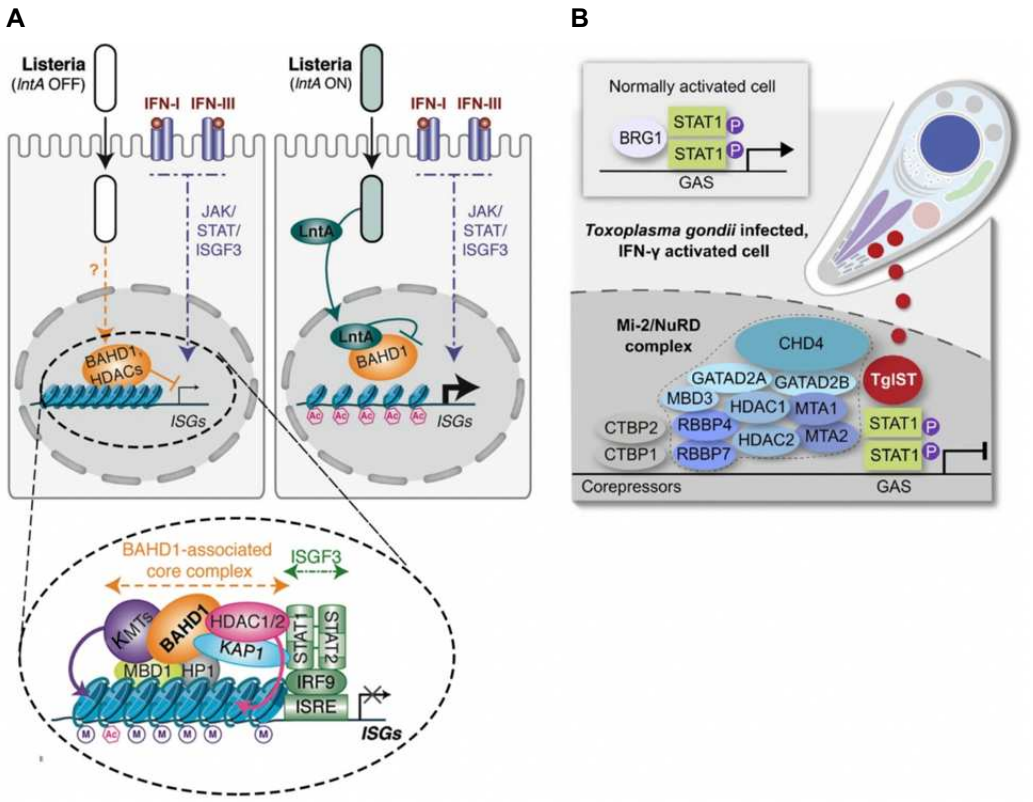

Figure 42 | Intracellular pathogen targeting of epigenetic complexes BAHD1 and NuRD.
(**A**) The proposed model for the recruitment of BAHD1 at ISG promoters during *Lm* infection in epithelial cells. The BAHD1-MIER complex could be recruited to ISRE promoter sequences through HDAC1-STAT1/2 or KAP1-STAT1 interactions, resulting in heterochromatin formation, through histone deacetylation and trimethylation at H3K9, and partially abrogated expression of ISGs (left). LntA secreted by *Lm* interacts with BAHD1 and alleviates BAHD1-mediated ISG repression (right) (Source: Lebreton et al., 2012). (**B**) Schematic representation of NuRD complex recruitment to STAT1 dependent promoters during *T. gondii* infection in macrophages (Source: Olias et al., 2016).

Interestingly, another hepatotropic pathogen, the protozoan *Toxoplasma gondii*, targets the epigenetic complex NuRD through the secretion of a specific virulence factor (Olias et al., 2016). NuRD is recruited to STAT1 dependent promoters, resulting in the partial abrogation of ISG expression in macrophages, in a similar manner to the BAHD1-mediated repression of ISGs in epithelial cells. In the case of *Lm*, however, a virulence factor (LntA) has been identified that alleviates BAHD1-mediated repression of ISG expression, and it is not known whether a distinct virulence factor acts in a divergent manner to activate BAHD1 repression, as is the case in *T. gondii*-mediated ISG repression (Figure 42).

The role of BAHD1 in the transcriptional repression of APP-coding genes is also plausible and merits examination. As described above and in section B.2.2.1, APP gene expression is regulated by NF-κB and STAT3, among other TFs (Asselin and Blais, 2011; Bode et al., 2012). The chromatin environment at both NF-κB and STAT3 binding sites is a key regulatory mechanism governing gene expression through promotor accessibility. At the same time, both STAT3 and NF-κB can interact with epifactors to influence chromatin topology (Bhatt and Ghosh, 2014; Wingelhofer et al., 2018; Zhao et al., 2013), including components of the BAHD1-MIER complex. STAT3, like STAT1, interacts with KAP1 (Tsuruma et al., 2008) as well as with the KMT G9a to form a repressor complex that facilitates H3K9 dimethylation (Wingelhofer et al., 2018). The p50 subunit of NF-κB can attract and form complexes with HDACs, and RELB interacts with KMTs G9a and SETDB1 (Papoutsopoulou and Campbell, 2021). The siRNA silencing of *BAHD1* in the human colon epithelial cell line Caco-2 led to increased activation of NF-κB and increased STAT3 phosphorylation (Zhu et al., 2015), suggesting a direct or indirect role for BAHD1 in regulating NF-κB- and STAT3-dependent gene expression. Interestingly, MIER1 has also been reported to interact with and inhibit the activity of CREBBP (Blackmore et al., 2008), a histone acetyltransferase implicated in the positive regulation of STAT3-dependent APP expression (Wang et al., 2005; Wingelhofer et al., 2018).

The role of BAHD1 in cholesterol metabolism and the metabolic defects observed in *Bahd1* KO mice, leads to the question of whether BAHD1 could be implicated in the repression of cholesterol metabolic genes in hepatocytes during long-term infection. Full KO of the *Bahd1* gene in mice results in phenotypic defects in the placenta and brain (Lakisic et al., 2016; Pourpre et al., 2020) – both secondary *Lm* target organs – raising the question of BAHD1's role in maternal-neonatal listeriosis and/or neurolisteriosis. The role played by BAHD1 in the

liver, brain and placenta, under physiological and pathophysiological conditions merits more extensive exploration.

Hepatocytes as a niche for long-term intracellular *Lm* persistence

We revealed an immunomodulatory signature upon long-term infection of hepatocytes with *Lm*, at an infection time point at which *Lm* enters a slow-growth phase in LisCVs. LisCVs are partially degradative, but a subpopulation of bacteria survives in a dormant state, suggesting the possibility of long-term, low-level persistence of this pathogen in the liver parenchyma. For *Lm* to persist in hepatocytes, we hypothesized that the modulation of the host transcriptional landscape would promote tolerance to this intracellular dormant parasite. This tolerance could be perceived as a "trade-off" between the host that avoids excessive inflammation and associated tissue damage, and the bacteria, a small population of which could survive in a niche protected from detection by the immune system.

The liver is naturally biased towards immunotolerance, and this tolerance extends to the induction of systemic immune tolerance. Not only do liver transplants eschew rejection (the outcome for all other organs), the transplanted liver can confer tolerance to another transplant from the same donor (Calne et al., 1969; Cunningham et al., 2013). Persisting high-level antigen expression by hepatocytes silences CD8 T cell function (Tay et al., 2014a), a characteristic that is exploited by hepatotropic viruses and parasites resulting in long term chronic infection (Crispe, 2014; Protzer et al., 2012; Zheng and Tian, 2019). Hepatotropic viruses HBV and HCV can asymptomatically infect a very large proportion of hepatocytes while adaptive immunity and viral clearance are delayed for weeks, months, or even years. The immune response is not always sufficient to clear the virus, and chronic infection ensues (Bertoletti and Kennedy, 2015; Kennedy et al., 2017; Mondelli et al., 2005). The obligatory liver stage of *Plasmodium* spp. is also asymptomatic with sporozoite multiplication taking place within a cytosolic parasitophorous vacuole to evade immune sensing, with some species remaining in a quiescent state in hepatocytes for up to several years (Prudêncio et al., 2006; Zuzarte-Luis and Mota, 2020). The hypothesis of the liver as a long-term niche for LisCV-contained *Lm* deserves further attention and exploration in an *in vivo* context.

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ANNEXES

Chapter 16

Microscopy of Intracellular *Listeria monocytogenes* in Epithelial Cells

Hélène Bierne, Mounia Kortebi, and Natalie Descoeudres

Abstract

The pathogen *Listeria monocytogenes* is a facultative intracellular bacterium, which targets a large range of cell types. Following entry, bacteria disrupt the invasion vacuole and reach the cytoplasm where they replicate and use the actin cytoskeleton to propel themselves from cell to cell. Mammalian epithelial cells grown in vitro can be used to study the different steps of the intracellular life of *Listeria*. However, rapid multiplication and dissemination of bacteria can induce important cell death and detachment, resulting in the formation of lytic plaques. Thus, in vitro infections with *L. monocytogenes* are usually restricted to short time courses, from a few minutes to one day. Here, we present a method to study long-term *L. monocytogenes* infections in epithelial cells using epifluorescence microscopy. This protocol enables the observation of actin-based motility, intercellular dissemination foci, and entrapment of *L. monocytogenes* within vacuoles of persistence termed "*Listeria*-Containing Vacuoles" (LisCVs). We also describe a protocol to study the recruitment of cytoskeletal proteins at *Listeria* actin comet tails, as well as a method to assess the membrane integrity of intracellular bacteria using a LIVE/DEAD viability assay.

Key words Fluorescence microscopy, Intracellular bacteria, Persistence, LIVE/DEAD assays

1 Introduction

Intracellular bacterial pathogens are generally classified into two groups: those that exploit host membrane trafficking to construct specific niches in vacuoles (i.e., "vacuolar pathogens") and those that escape from vacuoles to target the cytosol, where they proliferate and often spread to neighboring cells (i.e., "cytosolic pathogens"). However, the boundary between these distinct intracellular phenotypes is tenuous as the duration of infection and the host cell type can be decisive. *Listeria monocytogenes* is a paradigm for cytosolic pathogens, and the different stages of its intracellular life have been highly characterized in tissue-cultured cells [1]. Following phagocytosis (e.g., in macrophages) or receptor-mediated endocytosis dependent on the *L. monocytogenes* invasion proteins InlA

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and/or InlB (e.g., in epithelial cells), bacteria disrupt the primary vacuole and enter the cytosol. There, they replicate and use the force generated by actin polymerization, driven by the actin assembly-inducing protein ActA, to propel themselves and form membrane protrusions that project them into neighboring cells (Fig. 1). The resolution of such protrusions into double-membrane vacuoles and the subsequent membrane rupture of these secondary vacuoles allow bacteria to enter the cytosol of the adjacent cells and start a new cycle of actin polymerization and intercellular dissemination.

Besides these well-known intracellular stages, there is strong evidence to suggest that *Listeria monocytogenes* has also developed mechanisms to nest in vacuoles. For instance, in intestinal goblet cells, *Listeria* is transcytosed in a vacuole allowing rapid translocation of bacteria across the intestinal barrier [3]. In macrophages, subpopulations of *Listeria* remain confined to non-degradative phagosomes, known as "Spacious *Listeria*-containing Phagosomes" (SLAPs), which are thought to provide a niche for persistent *L. monocytogenes* infection in phagocytic cells [4, 5] (Fig. 1). Additionally, during long-term infections in a subset of epithelial cells, such as human hepatocytes and trophoblastic cells, *L. monocytogenes* are engulfed in lysosomal vacuoles, termed "*Listeria*-Containing Vacuoles" (LisCVs) [6]. While SLAPs are coupled to phagocytosis at the onset of infection and harbor bacteria that never enter the cytosol, LisCVs are formed later, after the phase of actin-based motility and intracellular spread and upon capture of cytosolic bacteria by endomembranes (Fig. 1). In SLAPs, as in LisCVs, bacteria enter a state of slow replication; in addition, in LisCVs, subpopulations of *L. monocytogenes* are degraded while others appear to reach the dormant state known as Viable But Non-Culturable (VBNC) [6]. In this chapter, we provide three protocols to observe the different intracellular stages of *L. monocytogenes* in epithelial cells using JEG3 placental cells as a model system. The first protocol enables the observation of the switch between the active cytosolic phase and the vacuolar persistence phase of *L. monocytogenes*: bacteria progressively cease to polymerize actin, and cytosolic bacteria are engulfed in membrane structures marked by the late endosome/lysosome marker LAMP1. The second protocol (adapted from [7]) focuses on imaging the recruitment of host cytoskeletal proteins at the actin-rich comet tail of *Listeria*. The third protocol (adapted from [6]) describes an assay to assess bacterial membrane integrity during intracellular infections.

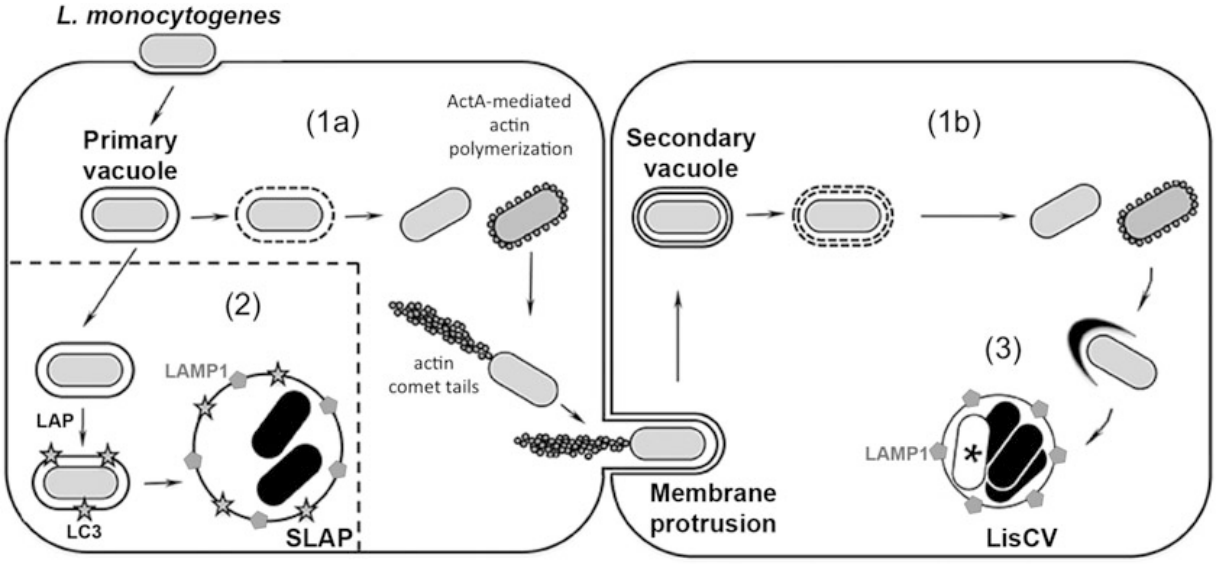

Fig. 1 Different stages in the intracellular life of *L. monocytogenes*. (Adapted from Bierne et al. [2]) (**1**) Intracellular invasion (**1a**) and dissemination (**1b**) of *L. monocytogenes* in mammalian cells. After bacterial entry into the host cell and transient residence within a primary vacuole, bacteria escape into the cytoplasm, multiply, and induce expression of the actin-polymerizing factor ActA. Actin polymerization promotes bacterial motility and cell-to-cell spread via the generation of membrane protrusions from the primary infected cell to neighboring cells. After the resolution of these protrusions into double-membrane secondary vacuoles, from which the bacteria escape, a new cycle of infection is initiated. (**2**) Model of SLAP formation in murine macrophages. After bacterial phagocytosis, subpopulations of bacteria expressing low amounts of the cytolysin LLO remain in phagosomes. A LC3-associated phagocytosis (LAP) process promotes formation of SLAPs, marked by LAMP1, in which bacteria enter a slow/non-replicative state (bacteria represented in black). (**3**) LisCV formation in a subset of epithelial cells. Following the active stage of bacterial cell-to-cell spread, bacteria stop expressing ActA. ActA-free bacteria multiply in the cytosol and are captured by endomembranes, forming *Listeria*-containing vacuoles (LisCV) marked by LAMP1. In these lysosome-like compartments, subpopulations of bacteria resist stress and degradation and enter a slow/non-replicative state (bacteria represented in black), while others are sensitive to stress and die (bacteria represented in white with *)

2 Materials

All experiments with *L. monocytogenes* must be conducted within a laminar flow class II Biological Safety Cabinet (BSC). Personal Protective Equipment (PPE) includes laboratory coat, disposable sterile gloves, and safety glasses. All work surfaces and materials must be disinfected both prior to and immediately following all procedures. Operations involving paraformaldehyde must be carried out in a certified chemical fume hood and waste eliminated in a container for hazardous waste.

2.1 Long-Term Infection Immunofluorescence Assay

1. *L. monocytogenes* strain 10403S [8]. Other strains can be used, including derivative strains expressing fluorescent proteins (*see* **Note 1**). Maintain strains at −80 °C in 20% glycerol.
2. Bacterial growth medium: Brain-Heart Infusion (BHI), as liquid medium or in agar plates.

3. Static incubator at 37 °C.
4. Shaking incubator at 37 °C.
5. Benchtop centrifuge.
6. JEG3 trophoblastic cells (ATCC HTB-36). Maintain frozen stocks in cell preservation medium in liquid nitrogen.
7. Cell preservation medium: fetal calf serum (FCS), 10% DMSO.
8. Cell culture medium: Minimum Essential Medium Eagle (MEM), 10% FCS, 1% non-essential amino acids (NEAA), 1 mM sodium pyruvate (NaP), 2 mM L-glutamine.
9. Inoculation and washing cell medium: MEM.
10. Cell incubator with a humidified 10% CO_2-containing atmosphere at 37 °C.
11. 6-Well cell culture plates.
12. Centrifuge for 15 mL polystyrene tubes and cell culture plates.
13. 37 °C water bath.
14. 22 mm square glass coverslips sterilized for 2 h at 180 °C in a dry-heat sterilizer (do not use a steam autoclave).
15. Cell counter and Trypan Blue solution (0.4%).
16. 1× phosphate-buffered saline (PBS).
17. 0.05% trypsin, 0.02% EDTA solution.
18. Gentamicin.
19. Fixation buffer: 4% paraformaldehyde (PFA) in 1× PBS.
20. Permeabilization buffer: 0.4% Triton X-100 in 1× PBS.
21. Blocking buffer: 2% bovine serum albumin (BSA) in 1× PBS.
22. Whatman filter paper.
23. Parafilm.
24. Distilled water.
25. Antibodies against *L. monocytogenes* [6] (unless using fluorescent bacteria, *see* **Note 1**).
26. Antibodies against human LAMP1.
27. Fluorescently labeled secondary antibodies (e.g., Alexa Fluor 488-conjugated, Cy3-conjugated or Alexa Fluor 555-conjugated goat antibodies).
28. F-actin stain (e.g., Alexa Fluor 647 Phalloidin Dye).
29. DNA stain: 1 mg/mL DAPI or Hoechst (prepared in water).
30. Finely pointed and curved forceps and a slightly curved needle.
31. Glass slides.
32. Mounting medium.
33. Inverted motorized fluorescence microscope with 10× (or 20×) and 63× (or 100×) objectives.

2.2 Immunofluorescence Assay to Study the Recruitment of Cytoskeletal Proteins During Infection

1. Same materials as in Subheading 2.1.
2. *L. monocytogenes* strain EGD-e [9]. Other strains can be used, including derivative strains expressing fluorescent proteins (*see* **Note 1**). Maintain strains at −80 °C in 20% glycerol.
3. CSK buffer: 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 10 mM PIPES (piperazine-*N*,*N*′- bis[2-ethanesulfonic acid]), pH 6.8, 1.2 mM PMSF (phenylmethylsulfonyl fluoride).
4. Permeabilization buffer: 0.4% Triton X-100 in CSK buffer.
5. Antibodies against *L. monocytogenes* [6] (unless using fluorescent bacteria, *see* **Note 1**) and cytoskeletal proteins (e.g., Arp3, Cofilin [7]).
6. Inverted motorized fluorescence microscope with a 63× or 100× objective.

2.3 LIVE/DEAD Viability Assay of Intracellular Listeria

1. Same materials as in Subheading 2.1.
2. *L. monocytogenes* strain 10403S [8] (*see* **Note 1**).
3. MOPS/$MgCl_2$ solution (0.1 M 3-(N-morpholino)propanesulfonic acid (MOPS), pH 7.4; 1 mM $MgCl_2$).
4. LIVE/DEAD® BacLight(TM) Bacterial Viability Kit.
5. Triton X-100.
6. LIVE/DEAD staining and permeabilization solution: mix 1.6 μM SYTO9, 20 μM propidium iodide, and 0.1% Triton X100 in MOPS/$MgCl_2$. Vortex briefly. Keep the solution in the dark.
7. Fine tip forceps.
8. Glass slides.
9. Clear nail polish.
10. Inverted motorized fluorescence microscope with a 63× or 100× objective.

3 Methods

We present protocols for a 6-well plate format, but they can be scaled down to 12-well or 24-well plate formats. These protocols are optimized for monolayers of JEG3 trophoblastic cells, which are permissive for InlA- and InlB-dependent entry of *L. monocytogenes*. The protocols can also be adapted to other cell types (*see* **Note 2**).

3.1 Long-Term Infection Immunofluorescence Assay

1. At least 1 week prior to the day of infection, thaw JEG3 cells by resuspending a frozen cellular aliquot in 10 mL of cell culture medium in a 15 mL polystyrene tube. Centrifuge for 5 min at 300 × *g* at room temperature to harvest the cells. Discard the

supernatant, and resuspend the cellular pellet in 10 mL of cell culture medium heated to 37 °C in a water bath. Seed the cells in a 75 cm^2 flask, and transfer to a cell incubator with a humidified 10% CO_2-containing atmosphere at 37 °C. Subculture cells when they reach 80% confluency.

2. At least 3 days prior to infection, streak *L. monocytogenes* strain 10403S from a −80 °C glycerol stock onto a BHI agar plate. Grow bacteria for 48 h at 37 °C (*see* **Note 3**).
3. Two to four days prior to infection, trypsinize a flask of JEG3 cells and seed cells (2 mL/well) into 6-well plates containing sterile coverslips, in order to reach about 90% confluency at the onset of infection (*see* **Note 4**). The number of plated wells will vary according to the number of different conditions that will be analyzed (*see* **Note 5**). Prepare a specific plate to count cells at the time of infection (2 wells), as well as at the end of infection (2 wells). Transfer the plates to the cell incubator where cells will attach and spread to become a monolayer.
4. In the evening of the day preceding infection, prepare a liquid culture of *L. monocytogenes* by inoculating several isolated bacterial colonies from the BHI agar plate into 5 mL of BHI liquid medium in a 15 mL polystyrene tube. Close the lid firmly, and allow the culture to grow overnight at 37 °C in a shaking incubator.
5. The day of infection, wash stationary-phase bacteria in PBS. For this, take 1 mL of the overnight *L. monocytogenes* culture, centrifuge for 5 min at 4000 × *g* in a tabletop centrifuge, discard the supernatant, and resuspend the pellet in 1 mL of PBS.
6. Prepare the inoculum by diluting the washed bacteria in the appropriate volume of infection medium (MEM). JEG3 cells are infected with 2 mL of inoculum per well to achieve a multiplicity of infection (MOI) of approximately 0.01 (*see* **Note 6**). To verify the MOI a posteriori, prepare serial dilutions of the inoculum in 1 mL PBS, plate 50 µL in duplicate on BHI agar plates, and allow bacteria to grow for 48 h at 37 °C. In two noninfected wells, add trypsin to detach and then count the JEG3 cells in an equal amount of Trypan Blue solution (**Note 7**).
7. Wash JEG3 cells by aspirating the medium and adding 2 mL of MEM per well.
8. Aspirate the MEM and add 2 mL of the inoculum to each well.
9. Centrifuge infected cells for 2 min at 300 × *g* to synchronize bacterial uptake.
10. Transfer the inoculated plate(s) to the cell incubator, and allow *L. monocytogenes* to invade cells for 1 h.

11. After the cellular infection has been completed for 1 h, gently wash cells with 2 mL per well of pre-warmed MEM. Be careful to pipet gently to avoid cell loss (*see* **Note 8**).
12. Replace MEM with 2 mL of complete cell culture medium supplemented with 25 μg/mL gentamicin, and transfer the 6-well plate(s) back to the cell incubator. Incubate the infected cells for 2, 6, 24, or 72 h.
13. At the chosen time point post infection, gently remove the medium and wash cells with 1 mL of PBS pre-warmed to room temperature.
14. Remove the PBS, and fix cells by adding 1 mL of the fixation buffer to each well. Incubate at room temperature for 30 min under a chemical fume hood.
15. Remove the fixative, and gently wash cells with 1 mL PBS per well. Renew the washing.
16. Permeabilize cells by adding 1 mL of 0.4% Triton X-100 in PBS and incubating at room temperature for 4 min.
17. Gently wash the coverslips in wells with 2 mL of PBS, three times.
18. Keep coverslips in 1 mL of blocking buffer per well for 15 min.
19. Prepare the primary antibody solution by diluting the *L. monocytogenes* antibodies and LAMP1 antibodies in blocking buffer (*see* **Note 9**). Maintain the antibody solution on ice.
20. Prepare an incubation chamber (Fig. 2): thoroughly wet a sheet of Whatman paper with distilled water, and firmly stick a sheet of parafilm onto it (ensure that there are no bubbles between the paper and the parafilm). Define a separate position for each coverslip by labeling the parafilm (*see* **Note 10**).
21. For each coverslip, dispatch a 40 μL drop of the antibody solution onto the parafilm (Fig. 2). Use fine tip forceps and a needle (or similar) to remove the coverslips from the cell culture plate. Carefully place each coverslip cell side down on the primary antibody drop. Cover the Whatman paper and parafilm with an inversed tray to maintain a humid environment. Incubate for 1 h.
22. Prepare the secondary antibody solution by diluting the following: Alexa Fluor 488-conjugated anti-rabbit antibody (1:400), Cy3-conjugated (or Alexa Fluor 555-conjugated) anti-mouse antibody (1:400), DAPI or Hoechst (1:1000), and Alexa Fluor 647 phalloidin (1:400) in blocking buffer (*see* **Note 11**).
23. Gently detach each coverslip from the parafilm by slowly injecting PBS under it using a pipette. Grip the coverslip using the fine tip forceps, and wash by dipping in three successive baths of PBS.

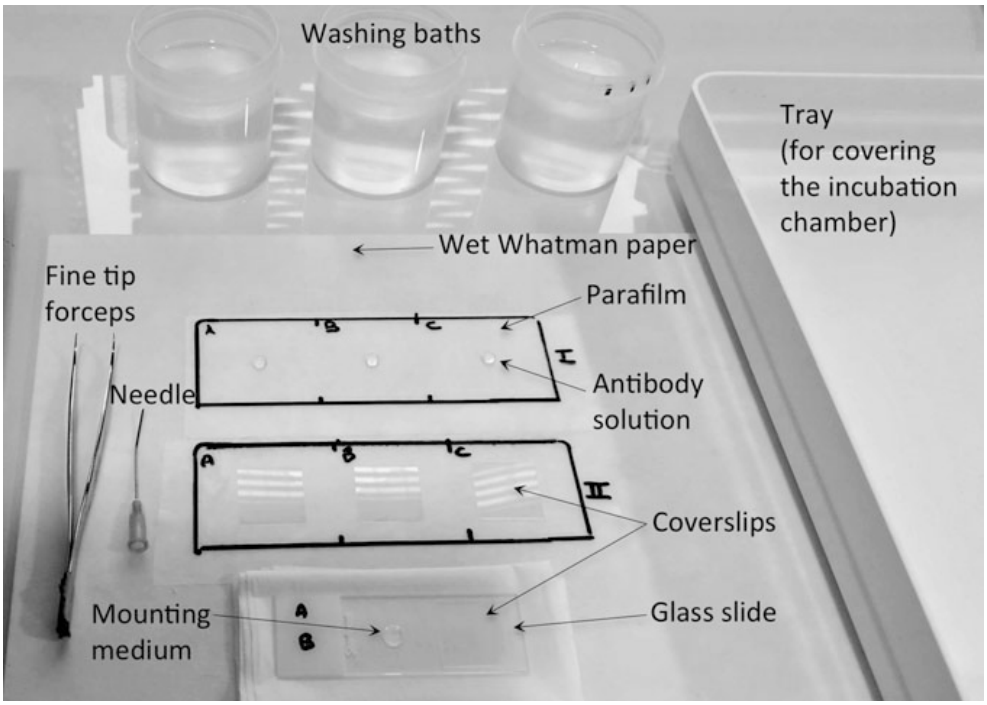

Fig. 2 Material for incubating coverslips with antibody solutions. Assemble a humidified chamber using a wet Whatman paper, onto which a sheet of parafilm is firmly stuck, making sure there are no bubbles between the paper and the parafilm. Label the position of each coverslip on the parafilm. Dispatch a drop of primary antibody solution for each coverslip (I). Carefully remove the coverslips from the cell culture plate using pointed curved forceps and a slightly curved needle and then upturn the coverslip onto the drop (II). Use a tray to cover the coverslips during the incubation period; ensure the cells never dry out. After incubation, gently detach each coverslip from the parafilm by slowly injecting PBS under it using a pipette. Grip the coverslip using the forceps, and wash by dipping in three successive baths of PBS. Repeat the process for the secondary antibody solution incubation. Pipette a drop of mounting medium onto a microscope slide (avoid bubbles). After washing each coverslip in two successive baths of PBS and one of distilled water, place the coverslip cell side down onto the drop. Incubate the slides at room temperature overnight in the dark. Protect the slides from light, and store at −20 °C until imaging is performed

24. For each coverslip, dispatch a 40 μL drop of the secondary antibody solution on a fresh sheet of parafilm in the incubation chamber.
25. Carefully place each coverslip cell side down onto the secondary antibody drop, as for the primary antibody solution, and cover with an inversed tray to maintain a humid environment and protect the fluorescent probes from the light. Incubate for 30 min at room temperature.
26. Prepare glass slides: wipe them down with a lint-free tissue moistened with a small amount of ethanol and label them. Two 22 mm coverslips can be mounted onto one glass slide.
27. After incubation, gently detach each coverslip by using the pipette to inject PBS under it.

28. Pipette a 10 µL drop of mounting medium onto a glass slide (one drop per coverslip) (Fig. 2). Ensure that there are no bubbles in the 10 µL drop.
29. Wash each coverslip in two successive baths of PBS followed by one bath of distilled water.
30. Remove any excess water by dabbing the edge of the coverslip on a paper towel before slowly placing the coverslip cell side down onto the drop of mounting medium on the glass slide (Fig. 2). It is important to avoid the formation of bubbles.
31. Let the coverslip dry overnight at room temperature, in the dark to protect the fluorescent probes.
32. Acquire images in the 350 nm (DAPI/Hoechst, to visualize nuclei and bacterial DNA), 488 nm (*L. monocytogenes*), 546 nm (LAMP1), and 647 nm (F-actin) channels on an inverted motorized fluorescence microscope, equipped with a digital camera. Use a 10× (or 20×) objective to observe bacterial spreading in the cell monolayer (Fig. 3a). Bacteria appear as dots within the cell monolayer stained with fluorescent phalloidin that labels F-actin. Use a 63× (or 100×) objective to observe *Listeria* associated with F-actin or entrapped in *Listeria*-Containing Vacuoles (LisCVs) (Fig. 3a). LisCVs appear as perinuclear LAMP1-positive compartments containing F-actin-negative bacteria (Fig. 3b).

3.2 Immunofluorescence Assay to Study the Recruitment of Cytoskeletal Proteins During Infection

This protocol has been adapted from Bierne et al. [7] to be used for JEG3 cells infected with strain EGD-e [9]. It is optimized to detect cytoskeletal proteins recruited by *L. monocytogenes* during the actin-based motility process.

1. Follow **steps 1–5** as described in Subheading 3.1.
2. Prepare the inoculum by diluting bacteria in the appropriate volume of infection medium (MEM). JEG3 cells are infected with 2 mL of inoculum to achieve a multiplicity of infection (MOI) of 1–5. To verify the MOI a posteriori, prepare serial dilutions of the inoculum in 1 mL PBS, plate 50 µL in duplicate on BHI agar plates, and allow bacteria to grow for 48 h at 37 °C. Use two noninfected wells to count the JEG3 cells, after detachment with trypsin.
3. Follow **steps 7–12** as described in Subheading 3.1.
4. After 3, 4, or 6 h of infection (*see* **Note 12**), carefully remove the medium and gently wash cells with 1 mL PBS.
5. Remove PBS with a pipette, and fix cells by adding 1 mL of the fixation buffer to each well. Immediately renew with fresh fixation solution, and incubate for 25 min at room temperature under a chemical fume hood (*see* **Note 13**).
6. Gently wash coverslips with 1 mL PBS. Renew the washing.

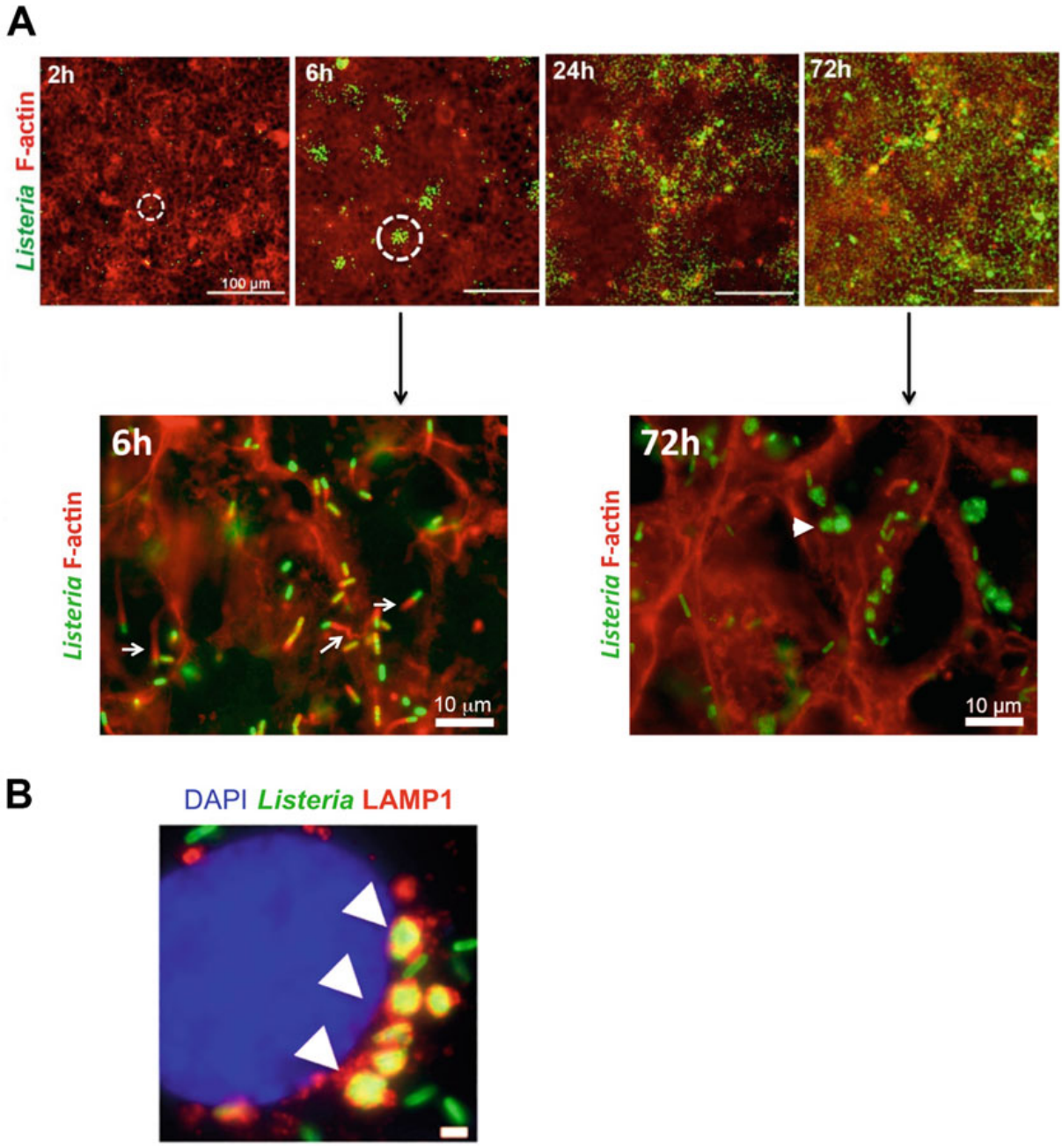

Fig. 3 Fluorescence microscopy analysis of a time course infection of *L. monocytogenes* in JEG3 cells (Adapted from Kortebi et al. [6]). JEG3 cell monolayers were infected with *L. monocytogenes* 10403S at MOI ~ 0.1. At the indicated time point post infection (p.i.), cells were fixed, permeabilized, and incubated with *Listeria* and LAMP1 antibodies for 1 h and then with secondary antibodies, fluorescent phalloidin (to stain F-actin), and DAPI (to stain DNA) for 30 min. (**a**) Micrographs of cells infected for 2, 6, 24, or 72 h and visualized with a 10× (top; bar: 100 µm) or 63× (bottom, bar: 10 µm) objective. Images are overlays of *Listeria* (green) and F-actin (red) signals. Circles highlight an individual bacterium at 2 h p.i., and an infection focus at 6 h p.i. Arrows point to examples of actin comet tails associated with Listeria, at 6 h post infection. The triangle shows a cluster of actin-free *Listeria* at 72 h post infection. (**b**) Micrograph of a cell infected for 72 h and visualized with a 100× objective (bar: 2 µm). Image is an overlay of *Listeria* (green), LAMP1 (red), and DAPI signals. Triangles show LAMP1-positive *Listeria*-containing vacuoles (LisCVs) at 72 h post infection

7. Permeabilize cells with 1 mL of 0.4% Triton X-100 in CSK buffer for 4 min (*see* **Note 13**).
8. Gently wash the coverslips with 2 mL CSK buffer and then twice with PBS.
9. Keep coverslips in 1 mL blocking solution (PBS with 2% BSA) per well for 15 min.
10. Prepare the primary antibody solution by diluting *L. monocytogenes* antibodies and antibodies against cytoskeletal proteins in blocking solution (*see* **Note 9**). Maintain the antibody solution on ice.
11. Follow **steps 20–31** as described in Subheading 3.1.
12. Acquire images on an inverted motorized fluorescence microscope, equipped with a digital camera and a 63× or 100× objective to observe *Listeria* associated with F-actin and the cytoskeletal protein of interest.

3.3 LIVE/DEAD Viability Assay of Intracellular Listeria

We adapted the Live/Dead BacLight Bacterial Viability assay from Kubica et al. [10] and Johnson and Criss [11] to assess whether live bacteria in LisCVs have intact or damaged membranes. This assay is based on the discriminative labeling of two fluorescent nucleic acid stains: the green membrane-permeant dye SYTO9 and the red membrane-impermeant dye propidium iodide (PI), which only crosses damaged cell membranes. Both dyes penetrate dead cells, but SYTO9 fluorescence is reduced in the presence of PI. As such, intact bacteria appear green, while those with damaged membranes appear red. To allow the dyes to reach vacuolar *Listeria*, this staining protocol is performed in the presence of 0.1% Triton X-100, which permeabilizes host cell membranes, including vacuoles containing bacteria [10]. The efficiency of permeabilization is assessed by the bright staining of host cell nuclei with PI as the dye crosses the double-membraned nuclear envelope. Cells are not fixed to avoid killing bacteria.

1. Follow **steps 1–12** as described in Subheading 3.1. (*See* **Note 1** regarding the choice of strains.)
2. At 72 h postinfection (*see* **Note 14**), gently wash cells twice with 1 mL of MOPS/$MgCl_2$ solution (0.1 M 3-(N-morpholino)propanesulfonic acid (MOPS), pH 7.4; 1 mM $MgCl_2$).
3. Gently add 1 mL of the LIVE/DEAD staining and permeabilizing solution (1.6 μM SYTO9, 20 μM propidium iodide, 0.1% Triton X100 in MOPS/$MgCl_2$) to each well.
4. Centrifuge for 10 min at 300 × *g* at room temperature (this enables dye staining while preventing cell detachment).
5. Incubate for further 5 min at room temperature in the dark.

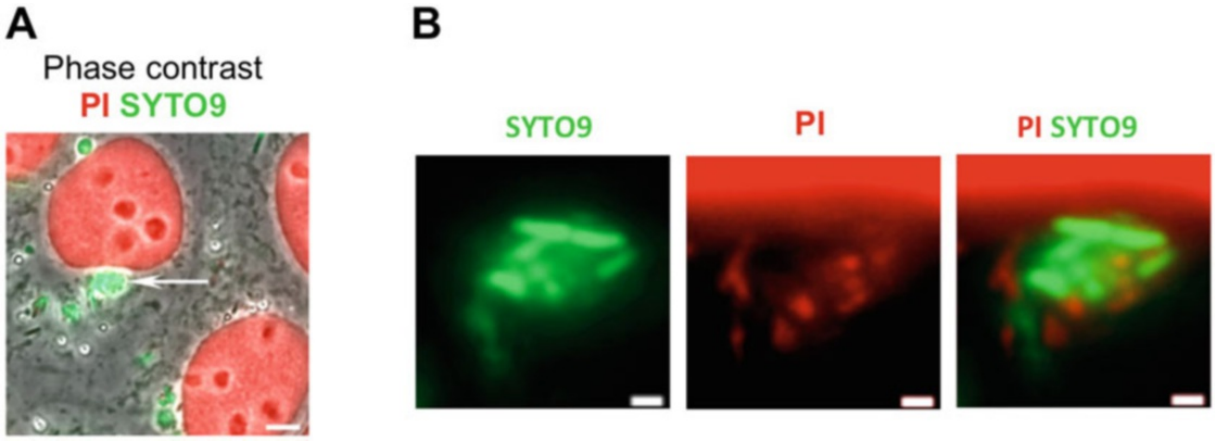

Fig. 4 LIVE/DEAD staining assay for intravacuolar *Listeria* (Adapted from Kortebi et al. [6]). JEG3 cells infected for 3 days with *L. monocytogenes* were permeabilized with 0.1% Triton X-100 and double-labeled with fluorescent nucleic acid stains SYTO9 and PI. This assay is based on the discriminative labeling of membrane-permeant SYTO9 (green) and membrane-impermeant PI (red), which only crosses damaged cell membranes. Both dyes penetrate dead cells, but SYTO9 fluorescence is reduced in the presence of PI. As such, live bacteria appear green, while those with damaged membranes appear red. (**a**) Overlay of phase contrast (showing groups of cells), SYTO9, and PI channels. The arrow points to a cluster of bacteria in a LisCV. Bar: 5 μm. (**b**) High magnifications of the region indicated by the arrow. Micrographs of the SYTO9 and PI individual channels and of the overlay of both channels are shown. Bacteria with intact membranes are stained in green, while the host cell nuclear DNA and damaged bacteria are stained in red. Bar: 1 μm

6. Gently wash cells twice with 1 mL of the MOPS/$MgCl_2$ solution (use a pipette to aspirate the buffer to prevent cell detachment).
7. Place the coverslips cell side down onto glass slides, and seal with clear nail polish. Make sure the nail polish only covers a small portion of the coverslip. Incubate for 2 min at 37 °C in the cell incubator.
8. Immediately examine the mounted coverslip under the microscope, with a 63× or 100× objective, for a maximum of 10 min. Bacteria appear as tiny green or red filaments (as the dyes stain the bacterial nucleoid) (Fig. 4) (*see* **Note 15**).

4 Notes

1. The protocols have been used with the *L. monocytogenes* strains 10403S [8], EGD-e [9], EGD-e expressing GFP [12], or 10403S expressing mCherry [13]. The use of fluorescent *Listeria* eliminates the need for labeling bacteria with primary antibodies, but the intensity of fluorescence is lower than when using dye-conjugated secondary antibodies. For the LIVE/DEAD assay, however, the use of fluorescent *Listeria* is not recommended.

2. The protocols have been successfully tested with human BeWo trophoblast cells (ATCC CCL-98), as well as the hepatocyte cell lines HuH-7 (Cellosaurus CVCL-0336) and Hep G2 (ATCC HB-8065). To obtain a uniform monolayer of Hep G2 cells, it is recommended to grow these cells in collagen-coated wells. Dilute type I collagen from rat tail to 50 μg/mL in PBS, and add 1 mL to each well of a 6-well plate. Incubate at room temperature for 1 h before aspirating the solution and rinsing each well three times with 1 mL of sterile PBS.
3. The BHI plate can be stored at 4 °C for up to 15 days.
4. Cell quantification may vary from one laboratory to another because of the use of different cell counter systems. Seeding concentrations must therefore be adapted in order to achieve approximately 90% confluency at the onset of infection.
5. We typically seed cells in triplicate (three independent wells) for each condition.
6. The specific MOI of the inoculum needs to be adjusted according to the *L. monocytogenes* strain that is examined in the assay. With strain 10403S, we classically use a MOI of 0.01 or 0.1.
7. Do not keep cells in Trypan Blue solution for more than 10 min before counting cells (after 10–15 min in Trypan Blue, cells start dying, thereby rendering the cell count inaccurate).
8. When using a MOI above 1 (e.g., 1–10), incubate cells for 10 min in 2 mL of MEM containing 100 μg/mL gentamicin to rapidly kill extracellular bacteria. This treatment prevents the lytic action of the LLO toxin, which is secreted by *L. monocytogenes*. After this treatment, wash cells with 2 mL of MEM before adding complete cell culture medium supplemented with 25 μg/mL gentamicin.
9. We generally use rabbit polyclonal *L. monocytogenes* antibodies and mouse monoclonal human LAMP1 antibodies. If using GFP- or mCherry-expressing *Listeria*, either rabbit or mouse LAMP1 antibodies (or antibodies against another host factor of interest) can be used.
10. It is imperative that coverslips never dry out. Ensure that the Whatman paper remains wet throughout the incubation (use distilled water).
11. Check the excitation and emission filters of the microscope to avoid spectral overlap; choose the secondary antibodies accordingly.
12. Determine the time point you would like to study: bacteria start to polymerize actin after about 2–3 h of infection, and start to form membrane protrusions after 4–6 h.

13. Some antibodies work better following fixation in methanol. In such cases, instead of using PFA for fixation, immerse coverslips or chamber slides in ice-cold methanol:acetone (1:1) and incubate at −20 °C for 10 min. Permeabilize with cooled acetone for 1 min at −20 °C (do not use Triton X-100). Air--dry the coverslips. Rinse them in 1× PBS.
14. This assay can be performed at different time points post infection.
15. To detect bacteria with SYTO9 and PI, one has to use a high intensity of light, which can become cytotoxic for bacteria. The examination of LIVE/DEAD intracellular bacteria must therefore be performed within 5–10 min. A field of infected cells needs to be found rapidly. The success of an experiment thus depends on the efficiency of infection.

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Synthèse en français

Introduction

Listeria monocytogenes est un agent pathogène d'origine alimentaire qui peut provoquer la listériose, l'une des zoonoses bactériennes d'origine alimentaire les plus dangereuses (European Food Safety Authority, European Centre for Disease Prevention and Control, 2021). Bien que rare, la listériose a un taux de létalité élevé et est particulièrement préoccupante pour les personnes immunodéprimées et les femmes enceintes (Radoshevich and Cossart, 2017). *L. monocytogenes* est une bactérie à Gram positif qui fait preuve d'une adaptabilité remarquable, étant capable de passer d'une vie de saprophyte vivant dans le sol à celle d'un pathogène invasif, très adepte à la survie et à la prolifération dans la cellule hôte (Schlech, 2019). Cette adaptabilité accroît le potentiel pathogène de *L. monocytogenes* dans le domaine alimentaire, car la bactérie peut résister aux concentrations élevées de sel et d'acide et aux faibles concentrations d'oxygène et aux basses températures utilisées pour la conservation des aliments (Allerberger et al., 2015). L'adaptabilité de *L. monocytogenes* a également facilité l'utilisation de cette bactérie en laboratoire et a permis à *L. monocytogenes* de devenir un pathogène modèle extrêmement polyvalent au cours des cinquante dernières années (Lecuit, 2020). L'analyse du processus infectieux de *L. monocytogenes*, tant *in vitro* qu'*in vivo* dans le modèle murin, est à l'origine de nombreuses avancées dans notre compréhension de la biologie cellulaire, des interactions hôte-pathogène et de l'immunologie innée et l'immunité à médiation cellulaire (Cossart, 2011 ; Radoshevich and Cossart, 2017).

La porte d'entrée principale de *L. monocytogenes* est le tube digestif, suite à l'ingestion d'aliments contaminés (Schlech et al., 1983). Une fois ingéré, *L. monocytogenes* traverse le tube digestif, rencontrant le faible pH de l'estomac et du duodénum, ainsi que des acides biliaires perturbateurs de la membrane, des bactéries commensales productrices de bactériocines et de peptides antimicrobiens, contre lesquels le pathogène a développé des mécanismes de survie très efficaces lui permettant de pénétrer le mucus intestinal (Gahan and Hill, 2014 ; Matereke and Okoh, 2020 ; Maudet et al., 2021). Néanmoins, la grande majorité de l'inoculum initial ingéré est tuée dans l'estomac ou éliminée dans les fèces en quelques heures. Une fois les entérocytes, les cellules de l'épithélium intestinal, envahis, cependant, une réplication intracellulaire extensive ainsi qu'une propagation de cellule à cellule ont lieu (Melton-Witt et al., 2012). *L. monocytogenes* peut ensuite traverser la barrière intestinale par plusieurs mécanismes, pour atteindre les ganglions lymphatiques puis la circulation sanguine

pour se disséminer dans les tissus cibles, notamment le foie et la rate (Radoshevich and Cossart, 2017) (voir le schéma de l'infection ci-dessous).

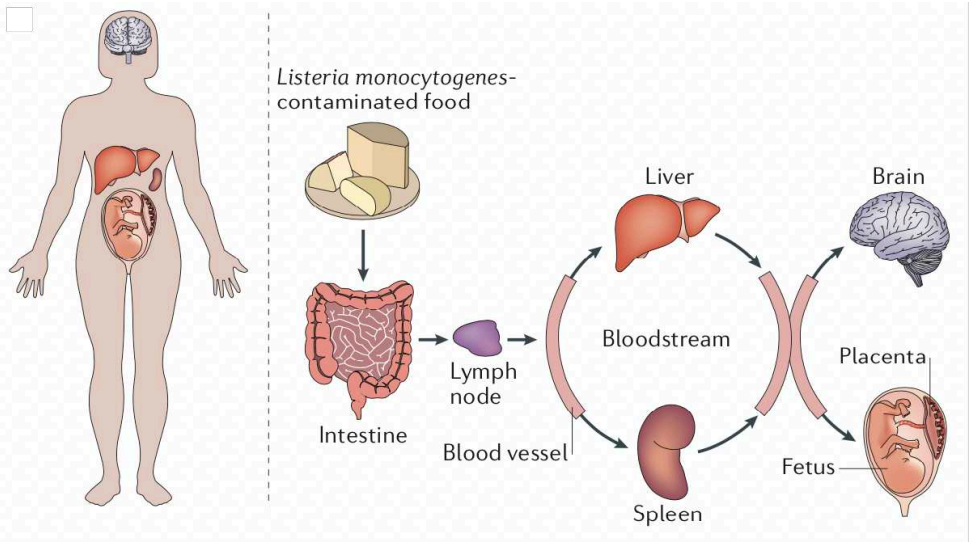

Schéma de l'infection par *L. monocytogenes* chez l'homme. Après l'ingestion d'aliments contaminés, *L. monocytogenes* peut traverser la barrière intestinale pour atteindre la circulation sanguine en passant par les ganglions lymphatiques pour se disséminer dans les tissus cibles, notamment le foie et la rate. Chez les personnes immunodéprimées, *L. monocytogenes* peut traverser la barrière hémato-encéphalique et provoquer une méningite ou une septicémie dont le taux de mortalité est très élevé. Chez les femmes enceintes, *L. monocytogenes* peut traverser la barrière fœtoplacentaire et provoquer une naissance prématurée ou l'avortement.
(D'après Radoshevich and Cossart, 2017)

Après la phase intestinale de l'infection, le foie joue un rôle central dans l'élimination des bactéries. Le foie a des rôles essentiels dans le métabolisme et la détoxification mais agit également comme une barrière aux infections systémiques, par son rôle majeur dans la détection, la capture et l'élimination des agents pathogènes circulant dans le sang (Kubes and Jenne, 2018 ; Protzer et al., 2012). Certains agents pathogènes, notamment les virus (par exemple, les virus de l'hépatite A, B, C, D, E), les parasites (par exemple, *Plasmodium falciparum*, *Toxoplasma gondii*, *Entamoeba histolytica*) et les bactéries (par exemple, *Listeria monocytogenes*, *Salmonella Typhimurium*, *Francisella tularensis*, *Brucella* spp., *Streptococcus pneumoniae*) sont capables d'envahir les hépatocytes, les cellules parenchymateuses du foie (Conlan and North, 1992; Protzer et al., 2012; Talwani et al., 2011; Wisplinghoff and Appleton, 2008). Parmi ces pathogènes, *L. monocytogenes* est un contaminant alimentaire bactérien capable d'atteindre et de se multiplier dans le foie après avoir

traversé la barrière intestinale. Chez la majorité des individus, l'invasion de *L. monocytogenes* est éliminée avec succès, mais si l'infection n'est pas contrôlée par une réponse immunitaire adéquate, la prolifération de *L. monocytogenes* peut conduire à la libération de bactéries intracellulaires dans le système circulatoire et à l'invasion d'autres sites, tels que le cerveau chez les personnes immunodéprimées, et le placenta et le fœtus chez les femmes enceintes, entraînant des septicémies, des méningo-encéphalites, des fausses couches et des infections néonatales (Schlech, 2019). Ces manifestations cliniques sévères font de la listériose l'une des infections d'origine alimentaire les plus mortelles (European Food Safety Authority, European Centre for Disease Prevention and Control, 2021).

La plupart de nos connaissances sur la phase hépatique de la listériose proviennent d'infections expérimentales sur des modèles animaux, principalement la souris, ce qui a produit un scénario d'infection très bien décrit, bien que certaines différences puissent provenir des différentes voies d'inoculation et/ou de la dose bactérienne utilisée (Pitts and D'Orazio, 2018). Après une inoculation intraveineuse, plus de 60% des bactéries sont éliminées de la circulation sanguine par le foie dans l'espace de 10 minutes. Les bactéries se fixent sur les cellules de Kupffer (les macrophages hépatiques résidents) et sont ensuite éliminées par une interaction complexe entre les cellules de Kupffer et les neutrophiles qui migrent rapidement vers le foie en réponse à l'infection (Conlan and North, 1991 ; Gregory et al., 1996 ; Gregory et al., 2002 ; Witter et al., 2016). Six heures post-infection, environ 90% des bactéries hépatiques sont associées aux hépatocytes, au sein desquels la réplication bactérienne a lieu pendant deux à trois jours. Ainsi, les charges de *L. monocytogenes* dans le foie augmentent de manière exponentielle avant d'atteindre un plateau après trois à quatre jours d'infection, puis diminuent avec le développement d'une immunité spécifique (Cousens and Wing, 2000). Il a été proposé que l'invasion bactérienne des hépatocytes peut avoir lieu par deux voies : l'internalisation directe ou la propagation de cellule à cellule à partir des cellules de Kupffer (Appelberg and Leal, 2000 ; Dramsi et al., 1995 ; Gaillard et al., 1996). Des observations en histologie (Conlan and North, 1991 ; 1992) et en microscopie électronique (Gaillard et al., 1996) suggèrent que les bactéries se propagent dans le parenchyme hépatique grâce au processus de motilité basé sur l'actine décrit dans des modèles cellulaires *in vitro* (Domann et al., 1992 ; Kocks et al., 1992 ; Welch et al., 1997 ; 1998). En accord avec cela, les souches mutantes de *L. monocytogenes* déficientes dans la production de la protéine ActA, qui induit la polymérisation des filaments d'actine nécessaire aux déplacements intra- et inter-cellulaires des *L. monocytogenes* cytosoliques (Tilney and Portnoy, 1989), sont de trois ordres de grandeur moins virulentes par

rapport à la souche sauvage dans le modèle de listériose murin (Domann et al., 1992). Le passage direct des bactéries d'un hépatocyte à un autre est proposé pour générer des foyers d'infection dans lesquels *L. monocytogenes* se dissémine à travers le parenchyme, évitant ainsi le contact avec les effecteurs humoraux du système immunitaire (Gaillard et al., 1996 ; Wood et al., 1993).

Après la phase de croissance active dans les hépatocytes, la charge bactérienne diminue fortement dans le foie grâce à de puissantes réponses immunitaires innées (Cousens et Wing, 2000). Plusieurs types de cellules contribuent à la défense du foie contre l'infection par *L. monocytogenes*, en particulier les neutrophiles, les cellules tueuses naturelles, les cellules dendritiques (Arnold-Schrauf et al, 2014 ; Conlan and North, 1991 ; Cousens and Wing, 2000 ; Gregory et al., 1996 ; Witter et al., 2016) et les cellules de Kupffer, dont la mort par nécroptose déclenche le recrutement de monocytes infiltrants, qui prolifèrent et se différencient en macrophages au site de l'infection (Bleriot et al., 2015). Ces cellules immunitaires collaborent via des contacts intercellulaires et la sécrétion de cytokines et de chimiokines pour tuer les bactéries ou inhiber leur réplication, et pour lyser les hépatocytes infectés. Les hépatocytes eux-mêmes participent activement à la réponse immunitaire innée en produisant et en sécrétant de manière constitutive une variété de protéines qui jouent un rôle important dans l'immunité innée, comme les facteurs du complément et les protéines impliquées dans l'hémostase (Zhou et al., 2016). Ces protéines, dont la production augmente rapidement et de manière substantielle en réponse à des stimuli inflammatoires sont connues sous le nom de protéines de la phase aiguë (Gabay and Kushner, 1999 ; Zhou et al., 2016). La production de cytokines pro-inflammatoires par les cellules de Kupffer, les monocytes et les neutrophiles, en réponse à une infection par *L. monocytogenes*, stimule la production des protéines de la phase aiguë par les hépatocytes (Kopf et al., 1994 ; Kummer et al., 2016). Cette première vague de défenses non spécifiques est essentielle à la survie de l'hôte ; l'inflammation contribue également au développement de la résistance acquise en stimulant l'amorçage et la prolifération des lymphocytes T cytotoxiques qui génèrent les réponses primaires et mémoires protectrices contre *L. monocytogenes* (Pamer, 2004 ; Qiu et al., 2018).

La capacité de *L. monocytogenes* à envahir et à endommager le foie est documentée depuis longtemps chez les rongeurs et les lagomorphes ; en effet, parmi les premiers noms proposés pour cette bactérie figuraient *Bacillus hepatis* (par Hülphers, en 1911) et *Listerella hepatolytica* (par Pirie, en 1927), sur la base d'observations de nécrose hépatique chez des lapins et des gerbilles (Gray and Killinger, 1966 ; Mitchell et al., 1927). Chez l'homme, cependant, alors

que des abcès hépatiques sont décrits dans les cas de listériose néonatale, les symptômes cliniques de lésions hépatiques dues à *L. monocytogenes* sont rarement rapportés au cours de listérioses invasives chez l'adulte. De plus, il existe un manque de connaissances sur le devenir des bactéries dans les organes, dont le foie, pendant la période d'incubation asymptomatique, qui peut être très longue dans les cas associés à la grossesse – jusqu'à soixante-dix jours (Goulet et al., 2013). En outre, le portage asymptomatique de *L. monocytogenes* existe chez l'homme en bonne santé (Painter and Slutsker, 2007), ainsi que chez de très nombreuses espèces de mammifères d'élevage ou sauvages (Allerberger et al., 2015 ; Gray and Killinger, 1966 ; Hurtado et al., 2017 ; Leclercq, 2021 ; Parsons et al., 2020 ; Yoshida et al., 2000), mais notre compréhension de ce portage asymptomatique, en particulier en ce qui concerne son stade hépatique, fait cruellement défaut.

Récemment, la notion a émergé que, en plus des phases bien connues de réplication active et de motilité dans le cytosol des cellules hôtes, *L. monocytogenes* peut entrer dans une phase quiescente dans les compartiments vacuolaires, allant d'une croissance lente à une dormance, qui pourrait jouer un rôle important dans les infections asymptomatiques (Bierne et al., 2018). Ces niches vacuolaires sont en particulier formées dans les cellules du foie, notamment les macrophages hépatiques (Birmingham et al., 2008) et les hépatocytes (Kortebi et al., 2017), par des mécanismes distincts. Plus spécifiquement, il a été montré que dans la lignée cellulaire d'hépatocytes humains HepG2 et dans les hépatocytes humains primaires, *L. monocytogenes* entre dans une phase de repos dans des vacuoles acides, appelées « *Listeria*-containing vacuoles » (LisCVs ; « vacuoles contenant *Listeria* ») (Kortebi et al., 2017). Les LisCV sont générées tardivement (c'est-à-dire après deux à trois jours d'infection), lorsque les bactéries cessent d'exprimer ActA et de polymériser l'actine, et sont des compartiments partiellement dégradatifs (Kortebi et al., 2017). Par conséquent, une sous-population de bactéries survit dans un état quiescent, ce qui soulève la possibilité d'une persistance à long terme de *L. monocytogenes* dans le parenchyme hépatique. Des stades de persistance dans les hépatocytes ont été décrits pour d'autres agents pathogènes, tels que les virus et les parasites hépatiques (Protzer et al., 2012). *Plasmodium vivax*, par exemple, peut entrer dans un état quiescent à l'intérieur d'une vacuole parasitophore et passer inaperçu pendant des années (Prudêncio et al., 2006).

Objectifs et résultats de la thèse : 1) Une signature transcriptionnelle immunomodulatrice associée à une infection persistante par *L. monocytogenes* dans les hépatocytes

Le premier axe de ma thèse consistait en la caractérisation de la réponse transcriptionnelle de l'hépatocyte à une infection à long terme par *L. monocytogenes* afin d'identifier une signature d'expression génique associée à la persistance bactérienne intracellulaire. Nous rapportons le développement de trois modèles cellulaires robustes d'infection persistante dans des lignées cellulaires d'hépatocytes humains HepG2 et Huh7 et dans des hépatocytes primaires de souris. Ces trois modèles ont permis l'obtention des populations homogènes d'hépatocytes hébergeant *L. monocytogenes* dans des LisCV. L'analyse transcriptomique par le séquençage de l'ARN (RNA-seq, de l'anglais « RNA sequencing ») dans les hépatocytes après trois jours d'infection par *L. monocytogenes* a permis d'identifier un paysage transcriptionnel profondément modifié, d'où émerge une signature commune de l'infection « à long terme » par *L. monocytogenes*, caractérisée par :

(*i*) l'augmentation de l'expression d'un ensemble de gènes impliqués dans l'immunité antivirale connu sous le nom de « gènes stimulés par les interférons » (« interferon stimulated genes »). La caractérisation de cette facette majeure de la réponse des cellules épithéliales à l'infection par *L. monocytogenes* a mis en évidence une divergence entre les hépatocytes humain et murins soulignant la nécessité des modèles animaux alternatifs pour mieux refléter la listériose humaine. Nous avons observé une réponse précoce chez la souris qui est caractérisée par l'expression de l'interféron de type I (IFN-β), et une réponse tardive concomitante à l'expression majoritairement de l'interféron de type III (IFN-λ1, IFN-λ2) dans les hépatocytes humains.

(*ii*) la diminution de l'expression de nombreux gènes codant pour des protéines de phase aiguë, en particulier celles impliquées dans les systèmes du complément et de la coagulation. Ce blocage transcriptionnel des gènes codant pour les protéines de la phase aiguë a été maintenu en présence d'une stimulation par des cytokines pro-inflammatoires. L'analyse protéomique quantitative du sécrétome des hépatocytes a révélé une diminution de l'abondance des protéines de phase aiguë dans le milieu extracellulaire, en accord avec les données transcriptomiques.

(*iii*) la régulation négative des gènes associés au métabolisme du cholestérol dans les hépatocytes humains infectés à long terme, indépendamment de la réponse à l'interféron.

Les signatures transcriptomiques et protéomiques observées se sont avérées indépendantes de la souche de *L. monocytogenes* utilisée. L'utilisation d'une souche de *L. monocytogenes* déficiente dans l'expression du facteur de virulence l'internaline C (InlC), un inhibiteur de NF-κB (Gouin et al., 2010), suggère un rôle pour cette protéine secrétée bactérienne dans l'inhibition de l'expression des gènes codant pour un sous-ensemble des protéines de phase aiguë. L'analyse transcriptomique par RNA-seq dans les hépatocytes Huh7 à la fois à 24 heures et à 72 heures post-infection suggère que l'inhibition de l'expression des gènes codant pour des protéines de phase aiguë, ainsi que la diminution de l'expression des gènes associés à la synthèse du cholestérol, augmente au cours de l'infection.

L'ensemble de ces résultats suggèrent une modification importante du programme transcriptionnelle de l'hépatocyte au cours de l'infection à long terme par *L. monocytogenes*. Le rôle de l'induction de la réponse interféron dans l'infection bactérienne en générale, et lors de l'infection par *L. monocytogenes* en particulier, reste sujet à controverse, mais pourrait participer à la persistance de *L. monocytogenes* dans les hépatocytes. L'inhibition de l'expression de gènes clés de l'immunité innée hépatique impliqués dans la réponse à la phase aiguë, notamment des gènes codant pour des composants du complément et de la coagulation, serait impliquée dans la diminution de la réponse immunitaire, et pourrait favoriser la survie de *L. monocytogenes* et le portage silencieux de *L. monocytogenes* dans le foie.

Objectifs et résultats de la thèse : 2) La réponse des hépatocytes à l'infection par *L. monocytogenes* dans des populations mixtes ou pures de cellules infectées : contribution des cellules « bystander » non-infectées et le rôle du complexe BAHD1-MIER.

Le deuxième axe de ma thèse consistait en l'étude des mécanismes susceptibles d'expliquer l'effet de l'infection persistante par *L. monocytogenes* sur la dérégulation des gènes hépatocytaires, en se focalisant sur le rôle des régulateurs épigénétiques BAHD1 et MIER.

Dans chaque cellule humaine, deux mètres d'ADN sont compactés dans le noyau, enroulés autour de protéines histones pour former des nucléosomes qui sont emballés dans des fibres de chromatine (Bierne and Hamon, 2020 ; Ou et al., 2017). L'état de compaction de la chromatine joue un rôle majeur dans la régulation de l'expression des gènes en contrôlant l'accessibilité de l'ADN à la machinerie transcriptionnelle. La régulation de la structure de la chromatine est un processus dynamique qui implique la méthylation de l'ADN, et une grande variété de modifications post-traductionnelles des histones telles que la méthylation, l'acétylation, la phosphorylation et l'ubiquitination, ainsi que le remodelage des nucléosomes (Bierne, 2017 ;

Bierne and Hamon, 2020). Ces différents mécanismes fonctionnent de concert pour coordonner la formation de structures chromatiniennes, qui sont soit peu compactes et actives, soit fortement condensées et silencieuses sur le plan transcriptionnel. La régulation transcriptionnelle basée sur l'état de la chromatine est contrôlée par des complexes macromoléculaires composés de protéines qui ont été baptisées « épifacteurs » (Medvedeva et al., 2015) et qui reconnaissent, ajoutent ou suppriment spécifiquement les modifications post-traductionnelles des histones ou la méthylation de l'ADN, ou qui ont des activités de remodelage de la chromatine (Bierne, 2017). L'assemblage combinatoire d'épifacteurs avec des facteurs de transcription active ou réprime la transcription ainsi modifiant l'expression génique en réponse aux stimuli développementaux, physiologiques ou environnementaux (Bierne, 2017). À leur tour, les voies de signalisation cellulaire influencent les niveaux d'expression, la localisation et l'assemblage des épifacteurs en complexes. Comme les complexes de remodelage de la chromatine ont un rôle crucial dans la régulation de la transcription, y compris pendant l'infection, ils peuvent faire de bonnes cibles des pathogènes, comme *L. monocytogenes* (Bierne, 2017 ; Dong and Hamon, 2020).

Une approche de microbiologie cellulaire visant à rechercher des protéines sécrétées par *L. monocytogenes* ciblant des organelles intracellulaires a permis d'identifier LntA (« *Listeria* nuclear targeted protein A ») comme une protéine bactérienne ciblant le noyau (Lebreton et al., 2011). De tels effecteurs bactériens exerçant leurs fonctions dans le noyau de la cellule hôte ont été définis par Bierne et Cossart comme des "nucléomodulines" et ont été identifiés chez plusieurs autres pathogènes intracellulaires (Bierne and Pourpre, 2020 ; Bierne et al., 2012a). La protéine humaine ciblée par LntA dans le noyau a été identifiée comme étant BAHD1 (« bromo adjacent homology domain-containing 1 ») (Bierne et al., 2009 ; Lebreton et al., 2011 ; 2014).

La caractérisation de la protéine BAHD1 a révélé qu'elle était un composant central d'un nouveau complexe associé aux histone désacétylases (HDACs). Sur le plan biochimique, une série d'expériences impliquant la technique de double hybride, la co-immunoprécipitation et la colocalisation (Bierne et al., 2009), ainsi que la purification par affinité en tandem des protéines associées à BAHD1 (Lakisic et al., 2016 ; Lebreton et al., 2011) ont montré que BAHD1 forme un complexe avec HDAC1/2, les histone-lysine méthyltransférases (par exemple G9a), les lecteurs de histone H3 triméthylée sur la lysine 9 (H3K9me3) et les lecteurs de l'ADN methylé (respectivement, HP1 « heterochromatin protein 1 », et MBD1 « methyl-CpG-binding domain protein 1 », ainsi que d'autres composants (par exemple, CDYL « chromodomain Y like », et

KAP1 « KRAB-associated protein 1 »). Parmi les principaux partenaires de BAHD1, les protéines MIER « mesoderm induction early response » ont été identifiés, en particulier MIER1 et MIER3 (Fan et al., 2021 ; Lakisic et al., 2016 ; Lebreton et al., 2011).

Une caractérisation approfondie de MIER1 par Gillespie et ses collègues a montré que cette protéine de régulation de la transcription et de la chromatine interagit avec HDAC1/2, le histone-lysine méthyltransférase G9a, et CREBBP (« CREB binding protein ») (Blackmore et al., 2008 ; Ding et al., 2003 ; Gillespie and Paterno, 2012 ; Wang et al, 2008), et présente des similitudes structurelles et fonctionnelles avec les membres de la famille des protéines MTA (« metastasis-associated protein ») du complexe de remodelage de la chromatine NuRD (« nucleosome remodelling and deacetylase »), en raison de ses domaines ELM2 et SANT (Derwish et al, 2017 ; Ding et al., 2003 ; 2004 ; Paterno et al., 1997). Le domaine ELM2 de MIER1 est responsable de l'interaction avec les HDACs (Derwish et al., 2017 ; Ding et al., 2003). MIER2 et MIER3 présentent une homologie avec MIER1, notamment dans leurs domaines ELM-SANT (Derwish et al., 2017). Il a été démontré que MIER2 recrute les HDACs, mais moins efficacement que MIER1 (Derwish et al., 2017). MIER3 ne recrute pas les HDACs (Derwish et al., 2017), mais est associée à la désacétylation des histones H3 et H4 par l'inhibition de l'histone acétyltransférase p300 (Zhang et al., 2020).

BAHD1, d'autre part, partage avec les protéines MTA un domaine BAH (« bromo adjacent homology ») (Bierne et al., 2009), qui est connu pour favoriser les interactions protéine-protéine et la liaison aux nucléosomes (Yang and Xu, 2013). Bierne et ses collègues ont proposé le modèle dans lequel les protéines BAHD1 et MIER coopèrent pour remplir une fonction d'échafaudage similaire à celle de MTA dans le complexe NuRD (Lakisic et al., 2016). L'échafaudage BAHD1-MIER établit un pont entre la méthylation de l'histone sur la lysine 9, la désacétylation de l'histone et la méthylation de l'ADN, qui sont toutes des marques épigénétiques favorisant l'extinction des gènes.

L'étude fonctionnelle de BAHD1 est cohérente avec cette caractérisation biochimique. BAHD1 déclenche la compaction de la chromatine en hétérochromatine et induit l'extinction des gènes (Bierne et al., 2009). L'inactivation du gène *BAHD1* modifie l'acétylation et la méthylation des histones et la méthylation de l'ADN au niveau des gènes cibles (Lakisic et al., 2016) et la surexpression de *BAHD1* remodèle le méthylome dans les cellules humaines (Libertini et al., 2015). Les gènes inhibés par BAHD1 diffèrent selon les types de cellules et les stimuli spécifiques. L'extinction complète du gène *Bahd1* chez la souris entraîne des défauts

phénotypiques dans le placenta et le cerveau (Lakisic et al., 2016 ; Pourpre et al., 2020) ainsi que des défauts métaboliques, notamment dans le métabolisme du cholestérol (Lakisic et al., 2016). Il a été démontré que BAHD1 régule l'inflammation dans le côlon au cours de la colite ulcéreuse (Zhu et al., 2015), et au cours de l'infection par *L. monocytogenes*, BAHD1 réprime l'expression des gènes stimulés par les interférons en aval de la signalisation des interférons de type I et de type III dans les cellules épithéliales de côlon LoVo (Lebreton et al., 2011 ; 2014).

Des travaux préliminaires par l'équipe ont identifié trois gènes (*BAHD1*, *MIER1* et *MIER3*) codant pour des sous-unités du complexe répressif de remodelage de la chromatine BAHD1-MIER comme étant spécifiquement surexprimés dans la lignée de cellules hépatiques HepG2 infectées. BAHD1 joue un rôle dans la répression de la réponse interféron pendant l'infection des cellules intestinales par *L. monocytogenes* (Lebreton et al., 2011) et dans la répression des gènes du métabolisme des lipides (Lakisic et al., 2016). Mon objectif était donc d'explorer le rôle du complexe BAHD1-MIER lors d'une infection persistante par *L. monocytogenes*, dans le cadre d'un projet de collaboration entre les laboratoires de H. Bierne et L. Gillespie.

Nous avons observé une augmentation de l'expression du gène *IFNL1*, codant pour l'interféron de type III IFN-λ1, ainsi que l'expression d'un groupe de gènes stimulés par les interférons dans des hépatocytes dont l'expression des gènes codant pour *BAHD1*, *MIER1* et *MIER3* a été inactivée par des petits ARN interférents (siRNA). Les résultats de ce présent travail élargissent donc les résultats publiés précédemment (Lebreton et al., 2011 ; 2014), en fournissant des données nouvelles sur l'inhibition de gènes stimulés par l'interféron par le complexe BAHD1-MIER dans les hépatocytes humains infectés. Les résultats suggèrent également que, dans une population mixte d'hépatocytes infectés et non infectés, les cellules voisines aux cellules infectées mais elles-mêmes non infectées (« bystander cells ») sont les principaux producteurs d'interférons et des protéines produites en réponse aux interférons, après trois jours d'infection. Ce phénomène d'activation secondaire est particulièrement important lorsque l'infection réprime la signalisation innée dans les cellules infectées, comme nous l'avons observé pour l'inhibition par le complexe BAHD1 de l'expression des gènes stimulés par les interférons dans les hépatocytes. Ces résultats, bien que nécessitant une confirmation par des approches complémentaires, fournissent la première preuve d'un rôle du complexe chromatino-répressif BAHD1-MIER dans l'atténuation des réponses interféron dans les hépatocytes hébergeant des *L. monocytogenes* intracellulaires, et suggèrent un rôle important de l'activation secondaire dans la réponse de l'hépatocyte à l'invasion par *L. monocytogenes*.

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ÉCOLE DOCTORALE
Structure et dynamique des systèmes vivants (SDSV)

Titre : Caractérisation de la réponse des hépatocytes à l'infection à long terme de *Listeria monocytogenes*

Mots clés : *Listeria monocytogenes*, hépatocytes, transcriptomiques, protéines de phase aiguë, interféron, immunité innée

Résumé : *Listeria monocytogenes* (*Lm*) est un pathogène intracellulaire facultatif provoquant de graves maladies d'origine alimentaire chez les femmes enceintes et les personnes immunodéprimées. Après la phase intestinale de l'infection, le foie joue un rôle central dans l'élimination des bactéries. C'est aussi un organe cible primaire de *Lm*, dans lequel les bactéries se multiplient efficacement dans les hépatocytes, les cellules parenchymateuses. Des données récentes suggèrent que, lors d'une infection à long terme des hépatocytes, une sous-population bactérienne peut échapper à l'éradication, en entrant dans une phase de persistance dans des vacuoles intracellulaires appelées LisCVs.

Le premier axe de ma thèse consistait à examiner la réponse de l'hôte à cette infection à long terme dans les hépatocytes, avec l'objectif d'identifier une signature transcriptomique commune à plusieurs modèles d'hépatocytes. Des modèles cellulaires d'infection persistante ont été établis dans des lignées cellulaires d'hépatocytes humains HepG2 et Huh7 et dans des hépatocytes primaires de souris. Les bactéries *Lm* sont systématiquement entrées dans la phase de persistance après trois jours d'infection dans ces cellules, tout en induisant une puissante réponse à l'interféron, de type I dans les hépatocytes primaires de souris, et de type III dans les HepG2, tandis que les cellules Huh7 sont restées sans réponse. L'analyse transcriptomique par RNA-seq a permis d'identifier un paysage transcriptionnel profondément modifié, d'où émerge une signature commune de l'infection à long terme par *Lm*, caractérisée par (i) l'augmentation de l'expression d'un ensemble de gènes impliqués dans l'immunité antivirale et (ii) la diminution de l'expression de nombreux gènes codant pour des protéines de phase aiguë, en particulier celles impliquées dans les systèmes du complément et de la coagulation. Ce blocage transcriptionnel des gènes codant pour les protéines de la phase aiguë a été maintenu en présence d'une stimulation par des cytokines pro-inflammatoires. L'analyse protéomique quantitative du sécrétome des hépatocytes a révélé une diminution de l'abondance des protéines de phase aiguë, en accord avec les données transcriptomique. L'infection a également modifié l'expression de nombreux gènes associés au métabolisme du cholestérol dans les hépatocytes humains, indépendamment de la réponse à l'interféron.

Le deuxième axe de ma thèse consistait en l'étude du rôle des facteurs épigénétiques BAHD1 et MIER dans l'infection à long terme par *Lm* dans les hépatocytes. L'association BAHD1-MIER forme l'échafaudage d'un complexe répressif chromatinien récemment décrit, appartenant à la famille des complexes histone désacétylase (HDAC). Il avait été montré précédemment que BAHD1 réprimait la réponse interféron lors de l'infection de cellules épithéliales de colon par *Lm*. Le présent travail élargit ces résultats, en fournissant des données nouvelles sur l'inhibition de gènes stimulés par l'interféron par BAHD1-MIER dans les hépatocytes. Les résultats suggèrent également que, dans une population mixte d'hépatocytes infectés et non infectés, les cellules auxiliaires non infectées sont les principaux producteurs d'interférons et des protéines produites en réponse aux interférons, après trois jours d'infection.

Ces travaux suggèrent fortement que l'infection à long terme par *Lm* dérégule profondément les fonctions sécrétoires et métaboliques des hépatocytes, ce qui pourrait générer un environnement favorable à l'établissement d'une infection persistante en réduisant l'abondance de protéines clés d'immunité innée d'origine hépatocytaire. En même temps, cela ouvre de multiples perspectives pour explorer les mécanismes de régulation transcriptionnelle de l'hôte pendant une infection persistante et le rôle des cellules auxiliaires non infectées dans la subversion de la répression transcriptionnelle médiée par l'infection.

Title: Characterisation of the hepatocyte response to long-term *Listeria monocytogenes* infection

Keywords: *Listeria monocytogenes*, hepatocytes, transcriptomics, acute phase proteins, interferon, innate immunity.

Abstract: *Listeria monocytogenes* (*Lm*) is a facultative intracellular pathogen that causes severe foodborne illness in pregnant women and immunocompromised individuals. After the intestinal phase of infection, the liver plays a central role in the clearance of bacteria. It is also a primary target organ, in which *Lm* replicates extensively in hepatocytes, the parenchymal liver cells. Recent data suggest that during long-term infection of hepatocytes, a bacterial subpopulation can escape eradication by entering a persistence phase in intracellular vacuoles called LisCVs.

The first axis of my thesis was to examine the host response to this long-term infection in hepatocytes with the objective of identifying a common transcriptomic signature in several hepatocyte models. Cellular models of persistent infection were established in HepG2 and Huh7 human hepatocyte cell lines and primary mouse hepatocytes. *Lm* consistently entered the persistence phase after three days of infection in these cells, while inducing a potent interferon response, of type I in primary mouse hepatocytes and type III in HepG2, while Huh7 cells remained unresponsive. RNA-sequencing analysis identified a profoundly altered transcriptional landscape from which a common signature of long-term *Lm* infection emerged, characterized by (i) the upregulation of a set of genes involved in antiviral immunity and (ii) the downregulation of many genes encoding acute phase proteins, particularly those involved in the complement and coagulation systems. This transcriptional block on acute phase protein coding gene expression was maintained in the presence of pro-inflammatory cytokine stimulation. Quantitative proteomics analysis of the hepatocyte secretome revealed reduced protein abundance that correlated with transcriptomic downregulation. Infection also altered the expression of cholesterol metabolism-associated genes in human hepatocytes that was independent of the interferon response.

The second axis of my thesis involved investigating the role of the epigenetic factors BAHD1 and MIER in long term *Lm* infection in hepatocytes. The BAHD1-MIER association forms the scaffold of a recently described chromatin-repressive complex, belonging to the histone deacetylase (HDAC) family. BAHD1 was previously shown to repress the interferon response upon *Lm* infection of colon epithelial cells. This work expands on these results, by providing novel data on the inhibition of interferon-stimulated genes by BAHD1-MIER in hepatocytes. The results also suggest that, in a mixed population of infected and uninfected hepatocytes, uninfected bystander cells are the major producers of interferon and interferon stimulated gene products in response to interferon after three days of infection.

This work strongly suggests that long-term infection with *Lm* profoundly deregulates the secretory and metabolic functions of hepatocytes, which could generate an environment favourable to the establishment of persistent infection through the reduced abundance of crucial hepatocyte specific innate immune proteins. At the same time, it opens up multiple avenues to explore the mechanisms of host transcriptional regulation during persistent infection and the role of uninfected bystander cells in subverting infection-mediated transcriptional repression.