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Departamento de Biologia Vegetal

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João Pedro Xavier dos Santos

Dissertação

Mestrado em Biologia Molecular e Genética

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RESUMO ALARGADO

A superfamília Ras de pequenas GTPases é dividida em cinco grandes famílias: Ras, Rho, Arf, Ran e Rab. Quando ligadas a GTP, estas proteínas interagem com diferentes efetores regulando uma grande variedade de funções a jusante, como, por exemplo, transdução de sinal, organização do citoesqueleto e tráfico de vesículas, através de um mecanismo chamado ‘’*GTP molecular switch”*. Vários estudos já demonstraram que estas GTPases, nomeadamente as Rab, exercem um papel central durante a fagocitose. As Arl são pequenas GTPases, identificadas com base na sua semelhança com as Arf (daí a sua designação de Arl – ‘’*ADP-ribosylation factor-like*’’), que ainda permanecem maioritariamente desconhecidas. Sabe-se, no entanto, que existem diferenças estruturais significativas entre as Arf e as Arl, nomeadamente nas regiões que rodeiam o local de ligação aos nucleótidos e nas regiões amino e carboxiterminais que se estendem do domínio da GTPase.

Salmonella é uma bactéria intracelular Gram-negativa que infecta humanos, causando febre tifóide e doenças gastrointestinais sendo, portanto, uma grande preocupação de saúde pública a nível global. *S. enterica* serovar Typhimurium tem sido extensivamente utilizado para estudar os mecanismos moleculares e celulares da virulência de *Salmonella*. Esta bactéria é capaz de infectar e de se replicar em vários tipos de células, mas encontra-se maioritariamente em macrófagos. Um dos mecanismos de controlo de inúmeras infeções é a fagocitose, que implica a ingestão dos patogénios por fagócitos profissionais formando-se um fagossoma. Este sofre, depois, um processo de maturação que envolve a fusão com diferentes organelos da via endocítica formando-se um fagolisossoma com um ambiente acídico rico em proteases que fornece condições favoráveis para a digestão das partículas. No entanto, *Salmonella* tira proveito da fagocitose pois, como já referido, ela replica-se intracelularmente (logo necessita de ser internalizada para se replicar). A replicação ocorre num compartimento rodeado por uma membrana conhecido como vacúolo-contendo *Salmonella* (SCV – ‘’*Salmonella-containing vacuole”*), dentro do qual a bactéria se encontra protegida das actividades antimicrobianas das células fagocíticas. Esta replicação intravacuolar depende de interacções espácio-temporalmente reguladas com compartimentos vesiculares celulares do hospedeiro, cuja regulação é feita por sistemas de secreção tipo III. *Salmonella* codifica dois sistemas de secreção tipo III, SPI1 e SPI2, que são respetivamente ativados durante a invasão da célula hospedeira e durante a replicação (mas não são independentes um do outro).

As interacções entre organismos multicelulares e microorganismos envolvem estratégias empregues pelos últimos a fim de sobreviver dentro das células hospedeiras. Uma destas estratégias é o combate à fagocitose, tendo como alvo as pequenas GTPases.

De facto, já foi demonstrado que vários microorganismos são capazes de manipular os níveis de Rab GTPases em seu benefício. Apesar das diferenças entre as Arl e as Rab GTPases, é plausível propor que as Arl possam também ser manipuladas por *Salmonella* (e também *Plasmodium berghei* e *Escherichia coli*), exercendo um papel importante na fagocitose, da mesma forma que as Rab o fazem. De facto, um membro das Arl, ARL8B, mostrou ser essencial durante infecções de células hospedeiras por *Salmonella*, sendo essencial para a maturação do vacúolo-contendo *Salmonella* e, consequentemente, para a replicação da bactéria.

Assim este projecto tem como principal finalidade estudar o papel das Arl GTPases na infecção por *Salmonella*, estudando também *E. coli*, visto que é uma bactéria não-patogénica e *P. berghei,* que é um parasita que mimetiza a infecção da malária. Desta forma estamos também interessados em comparar as infecções por estes três micróbios. O projecto está dividido em três objectivos:

1. Analisar a expressão das diferentes Arl GTPases em BMDMs (macrófagos derivados de medula óssea) recolhidos de ratinho

2. Analisar se a infecção de BMDMs pelos diferentes micróbios leva a uma alteração da expressão das Arl que são naturalmente expressas por essas células. Como objectivo adicional pretendemos também analisar se infecção dos BMDMs por bactérias patogénicas vs. bactérias não patogénicas e por bactérias vs. parasitas leva a diferenças na possível modulação da expressão dos genes das Arl GTPases

3. Se algumas Arls tiverem a sua expressão alterada pela infecção, estudar se interferir com os níveis dessas Arls (por silenciamento e/ou sobreexpressão) altera a fagocitose ou a replicação de *S.* Typhimurium.

Dos vinte genes que codificam Arl GTPases estudados verificou-se que apenas Arl4c e Arl9 não são expressos em BMDMs, pelo que se prosseguiu o estudo com as restantes 18 Arl GTPases. Na segunda fase do projecto, através de qRT-PCR, verificou-se que é relevante qual o sistema de secreção expresso por *Salmonella* Typhimurium aquando da infecção, pois observou-se que quando a bactéria expressa o SPI2-T3SS os efeitos são mais significativos, havendo um maior número de Arls que têm os seus níveis aumentados relativamente a bactérias que expressam o SPI1-T3SS. Com o parasita verificou-se também o aumento do nível de algumas Arls, mas tal aumento não foi tão significativo. Apenas Arl10, Arl11, Arl13b e Arl15 não viram os seus níveis aumentados por qualquer micróbio, enquanto Arl4d e Arl14 tiveram os seus níveis sempre aumentados. Deste modo podemos concluir que bactérias e parasitas modulam diferentemente a expressão das Arl GTPases.

Adicionalmente, mostramos que o facto da bactéria ser ou não patogénica também tem influência, visto que *E. coli* teve efeitos menos significativos que *S.* Typhimurium.

Para a terceira fase do projeto, através de silenciamento e/ou sobreexpressão de Arl3, Arl8b, Arl14 e Arl16, estudaram-se os efeitos na fagocitose ou replicação de *Salmonella* Typhimurium. Através de citometria de fluxo avaliámos a percentagem de células infetadas e a intensidade média de fluorescência de macrófagos infetados tratados com siRNAs específicos para as referidas Arls comparativamente a macrófagos infetados não tratados. Observou-se um aumento da fagocitose e um aparente aumento da replicação quando os macrófagos eram silenciados para Arl8b ou Arl14 quando a infeção por *Salmonella* durava 2h ou 24h, o que indica um aumento da fagocitose, mas também da replicação. Relativamente ao gene Arl3 parece haver um aumento também da replicação, embora o sucesso do silenciamento desta Arl tenha sido menor, enquanto que na Arl16 não se notam diferenças. Já, por imunofluorescência confirma-se o efeito do silenciamento de Arl8b e de Arl14 no aumento da fagocitose da bactéria e também a ausência de efeitos aquando do silenciamento de Arl16.

Os resultados obtidos mostram que *P. berghei* e *E. coli* aumentam os níveis de algumas Arl GTPases. Possivelmente, tendo em conta o que se sabe sobre as Rab GTPases, isto servirá como mecanismo de evasão imunitária, nomeadamente como forma de evitar a fagocitose pelos macrófagos. Relativamente à infeção por *Salmonella* o silenciamento de Arl8b ou de Arl14 conduz a um aumento da fagocitose e, aparentemente, a uma aumento da replicação. Nós propomos uma hipótese em que o aumento da fagocitose justifica o aparente aumento da replicação da bactéria com o silenciamento: na realidade o que acontecerá será que o aparente aumento da replicação é um reflexo da maior fagocitose aquando do silenciamento (estes resultados são suportados por ensaios de citometria de fluxo e imunofluorescência). Na verdade o silenciamento de Arl14 e de Arl8b não conduz a uma alteração da replicação da bactéria, apenas da fagocitose. A Arl8b é essencial para a maturação do SCV, sendo, consequentemente, a sua presença importante para a replicação. Nós justificamos a existência de replicação na ausência da Arl8b devido a um possível mecanismo de compensação com a sua isoforma Arl8a. Desta forma, a Arl8a vai, na ausência da Arl8b, assumir as suas funções. Por sobeexpressão do gene Arl8b não se observaram diferenças na replicação comparativamente com a amostra controlo. Mais uma vez, isto seria um reflexo da menor taxa de fagocitose que se esperaria ocorrer aquando da sobreexpressão desta Arl (as poucas bactérias internalizadas replicar-se-iam, devido à presença de Arl8b, mas não o suficiente para o seu aumento ser maior quando comparado com a amostra controlo). Assim, nós propomos uma hipótese em que o silenciamento de Arl8b ou de Arl14 conduz a um aumento da fagocitose sem, no entanto,

afetar a taxa de replicação da bactéria. Adicionalmente, também propomos a possível existência de um mecanismo de compensação entre Arl8a e Arl8b, em que, na ausência de Arl8b, Arl8a pode assumir as suas funções.

Resumidamente, as principais conclusões a tirar deste trabalho são: a modulação distinta da expressão das Arl GTPases pelos diferentes micróbios, tal como se verifica nas Rab GTPases; a confirmação de que a Arl8b está envolvida na infeção por *Salmonella*; pela primeira vez é descrito um envolvimento da Arl14 (e talvez da Arl3) na infeção por *Salmonella*. Devido ao facto das Arl GTPases ainda serem relativamente desconhecidas estes resultados abrem novos possíveis percursos a investigar, como seja aprofundar o papel da Arl8b e da Arl14 (sem ignorar as restantes GTPases) na infeção pelos três micróbios e, seguidamente, estudar possíveis efetores destas Arls e como eles interagem com moléculas do hospedeiro para benefício do micróbio. Assim, este estudo mostra que o estudo das Arl GTPases é bastante promissor pelo que este deverá ser um campo em expansão nos próximos anos.

Palavras-chave: Macrófagos derivados de medula óssea, Arl GTPases, *Salmonella* Typhimurium, Vacúolo contendo-*Salmonella,* Fagocitose, *Plasmodium berghei*, Interacções micróbio-hospedeiro

ABSTRACT

Microbes like *S.* Typhimurium, *E. coli* and *P. berghei* have developed mechanisms of immune evasion. To achieve this, these microbes subvert host small proteins, like members of the Rab and Arl families. Therefore, members of the Arl subfamily could be targeted by these pathogens. We confirmed that eighteen of the twenty studied Arls are expressed in macrophages. By qRT-PCR, we also analyzed the expression levels of these eighteen Arls in infected macrophages and observed that all microbes differentially up-regulated the levels of specific Arls. By silencing Arl8b and Arl14 in SPI2-expressing *Salmonella*-infected macrophages we verified an increase in phagocytosis of the bacteria and an apparent increase in replication. However, we propose that this apparent increase in replication is a consequence of the previous increase in phagocytosis that we also observed. In fact we confirmed that the silencing of Arl8b and Arl14 does not affect replication. Overexpression of Arl8b supports this idea. In the absence of Arl8b replication would occur because its isoform Arl8a would assume its functions. Thus, our results suggest that Arl8b and Arl14 are needed for the avoidance of phagocytosis by *Salmonella*, but we failed to explain why the bacteria would want to avoid this process, since it is essential for their entry and into macrophages and subsequent replication. In the case of Arl16 silencing, no effects on phagocytosis or replication were found. Arl3 silencing was less efficient, but nevertheless there seems to exist some effect on replication. Surprisingly, we observed no changes in phagocytosis upon Arl3 silencing. Overall this work provides a new insight into the involvement of Arl14 in *Salmonella* infection and suggests that parasites and bacteria modulate differentially the expression of Arl proteins, possibly as a mechanism to evade the host immune responses. Moreover, this work confirms the importance of Arl8b in *Salmonella* infection. Furthermore, we also confirmed the fact that Arl proteins are important molecules, whose study will only expand in the future.

KEYWORDS: Bone marrow-derived macrophages, Arl GTPases**,** *Salmonella* Typhimurium**,** *Salmonella*-containing vacuole**,** Phagocytosis**,** *Plasmodium berghei***,** Host-microbe interactions

ABBREVIATIONS

Arf GTPase – ADP-ribosylation factor GTPase

Arl GTPase – ADP-ribosylation factor-like GTPase

BLAST – Basic Local Alignment Search Tool

BMDMs – Bone marrow-derived macrophages

cDNA – complementary DNA

DAPI – 4',6-diamidino-2-phenylindole

dNTP – Deoxyribonucleotide

DTT – Dithiothreitol

EEA1 – Early Endosome Antigen 1

FBS – Fetal Bovine Serum

GAP – GTPase-activating protein

GAPDH – Glyceraldehyde-3-phosphate dehydrogenase

GDI – Guanine nucleotide dissociation inhibitor

GEF – GDP/GTP exchange factor

GFP – Green Fluorescent Protein

GTP – Guanosine Triphosphate

HK – Heat-killed

LAMP – Lysosome-associated membrane protein

LPS – Lipopolysaccharide

MFI – Mean Fluorescence Intensity

$MgCl_2$ – Magnesium chloride

***Pb* –** *Plasmodium berghei*

PBS – Phosphate buffered saline

PCR – polymerase chain reaction

PFA – Paraformaldehyde

qRT-PCR – quantitative real-time polymerase chain reaction

RBC – red blood cells / erythrocytes

RPMI medium – Roswell Park Memorial Institute medium

***S.* Typhimurium –** *Salmonella enterica* serovar Typhimurium

Sif – *Salmonella*-induced filaments

SifA – *Salmonella*-induced filament gene A

siRNA – small interfering RNA

SKIP – SifA and kinesin-interacting protein

SPI1 T3SS – *Salmonella* pathogenicity island 1-type III secretion system

SPI2 T3SS – *Salmonella* pathogenicity island 2-type III secretion system

TfR – Transferrin Receptor

INTRODUCTION

Throughout the evolution of interactions between eukaryotic cells and microorganisms, such as bacteria and parasites, there was the need for the host cells to develop mechanisms that allow them to efficiently eliminate those microorganisms. One of such mechanisms is phagocytosis, which is the process by which professional phagocytes engulf particles after being chemotactically attracted to them and performing a transient and localized remodeling process that leads to the formation of a phagosome [1]. This phagosome then undergoes a complex series of changes, known as phagosomal maturation, that involve fusion with different organelles of the endocytic pathway. This leads to the formation of a phagolysosome which is an acidic compartment enriched in proteases that provides favorable conditions for particle digestion [2]. Phagocytosis is a highly conserved and complex process that has evolved to counter the constant threat posed by pathogens. However, several microorganisms, including *Salmonella enterica* serovar Typhimurium, *Escherichia coli* and *Plasmodium berghei,* have co-evolved strategies that allow them to escape the host cell defenses such as phagocytosis. One of such strategies involves the modulation of host small GTPases, such as Rab proteins and, as it has been more recently proposed, also Arl proteins.

Section 1: Small GTPases and the GTP molecular switch mechanism

Small GTPases are globular hydrolase enzymes, with ~21 kD that bind and hydrolyze guanosine triphosphate (GTP) and are present only in eukaryotes from yeast to human [12]. The Ras superfamily of small GTPases has more than 100 members that, according to the primary aminoacid sequences and biochemical properties of the proteins, are divided into five main families: Ras, Rho, Rab, Arf and Ran.

Structurally, all small GTPases contain four conserved domains for guanine nucleotide binding and GTPase activities (I through IV) and a domain for interaction with downstream effectors, as can be seen in figure 1. Generally, these proteins have post-translational modifications such as myristoylation, prenylation, acetylation or palmitoylation that allow them to interact with intracellular membranes. Rab, Ras, and Rho GTPases contain a C-terminal motif for prenylation [10]. The motif can be CAAL (C, cysteine; A, aliphatic amino acid; L, leucine) for geranylgeranylation by geranylgeranyltransferase I (GGTase I), CC/CXC for geranylgeranylation by GGTase II, or CAAX (X indicates any amino acid except for leucine and phenyalanine) for farnesylation. Arf GTPases are myristoylated [9] at the N terminus and Ran GTPases have no known modification.

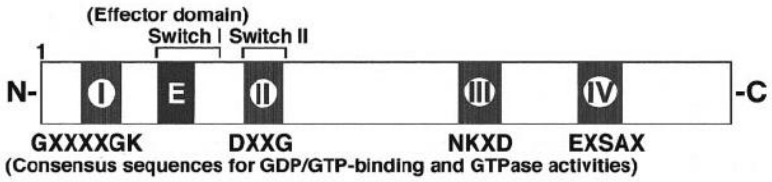

Figure 1: Consensus region of small GTPases (I –IV – domains for guanine nucleotide binding and GTPase activities; E – effector domain). The switch regions I and II change conformation upon exchange of GDP for GTP. *Figure adapted from Takai, Sasaki and Matozaki 2001* [12]

The activity of small GTPases is regulated trough a conserved mechanism called GTP molecular switch (figure 2) that involves major conformational changes in two variable regions, termed switch I and switch II. This mechanism consists in a cycle of GTP binding and GTP hydrolysis, which activates or inactivates the protein, respectively [5]. Generally, GTP-bound forms are able to interact with downstream effectors to perform a wide variety of functions, hence the classification of GTP bound form as the active form [2, 6]. The activity of these proteins is highly regulated by a set of factors:

- GDP/GTP exchange factor (GEF) which catalyzes the exchange from GDP to GTP. GEFs recognize specific residues in the switch regions and facilitate GDP release [3].
- GTPase-activating protein (GAP) which catalyzes GTP hydrolysis into GDP. It also catalyzes the intrinsic GTPase activity of small GTPases.
- Guanine nucleotide dissociation inhibitor (GDI) which acts as a chaperone for the inactive form in the cytosol, stabilizing it. Arf GTPases do not have associated GDIs.

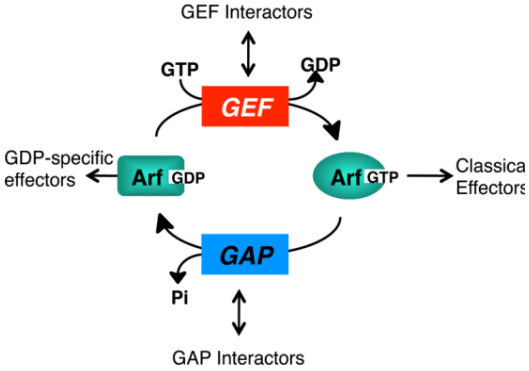

Figure 2: The GTP molecular switch. GTPases undergo a cycle of GTP binding and hydrolysis, mediated by GEFs and GAPs, respectively. The GTP-bound form carries out G protein functions by interaction with classical effectors. Currently, only a few unique effectors are known that interact specifically with the GDP-bound form. *Figure adapted from Donaldson and Jackson (2011)* [4]

GEF stimulate the dissociation of GDP from the GDP-bound form and, because the cytosolic GTP concentration is high, this nucleotide binds the molecule as soon as the GDP has been released. This leads to the conformational change of the downstream effector-binding region so that it can interact with downstream effector(s). The GTP-bound form is converted by the action of GAP to the GDP-bound form, which then releases the bound downstream effector(s) [11, 12]. In this way, one cycle of activation and inactivation is

achieved, and GTPases serve as molecular switches that transduce an upstream signal to a downstream effector.

Rab GTPases are the largest and most studied family of small GTPases and play a key role in regulating all steps of membrane trafficking [1], phagosome maturation [7] and internalization. The ADP-ribosylation factor (Arf) family controls membrane traffic and organelle architecture, such as structural integrity of the Golgi [8]. Besides six Arfs, divided into three classes, this family also includes more than 20 Arf-like proteins (Arl), which seem to have broader roles than Arf proteins.

Section 2: The Arl GTPases

A unique feature of the Arf GTPases is a segment of 10-25 aminoacids containing a myristoylated, amphipatic α-helix that extends from the N-terminus of the GTPase domain, assuring tight membrane association. However, many Arl proteins seem to lack this myristoylation motif. So, despite some Arls being myristoylated (Arl4a, Arl4c, Arl4d, Arl11, Arl14) the others are either acetylated (Arl8a, Arl8b), palmitoylated (Arl13b, Arl15) or even prenylated [8, 14].

Figure 3: Comparison between GTPases structures and conformation changes a) Schematic of Arf, Sar1a and Arl GTPases, indicating the conserved amino-terminal amphipatic helix and the protein-specific lipid modifications at the N-terminus. Note that both Arfs are myristoylated, while one of the Arls (Arl8b) is acetylated at the N-terminus. The switch and interswitch regions are also depicted. b) The Arf-GDP form reversibly associates with the membrane surface and the myristoylated N-terminal helix ensures tight membrane association of the Arf-GTP form. Switch and interswitch regions undergo a conformational change upon GTP binding to enter the hydrophobic pocket that the N-terminal amphipatic helix occupies in the GDP-bound form. *Figure adapted from Donaldson and Jackson (2011)* [6]

Throughout this work, as will be explained later, we decided to focus our study with four Arls: Arl3, Arl8b, Arl14 and Arl16.

Arl3 is known to localize to the primary cilia and also to the cytosol and microtubules. Primary cilia are organelles constituted by microtubules, present at the surface of most eukaryotic cells, responsible for the detection and transmission of chemical and mechanical signals from the extracellular medium. Effectors of this GTPase include phosphodiesterase 6 delta (PDE6δ) and Uncoordinated 119 protein (UNC119), also known as human retinal gene

4 (HRG4) [10, 18]. These proteins have similar hydrophobic lipid binding pockets and were recently shown to co-operate with Arl3 to regulate the membrane association of lipid-modified proteins. Moreover, X-linked retinitis pigmentosa protein 2 (RP2) is a known GAP for this GTPase [17] and appears to regulate the assembly and traffic of membrane associated protein complexes.

Arl8b is one of the most studied Arl GTPases and is known to be present at late endosomes and mature lysosomes [1, 13]. Arl8b overexpression stimulates lysosomes motility along microtubules [1] through the binding to the soluble protein SKIP (SifA and kinesin-interacting protein), which is also a target of the *Salmonella* protein SifA (see below for details). Arl8b and SKIP act together to recruit the motor protein kinesin-1 to lysosomes and hence direct their movement towards microtubule plus ends, away from the microtubule organizing-center (anterograde movement) [16]. Another study [7] identified HOPS (homotypic fusion and vacuole protein sorting) complex members as effectors of Arl8b and as being dependent on this GTPase for recruitment to the lysosomes, suggesting that Arl8b-HOPS plays a general role in directing traffic from endosomes to lysosomes. Moreover, this same study, showed that the formation of CD1 antigen-presenting complexes in lysosomes, their delivery to the plasma membrane, and phagosome-lysosome fusion were all markedly impaired in Arl8b-silenced cells resulting in corresponding defects in T cell activation and microbial killing. Thus, Arl8b seems to have an important role in cellular trafficking, especially lysosomal trafficking, and in immunological functions, such as antigen presentation and microbial killing. These results are supported by other studies that found that Arl8b is necessary during the formation of the phagolysosome for clearance of apoptotic cells in *Caenorhabditis elegans* [17] and for tubulation of lysosomes in macrophages [12]. Also, as will be described later, Arl8b has been described to be subverted by *Salmonella.*

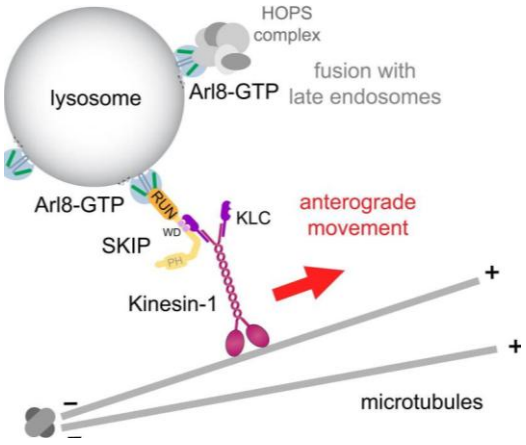

Figure 4: Arl8b known effectors. Arl8b is a marker of lysosomes and interacts with HOPS complex and SKIP proteins. Arl8b recruits kinesin-1 to lysosomes by binding to SKIP that then binds via its WD motifs to kinesin light chain (KLC), allowing the anterograde movement of the lysosome. Moreover, HOPS complex is dependent on binding to Arl8b for recruitment to the lysosomes. *Figure adapted from Rosa-Ferreira and Munro (2011)* [16]

In one study, Arl14 has been found to control MHC-II transport along the actin cytoskeleton in human dendritic cells [15]. The authors of this study found that Arl14 recruits

the motor myosin 1E via an effector protein Arf7EP, forming a complex that controls the movement of MHC-II along the actin cytoskeleton.

Like Arl14, almost nothing in known about Arl16. One study [19] found that Arl16 binds to the C-terminal domain of retinoic acid-inducible gene I (RIG-I), which recognizes RNA virus-derived nucleic acids that lead to the production of type I interferon (IFN) in most cells. Moreover, the authors show that overexpression of this Arl, but not Arl1 or Arf1, inhibits RIG-1-mediated downstream signalling and antiviral activity. The interaction between the two molecules only happens when Arl16 is in the GTP-bound form, suggesting that the viral infection triggers the binding of GTP to Arl16 that, consequently, the inhibition of RIG-I. The cellular localization of this GTPase is still unknown.

Among the other Arl GTPases the best studied cases are the known roles of Arl6 and Arl13b in ciliopathies, which are ciliary dysfunctions caused by defects in primary cilia [21]. Such dysfunctions include Bardet-Biedl syndrome [11, 20] and Joubert Syndrome [4, 9]. Arl2 has been identified to have a role in breast cancer, influencing microtubule dynamics and cell cycle progression in breast tumor cells [2] and invasiveness of the tumor [3].

Section 3: Infection by *S.* Typhimurium, the *Salmonella* pathogenicity islands (SPI) encoding Type III Secretion Systems (T3SS) and the *Salmonella*-containing vacuole

Salmonella spp is a genus of gram-negative intracelullar and enterobacterial pathogens that cause gastrointestinal infections commonly known as salmonelloses [3]. The genus *Salmonella* includes two species: *Salmonella bongori* and *Salmonella enterica*; and this last one comprises more than 2500 serovars, including serovars Typhi and Typhimurium [24]. In humans *S. enterica* serovar Typhi causes typhoid fever, while in genetically susceptible mouse strains *S. enterica* serovar Typhimurium induces a systemic illness similar to typhoid fever [21]. For this reason, *S.* Typhimurium has been widely used as a model for systemic infection in mice to study the molecular and cellular mechanisms that underlie *Salmonella* virulence.

An essential feature of the pathogenicity of *Salmonella* is its interaction with both phagocytic (such as macrophages) and non-phagocytic (such as epithelial) cells [41]. *Salmonella* Typhimurium is initially confined to the intestine and specialized microfold (M) cells of the Peyer's patches are the principal gate of entry into the intestinal epithelium used by the bacteria [26, 37, 42]. M cells represent a small proportion of the specialized follicular-associated epithelium (FAE) but, shortly after *Salmonella* infection, the number of these cells increases [37]. It is believed that *Salmonella* is able to transdifferentiate primed epithelial cells into M cells [37, 42] using the effector SopB [40] and, in that way, can promote intestinal

invasion. *Salmonella* can also enter the intestinal epithelium through enterocytes by bacterial-mediated endocytosis [17]. Once the epithelial barrier has been breached, *Salmonella* serotypes associated with systemic infection enter intestinal macrophages through macropinocytosis [1]. Inside macrophages, the bacteria activate a series of virulence mechanisms in order to survive in the microbicidal environment of the phagosome, promoting replication and dissemination of the bacteria.

A pathogenicity island is a large region of genomic DNA that encodes genes that are associated with virulence. Pathogenicity islands are typically transferred horizontally between bacterial strains and are often inserted into tRNA genes within the genome [21]. *Salmonella* harbours two *Salmonella* pathogenicity islands (SPIs) each encoding a type III secretion system (T3SS) for virulence proteins. These T3SS mediate the transfer of virulence effectors from the bacterial cell into the host-cell cytoplasm [20]. *Salmonella* pathogenicity island 1-type III secretion system (SPI1 T3SS) is required for invasion of both non-phagocytic and phagocytic cells and *Salmonella* pathogenicity island 2-type III secretion system (SPI2 T3SS) is needed for intracellular bacterial replication and systemic infection [20], as shown in figure 5. These complexes are extremely important as they allow the bacteria to interact with the host and modify the host cell functions to their own advantage. The systems have structural and functional similarities but a significant number of proteins encoded by SPI1 and SPI2 have no homologues in the other system and may be linked to specific functions of either system. However, as various authors note, the systems do not operate independently from each other [7, 8, 23]. In fact, as one study showed, the expression of SPI2-T3SS effectors in mice might begin in early stages of infection, before intestinal penetration [8], but they do not seem to be directly involved in phagocytosis. The authors of this study propose that this might work as a preparation of the bacterium for the inhospitable intracellular environment of the macrophage and ease the transition to the later systemic phase of the illness. Conversely, some effectors of the SPI1-T3SS persist in host cells long after bacterial internalization [7, 23].

One of the main hallmarks of phagocytosis is the interaction with the endolysosomal pathway to form the phagolysosome and, consequently, digest the ingested particles. The endolysosomal pathway is essential for intracellular transport and degradation of extracellular cargo [14, 36] and involves various endosomal compartments such as early endosomes, late endosomes and lysosomes.

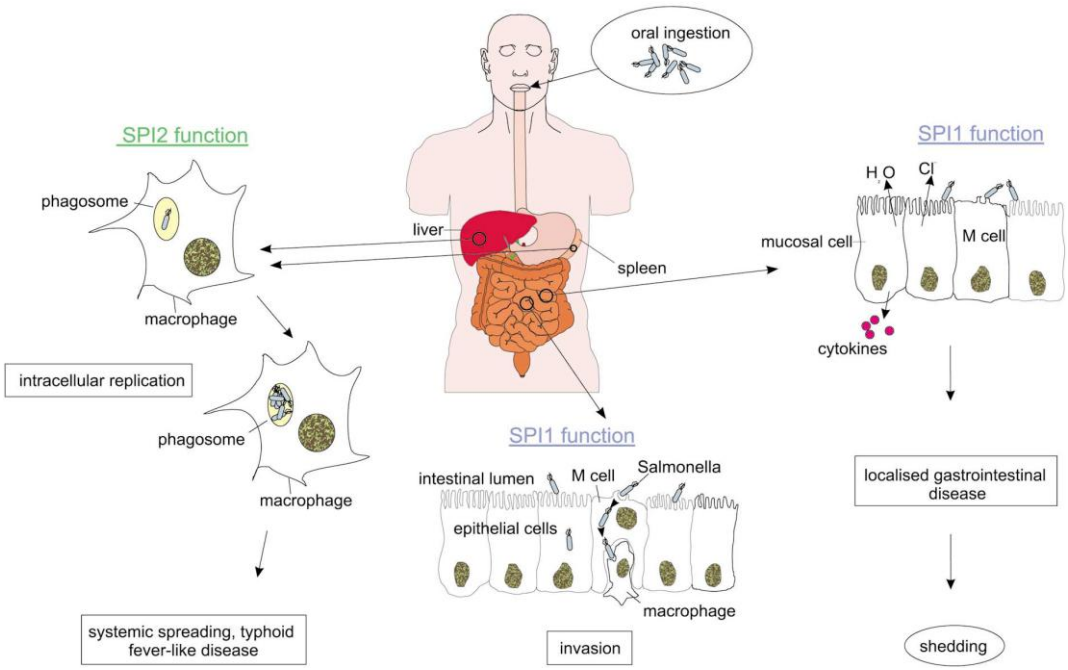

Figure 5: Schematic representation of host–pathogen interactions during pathogenesis of *Salmonella* infections. SPI1 function is required for the infection of non-phagocytic cells and penetration of the gastrointestinal epithelium. Furthermore, SPI1 is also needed for the invasion of phagocytic cells (such as macrophages). SPI2 is required for the later stages of the infection, characterized by the intracellular replication of the bacterium. *Figure adapted from Hansen-Wester and Hensel (2001)* [20]

Salmonella primarily replicates in macrophages [35] inside a unique organelle known as *Salmonella*-containing vacuole (SCV). The SCV can persist intracellularly from hours to days allowing the bacteria to escape phagolysosomal fusion. The SCV interacts with the endolysosomal pathway but the extent of this interaction is controversial. While various studies have confirmed an interaction between the SCV and early endosomes, shown by the presence of early endocytic markers, such as EEA1 [39], TfR [39], Rab5 and Rab11 [38] in the early SCV, the evidence of an interaction between SCV and late endosomes/lysosomes is still conflicting. Some groups reported that the SCV undergoes fusion with lysosomes [32, 13]. One study (30) demonstrated that SCV undergoes fusion with lysosomes whose potency has been reduced by interaction with SifA-SKIP. However, this study was done with HeLa cells, rather than macrophages. Other studies, done with macrophages, showed that the majority of SCVs do not fuse with lysosomes and late endosomes [11, 19, 33]. It is clear, though, that the SCV retains late endosome or lysosome markers such as LAMP-1, LAMP-2, LAMP-3 (15, 30, 34) and Rab7 [31].

After internalization, the bacterium remains inside a phagosome [1] that rapidly shrinks and evolves to the SCV. Then, *Salmonella* undergoes several surface remodeling processes, repressing bacterial molecules that could be recognized by the host as markers of an infection [16]. Many hours after uptake, *Salmonella* induces the formation of vast

tubular structures, enriched in LAMP-1, called *Salmonella*-induced filaments (Sifs), which originate from the SCV and extend throughout the cell [18]. Sifs are formed along microtubules and, although these filaments can have actin (10), their formation does not involve the actin cytoskeleton. Moreover, Sif formation is dependent on the SPI2 T3SS effector SifA [10] and, to a lesser extent, on SseF, SseG, SopD2 and PipB2 [10, 25, 28, 22]. SifA is known to bind the host effector SKIP and the depletion of SKIP in *S.* Typhimurium-infected cells leads to the abrogation of Sifs [6]. As SifA belongs to the WxxxE (tryptophan (W)-variable (x)-x-x-glutamate) family of bacterial effectors that mimic GTPases [2] it is believed that this effector uses a GTPase-type mechanism for Sif formation [34]. The biological significance of Sif formation is largely unknown, but SifA might be needed to maintain the integrity of the SCV membrane and might have an essential role in pathogenesis [4]. So, the fact that *Salmonella* replicates inside a vacuole where it is safe from the host defense mechanisms, contributes to its virulence and pathogenicity.

While in macrophages *Salmonella* can only exclusively remain inside the SCV, because the cytosol is lethal, in epithelial cells the bacteria can also be found in the cytoplasm [5, 9]. It seems that this cytosolic stage serves as a transition step that precedes the exit into the extracellular environment [27]. In the cytoplasm bacterial replication far exceeds that occurring within the SCVs and the bacteria express different virulence genes. Specifically, instead of expressing genes encoding for SPI2-T3SS, they are induced for SPI1-T3SS and flagellar motility, which facilitates the invasion of adjacent cells after extrusion [29]. Thus, despite the essential role of the SCVs, sometimes growth of *Salmonella* in the cytosol is just as important for the infectious cycle.

Overall, *Salmonella* has evolved many strategies that allow it to escape the host defense mechanisms and to successfully persist inside the host in a controlled way. One of such strategies involves taking advantage from being phagocytosed, as a way to replicate inside the SCV. Figure 6, depicting *Salmonella* infection of a phagocytic cell is presented next.

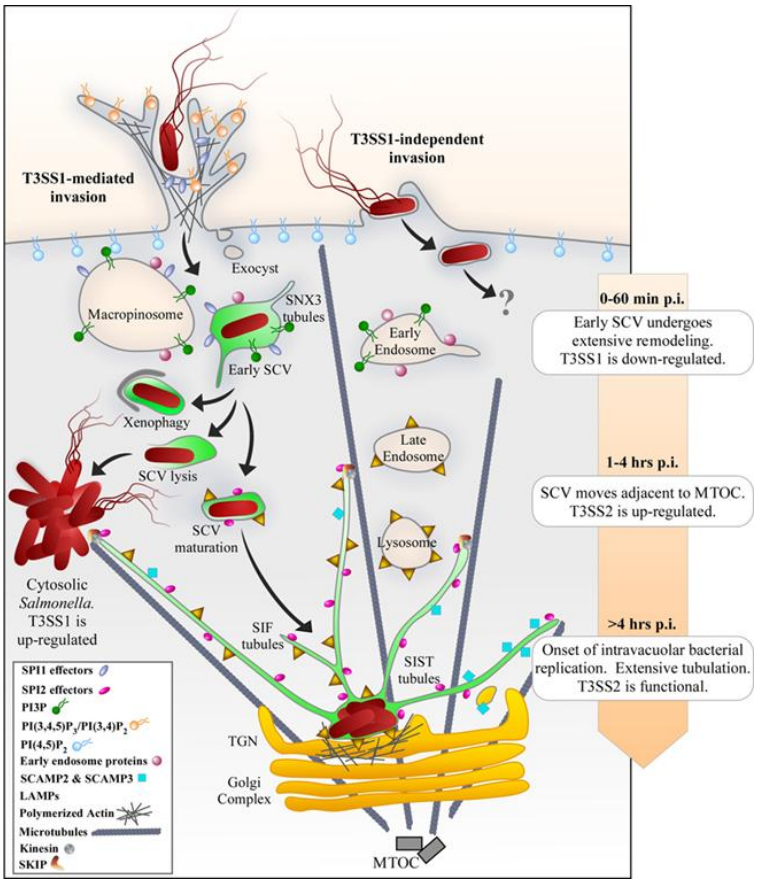

Figure 6. Infection of a phagocytic cell by *Salmonella*. Invasive *Salmonella* uses SPI1-T3SS to invade the host cell, by actin-madiated ruffling. SPI1 effectors are also present on the SCV membrane. The early SCV has many characteristics of early endosomes. Dynamic tubular networks containing SNX3 are involved in maturation of the SCV [12]. During this initial phase of infection *Salmonella* down-regulates SPI1 T3SS and induces SPI2 T3SS, which is required for subsequent steps in SCV biogenesis. The majority of SCVs relocate to a juxtanuclear location within 1–2 h and become enriched in proteins that are normally found in late endosomes and lysosomes. However, some SCVs do not undergo this maturation process and instead either lyse and release the bacteria into the cytosol or are targeted by the autophagy system. In the mature SCV, replication is initiated 4–6 h post invasion and is accompanied by the formation of the Sifs enriched in Lamp1. *Salmonella* can also invade cells via T3SS1-independent mechanisms, although biogenesis of the SCV under these conditions has not been well studied. *Figure adapted from Malik-Kale et al. 2001* [29]

<u>Section 4:</u> Overview of *Escherichia coli* and *Plasmodium berghei* infections

Enteric *E. coli* is not only present in the natural flora of the gut of many animals but can also represent an important pathogen causing significant morbidity worldwide. Human infections can occur through consumption of contaminated water or food products or through direct person-to-person spread due to poor hygiene [1]. In this project, *E. coli* was used as a non-pathogenic bacterium in contrast to the pathogenic *S.* Typhimurium and *P. berghei. E. coli is* a gram-negative, facultative anaerobic and non-sporulating bacterium. Some pathotypes of *E. coli* employ a T3SS to translocate bacterial effectors directly into the eukaryotic host cell in order to subvert host cell processes. For these pathotypes the T3SS is a major, but not the only, contributor to virulence [3]. Contrary to *S.* Typhimurium, *E. coli* does not replicate intracellularly.

Malaria is a mosquito-borne infectious disease that affects humans and other mammals manifested by symptoms that typically include fever and headaches that, in severe cases, can progress to coma or death. It is caused by intracellular protozoan parasites of the genus *Plasmodium*. Details of the life cycle of the parasite differ between different species of *Plasmodium*, with *P. falciparum* causing the most virulent form of human malaria (figure 7). In this work, we used *P. berghei* that is not of direct concern to humans, as it only infects mice. *P. berghei* is used mainly as an experimental model in laboratory for the study of malaria, as it mimics the infection by *P. falciparum*. Moreover, it replicates intracellularly inside hepatocytes and erythrocytes [9, 11].

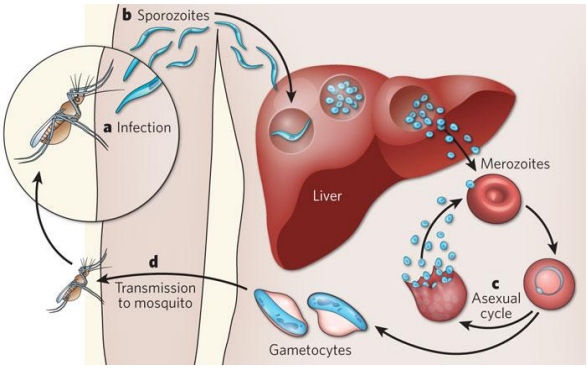

Figure 7: Basic features of the *Plasmodium* life cycle. a) Egg development in female *Anopheles* mosquitoes requires a blood meal. In the process, infected females inject the sporozoite form of the parasite into a human host. b) Sporozoites are carried in the bloodstream to hepatocytes, where they proliferate asexually (tissue schizogony), and then, as merozoites (extracellular form of the parasite), invade red blood cells. c) An asexual cycle (blood or intraerythrocytic stage) in which merozoites invade RBCs and progress through the ring, trophozoite (growing) and schizont (dividing) stages, eventually rupturing the cells and releasing male and female gametocytes [10]. d) These are transmitted back to a mosquito during a blood meal, where they fuse to form oocysts that duly divide to create sporozoites. These migrate to the salivary glands, where the cycle of infection starts again. *Figure adapted from Michalakis and Renaud (2009)* [6]

In this work we studied the blood stage of the infection (using schizonts), as it is the stage when the symptoms of the disease appear [2, 7]. The mammalian erythrocyte lacks a nucleus, so unlike many other pathogens, blood stage malaria parasites do not take over transcriptional control of the host cell. Rather, they alter transport and antigenic functions to obtain nutrients as well as avoid the host immune system, by exporting effector proteins to the erythrocyte cytoplasm and membrane. This occurs early in intraerythrocytic development and the processes by which effector proteins are exported provide fundamental mechanisms that can be targeted to control both infection and disease [2]. Although an effective malaria vaccine will need to be multivalent, incorporating antigens from different lifecycle stages, the blood-stage has been one of the most studied as a promising target. This is due to the fact that pathogenesis of malarial disease results from blood-stage infection and immune responses targeting blood-stage antigens can protect against disease or facilitate control of parasitemia [4, 5, 8].

Section 5: Arl GTPases in *S.* Typhimurium, *E. coli* and *P. berghei* infections

Bacteria and protozoa are able to modulate Rab protein expression [3, 4, 5]. A study from our group, by Seixas *et al.* [3], found that *P. berghei* induces the expression of different Rab proteins when compared to *E. coli* and *S.* Typhimurium, suggesting that this modulation could be necessary for the success of microbial infection. Moreover, this modulation happened during phagocytosis. Specifically, Seixas *et al.* found that Rab14 is modulated by *P. berghei*, but not by *S. Typhimurium* or *E. coli*. The reverse was obtained for Rab9 which, as they found, is modulated by the two strains of bacteria but not by the parasite. Wong *et al* [6] showed that pathogenic strains of *E. coli* are able to control Rho GTPase activity by translocating one effector to inactivate mammalian RhoGEFs, replacing them with bacterial RhoGEFs. Another work [2] showed that Rho GTPases can be manipulated by *Salmonella*.

Given the previous information, it is plausible to hypothesize that Arl GTPases can also be targets for these pathogens and play an important role in phagocytosis, the same way as Rab GTPases do. Indeed, Arl8b has been shown to be essential during *Salmonella* infection of host cells [1]. Moreover, these authors shown that, following infection, Arl8b localizes to SCVs and to tubulated endosomes that extend along microtubules in the host cell cytoplasm. They also found that Arl8b is necessary for kinesin-1 recruitment to SCVs (as it is for the recruitment of kinesin-1 to lysosomes) assisting in Sif formation. Thus, *S.* Typhimurium takes advantage of this Arl to promote the maturation of the SCV and consequent replication and spread of infection within the host. Until now, this is the only study that showed a direct interaction between Arl GTPases and a microbe.

OBJECTIVES

Considering the fact that *S.* Typhimurium, *E. coli* and *P. berghei* are able to manipulate Rab GTPases expression levels and that Arl and Rab GTPases are mechanistically similar the main aims of this work are:

1. Analyze the expression of Arl GTPases in BMDMs;

2. Analyze the changes in Arl genes expression upon infection of BMDMs by *Salmonella* Typhimurium, *E. coli* and *P. berghei*. As an additional aim we also want to study if the infection of BMDMs by pathogenic bacteria vs. non-pathogenic bacteria and by bacteria vs. parasite leads to differences in the modulation of Arl expression levels;

3. Study phagocytosis and replication of *S.* Typhimurium upon interference with the expression levels of some of the Arls modulated by infection with this bacterium.

Therefore, this work was divided in three phases and, in each of the phases, we tried to answer to each of the presented objectives. In the first phase, by Polymerase Chain Rection (PCR), we answered the first aim. The second aim was studied through quantitative Real-Time PCR (qRT-PCR). The third aim was achieved through silencing and overexpression assays for Arl3, Arl8b, Arl14 and Arl16 and the results were then obtained applying flow cytometry and immunofluorescence techniques.

MATERIALS AND METHODS

Mice, Parasites and Bacterial Strains

Balb/c mice were infected intraperitoneally with 10^5 infected red blood cells of *Plasmodium berghei* ANKA strain. Parasitemia was monitored by Giemsa-stained blood smears.

Relatively to bacteria, *E. coli* M61655 K12 strain (genetically engineered to express the fluorescent protein Ds-red) and *S.* Typhimurium strain NCTC 12023 were inoculated in Luria Bertani broth with the relevant antibiotics and incubated overnight (for *E. coli* and SPI2-T3SS expressing *S*. Typhimurium) or at the day of the infection (SPI1-T3SS expressing *S*. Typhimurium) at 37ºC with vigorous shaking.

Culture and Purification of Parasite Schizonts

At day 6 after infection the mice were bled and the blood used for *in vitro* culture for 18–24h so that parasites could develop into schizonts. This is achieved after overnight culture at 37ºC in RPMI medium containing FBS and gassed with a mixture of 10% CO_2, 5% O_2 and 85% N_2. Schizonts were enriched by magnetic isolation as described previously [1]. In all experiments purity was greater than 90%.

1st aim – Analyze the expression of Arl GTPases in BMDMs

RNA extraction, cDNA production and PCR

Total RNA was extracted using RNeasy kit (Qiagen) as per manufacturer's instructions. 1µg of total RNA was reverse-transcribed to synthesize complementary DNA (cDNA) using SuperScriptII RNase H-reverse transcriptase (Invitrogen, CA) and random hexamer primers. Reactions were incubated at 65ºC for 5 min, then at 25ºC for 10 min, followed by 42ºC for 50 min and finally at 70ºC for 15 min. To establish the expression pattern of the different Arls in the different cells we performed PCR with one cycle of 94ºC for 3 min and 32 cycles of 94ºC for 40 seconds, 58ºC for 40 seconds and 72ºC for 1 min using a 1:10 diluted Taq DNA polymerase (Invitrogen) and Arl-specific primers (Metabion) (Table S1). PCR products were resolved, by electrophoresis, in a 1,2%-agarose gel using TAE1X concentrated and stained with 2µL of GelRed. We determined the expression pattern with a ladder of 100bp and using tubulin as a positive control. Detailed protocols for this methodology are presented in supplementary data (Protocol S1 and Procotol S2).

2nd aim – Analyze the changes in Arl genes expression upon infection of BMDMs by *Salmonella* Typhimurium, *E. coli* and *P. berghei*

Differentiation of Primary Macrophages

Cells were collected from the bone marrow of mice and differentiated in vitro for 8 days in Iscove's medium supplemented with 10% Fetal Bovine Serum (FBS), 0.5 mM sodium pyruvate, 100 units/mL of penicillin, 100 mg/mL streptomycin, $5x10^{-5}$ M 2-mercaptoethanol and 30% L929-cell conditioned medium.

Macrophage and Pathogen Infections

Primary macrophages were counted and plated in 24 well plates at $5x10^5$ cells per well. *Plasmodium*-infected erythrocytes were added at a ratio of 30:1 (iRBC:Macrophage), the cultures incubated for 30 min., washed with medium to eliminate the parasites that were not internalized, and incubated for 4 hours, after which samples were frozen, in 300µL of lysis buffer RLT, for qRT-PCR analysis. *Salmonella* was grown in SPI1-T3SS or SPI2-T3SS conditions and added to the macrophages at a ratio of, respectively, 2:1 or 10:1 and the incubation followed the same protocol as for *Plasmodium. E. coli* was grown overnight and infections were done as described. Bacteria or parasite were also exposed to 95ºC, for 15min, so we could also use heat-killed microbes. For all infections with *Salmonella* and *E. coli*, gentamicin was applied after the infection of the macrophages as it is an antibiotic used against gram-negative bacteria in order to eliminate all the bacteria that were not internalized.

Real-time Quantitative PCR (qRT-PCR)

We extracted the RNA from the frozen samples and produced cDNA as previously described. Subsequently, we used the cDNA to perform quantitative real-time PCR (qRT-PCR) in ABI Prism 7900HT system using ABI Power SYBR Green PCR Master Mix. We used specific primers (Metabion) for the studied Arls (Table S1). cDNA levels were normalized against Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Sigma). For each Arl we used *medium* (in which macrophages were not infected), *heat-killed microbe* and *live microbe* conditions. Experiments were performed in quadruplicates, using 4µL of 1:10 diluted cDNA and 6µL of primer mix solution per well. A 2.0 fold increase was considered as the cut-off from which there was a significant increase of the gene expression level.

3rd aim – Study phagocytosis and replication of *S.* Typhimurium upon interference with the expression levels of some of the Arls modulated by infection with this bacterium

Silencing assays

Arl3, Arl8b, Arl14 and Arl16 silencing was achieved using siGENOME SMARTpool (Dharmacon) specific for *Mus musculus* Arl3, Arl8b, Arl14 or Arl16 (Table S2). Control siRNA was done with non-targeting siRNA pool (Dharmacon). Primary macrophages were transfected with 80pmol siRNA in a nucleoporator buffer supplied by the manufacturer (Amaxa Biosystems).

BMDMs were nucleoporated according to the manufacturer's protocol. The cells were then plated and incubated at 37ºC and bacterial infections with SPI2-expressing *Salmonella* were done accordingly with the referred in 2nd aim. We did not use SPI1-expressing bacteria in this aim. The optimum concentration of siRNA and post-transfection time-points after which samples were collected were previously confirmed (figure S1). Three different time-points were used:

- siRNA 24h + *Salmonella* 24h (siRNA transfection and *Salmonella* infection were made on the same day, so that cells were collected 24h post-infection and 24h post-transfection)
- siRNA 24h + *Salmonella* 2h (infection was done on the day after the transfection, so that transfection lasted for 24h and infection for 2h)
- siRNA 48h + *Salmonella* 24h (infection was made 24h after the transfection, so that transfection lasted for 48h and infection for 24h)

Overexpression assays

For overexpression assays we started by using human constructs, despite the fact that we were working with murine macrophages, as they were the ones available. However, the transfection results obtained were low and this is explained by the fact that the identity between the human and mouse sequences is not 100%, as we verified later (Table S3). Thus, we decided to produce new murine constructs for Arl8b and Arl14. After testing the new constructs we concluded that for Arl8b the results were good (around 11% of transfection) so we pursued the overexpression assay with infected macrophages.

After production of the new constructs, DNA was extracted using Midiprep kit (Sigma). BMDMs were transfected with 4µg of Arl8b-GFP or GFP alone, as described for silencing, plated and allowed to express the construct for 8 hours prior to 24 hours infection. After this, samples were collected for flow cytometry or treated for immunofluorescence assays.

Production of the wild-type mouse Arl GTPases-specific constructs

Wild type mouse *Arl8b* and *Arl14* cDNAs were amplified from murine BMDMs using primers to target mouse *Arl8b* (5'…GATCGGATCCGCCATGCTGGCGCTCATCTCCCG…3' and 3'…CGAATAAGTTGTGAGTTTTAGGGCCTCTTCGTTCGAACTAG5') or *Arl14* (5'…GATCGGATCCAAAATGGGTCTGCTGAATTCTAAAAACCC…3' and 3'…GGAATCGTTAGAAGTTCGTCTTTTTCGAACTAG…5') and the following PCR program: 1 step at 98ºC for 30 seconds, followed by 30 cycles of 10 seconds at 98ºC, 15 seconds at 52ºC, 30 seconds at 72ºC, and a final step at 72ºC for 10 minutes. Correct gene amplification was confirmed by running a small portion of the PCR product on a 1%-agarose gel. PCR products were purified with DNA Clean and Concentrator^{TM}-5 (Zymo Research) and simultaneously digested with BamHI and HindIII in Tango Buffer 2X using the primer-introduced restriction sites BamHI-HindIII in a water bath at 37ºC for at least 3 hours. Digested PCR products were purified and ligated into BglII-HindIII sites of pEGFP-N1 (Clonetech) by overnight incubation with T4 phage ligase at room temperature. *E.coli* TOP10 competent cells were transformed with 5µL of the dialysed ligation and plated on kanamycin-supplemented LA plates (50µg/mL). An isolate was grown overnight at 37ºC in LB supplemented with kanamycin 50µg/mL and DNA isolated using GenEluteT^{M} HP Plasmid Miniprep kit (Sigma). Constructs were verified by DNA sequencing and sequences were aligned to the NCBI accession numbers with BLAST to confirm correct cloning and in-frame insertion into the plasmid.

Flow Cytometry (detailed protocol at supplementary data – Protocol S3)

Cells were incubated with Alexa647-conjugated anti-CD11b antibody (as CD11b is expressed in macrophages) diluted in PBS containing 2% FBS and 0.01% NaN_3. Data were acquired on a FACScalibur and analyzed in FlowJo software (Becton Dickinson).

Immunofluorescence assays (detailed protocol at supplementary data – Protocol S4)

Macrophages transfected with siRNA were allowed to adhere to coverslips and infected with *Salmonella*. Cells were fixed with 2% PFA and stained with DAPI (stains the nucleus) and Phalloidin Alexa Fluor 488 (stains actin) (Molecular Probes-Invitrogen) and the coverslips mounted with Mowiol. Images were acquired with an Eclipse TE2000-S Widefield Fluorescence Microscope (Nikon) using a 63X objective and treated with ImageJ software.

Statistical Analysis

Experimental data were analyzed using the GraphPad Prism statistical analysis package (GraphPad Software, Inc., USA). Statistical differences were analyzed using Student's t-test. A *P value* ≤ 0.05 was considered statistically significant.

RESULTS

1st aim – Analyze the expression of Arl GTPases in BMDMs

We started by addressing the expression of the twenty Arl genes known to be present in mice, in BMDMs. With the exception of Arl9 and Arl4c (or Arf7), all the Arls were expressed in primary macrophages (figure 8). Also, accordingly to UniGene, Arl9 should, at least, be expressed in the mouse liver. However we were never able to obtain amplification, even when using three different sets of primers in cDNA collected from mouse liver (data not shown).

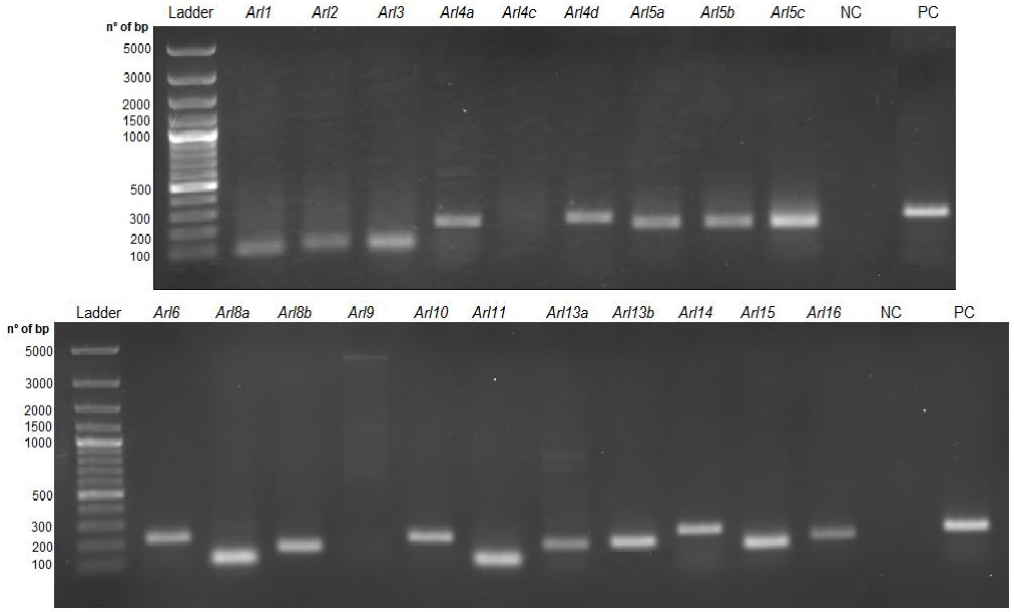

Figure 8: Arl GTPase genes expression in BMDMs collected from mice, determined through PCR. NC – negative control (sample without cDNA); PC – positive control. This image is representative of 4 experiments.

2nd aim – Analyze the changes in Arl genes expression upon infection of BMDMs by *Salmonella* Typhimurium, *E. coli* and *P. berghei*

We pursued the study focusing on all Arls expressed in BMDMs. For the infections we considered two parameters:

- **Multiplicity of Infection (MOI):** infections with *Salmonella* were made with a lower multiplicity of infection (MOI): for the bacteria expressing SPI1-T3SS we used a rate of 2:1 because, if we used a MOI of 10:1 (as we did for the bacteria expressing SPI2-T3SS) the majority of macrophages would die. In fact, it was previously described that under SPI1 conditions, macrophage killing can occur more rapidly [1, 2]. *Plasmodium*-infected red blood cells were added to the macrophages at a MOI of 30:1, since at a MOI of 10:1 we did not detect

differences in the expression of Arl GTPases (data now shown). *E. coli* was used at both 10:1 and 30:1 MOI for comparison with both *S.* Typhimurium expressing SPI2-T3SS and *P. berghei*, as it is a non-pathogenic bacterium.

- **Time of infection:** *Salmonella*-infected macrophages were collected 4h or 24h after the infection. *E. coli* and *P. berghei* infected macrophages were collected 4h after infection as this is the necessary time for the appearance of effects in the gene expression and much longer times would lead to the destruction of the microbe by macrophages.

We used LPS as a positive control as it is a potent activation stimulus for macrophages. In response to LPS, macrophages activate different cellular pathways such as cytokine secretion that Arls are likely to regulate. Heat-killed microbes were used to study if the presence of the live microbe is crucial or, in contrast, if the dead microbes will also lead to alterations in the gene expression. The results obtained for all Arls are summarized in table 1 and the plots for Arl3, Arl8b, Arl14 and Arl16 gene expression in *Salmonella*-infected macrophages are shown in figure 9. Plots for Arl8b, Arl13a and Arl14 gene expression in *Plasmodium*-infected macrophages are shown in figure S2.

Arl14 and Arl16 showed an increase in their expression when BMDMs were infected with *S.* Typhimurium or *E. coli.* However, Arl3 and Arl8b only had their levels increased upon *Salmonella* infections (figure 9). Strikingly, we never observed any decrease in the levels of any of the Arls upon infection with the different microbes. Furthermore, the expression of Arl10, Arl11, Arl13b and Arl15 did not change in any of the conditions used, and so it seems that they are not a target of these microbes (table 1). Interestingly, *Salmonella* expressing SPI1 or SPI2-T3SS did not show the same pattern of changes in the expression levels of Arl GTPases. SPI2 T3SS-expressing bacteria are able to increase almost all Arl GTPase levels, when compared to the control. Also, the longer the infection with *Salmonella*, the more striking are the effects, as with 24h of infection more Arls had their expression altered when compared to 4h of infection, for both T3SS (table 1). Remarkably, only Arl2 and Arl6 did not show an increase in the expression after treatment with LPS, our positive control. Furthermore, *P. berghei* up-regulated Arl14 and Arl13a gene levels, among others, but not Arl3, Arl8b or Arl16 (table 1, figure S2). However, this effect was less striking than with *Salmonella*. Overall, it seems that bacteria and parasite differentially manipulate the levels of specific Arl genes and that, among others, *S.* Typhimurium up regulates the levels of Arl3, Arl8b, Arl14 and Arl16 (figure 9).

	SPI1-expressing *Salmonella*		SPI2-expressing *Salmonella*		*E. coli*		*P. berghei*	LPS
MOI	2:1		10:1		10:1	30:1	30:1	
Time-point	4h p.i.	24h p.i.	4h p.i.	24h p.i.	4h p.i.	4h p.i.	4h p.i.	
Arl1		↑		↑	↑	↑	↑	↑
Arl2				↑				
Arl3			↑	↑				↑
Arl4a				↑		*		↑
Arl4d	↑	↑	↑	↑	↑	↑	↑	↑
Arl5a			↑	↑			↑	↑
Arl5b		↑	↑	↑	↑	↑*		↑
Arl5c	↑	↑		↑	↑	↑*	↑	↑
Arl6			↑	↑				
Arl8a				↑*				↑
Arl8b	↑	↑	↑	↑				↑
Arl10								↑
Arl11								↑
Arl13a				↑*	↑*		↑*	↑
Arl13b								↑
Arl14	↑	↑	↑	↑	↑	↑	↑	↑
Arl15								↑
Arl16	↑	↑	↑	↑	↑	↑		↑

Table 1: Different microbes modulate differentially the expression of Arl GTPase genes. Arl GTPase gene expression levels in macrophages infected with *Salmonella* expressing SPI1 or SPI2 T3SS, *P. berghei* or *E. coli.* These results were obtained by qRT-PCR, being that an increase in gene expression level above 2.0 fold is represented by an arrow (↑), while no difference in expression level, as compared to macrophages incubated with uninfected red blood cells or medium alone, is represented by an empty square. (*) represents an increase in the expression when macrophages were infected with the heat-killed bacteria or parasite.

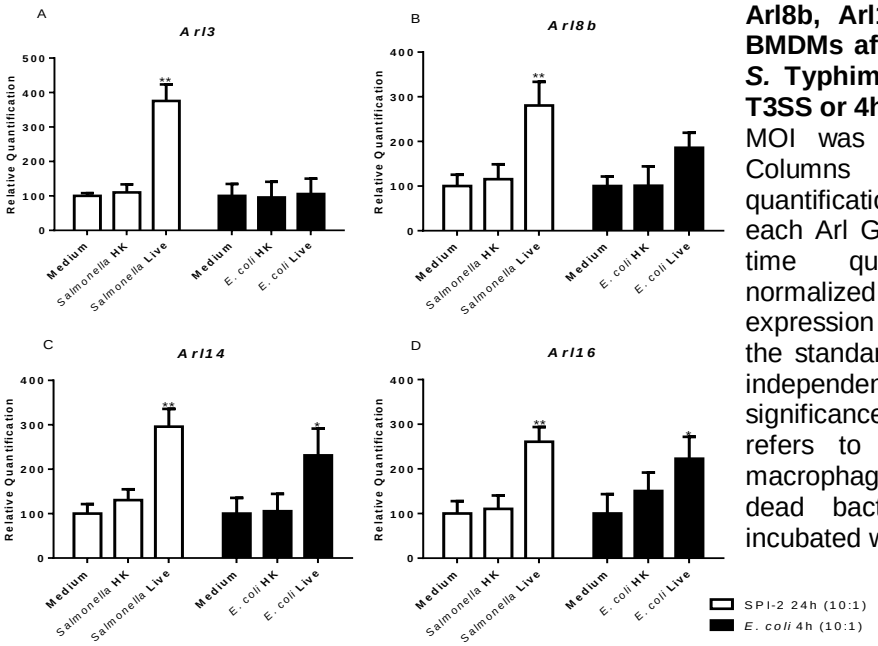

Figure 9: Expression of Arl3, Arl8b, Arl14 and Arl16 genes in BMDMs after 24h of infection with *S.* Typhimurium expressing SPI2-T3SS or 4h of infection with *E. coli*. MOI was 10:1 for both bacteria. Columns represent the relative quantification of the cDNA levels of each Arl GTPase, analyzed by real-time quantitative PCR and normalized against GAPDH gene expression levels. Error bars indicate the standard error of the mean of 3 independent assays. Statistical significance (*p<0.05, **p<0.01) refers to the difference between macrophages incubated with live or dead bacteria and macrophages incubated with medium alone.

3rd aim – Study phagocytosis and replication of *S.* Typhimurium upon interference with the expression levels of some of the Arls modulated by infection with this bacterium

We decided to pursue our work with Arl3, Arl8b, Arl14 and Arl16. We studied Arl14 and Arl16 as their levels were always consistent in qRT-PCR assays and their levels were always increased in all conditions of *Salmonella* infections. Arl8b was chosen as it is one of the most studied Arl GTPases, and already known to be involved in *Salmonella* infection, thereby providing a positive control. Arl3 was analyzed because its levels were only altered in infections by *Salmonella* harbouring the SPI2-T3SS. We did not study Arl4a, Arl4d, Arl5a, Arl5b or Arl5c as their expression levels, in qRT-PCR assays, were always very variable.

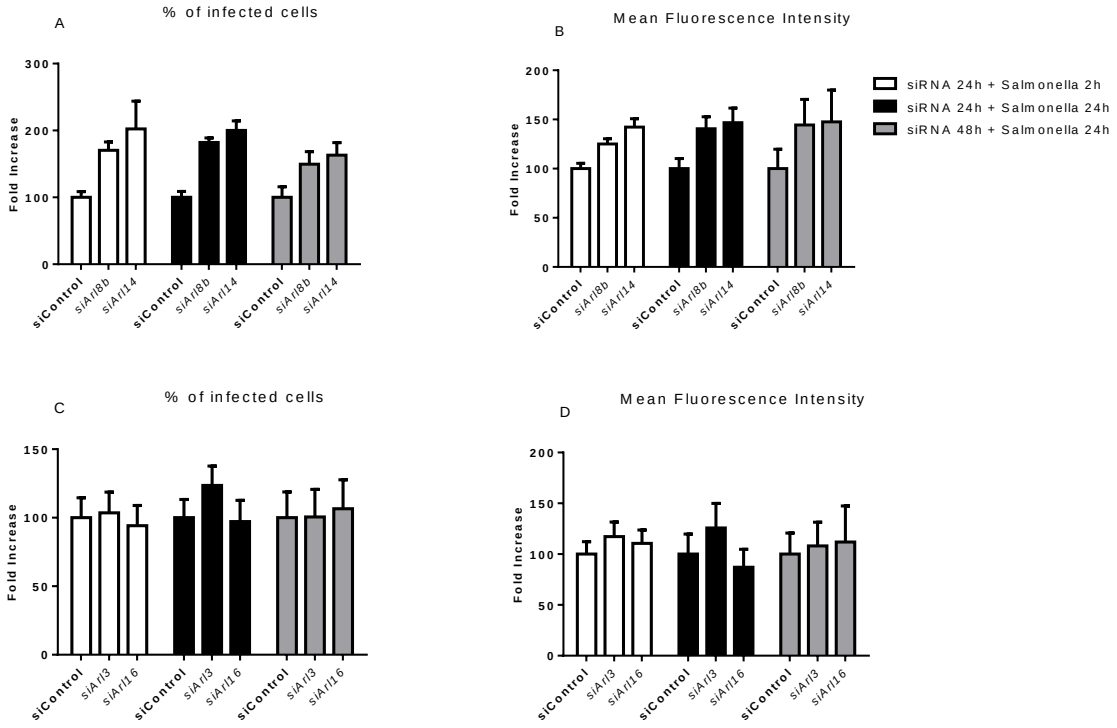

Figure 10: Arl3, Arl8b, Arl14 and Arl16 silencing using specific siRNAs in SPI2-expressing *Salmonella*-infected macrophages. Silencing of Arl8b and Arl14 was performed in the same experiments, and the same happened for Arl3 and Arl16. After transfection with the siRNA, cells were infected with the bacteria for 2h or 24h in order to achieve the desirable time-points. Samples were then treated for flow cytometry to evaluate possible alterations in phagocytosis (2h p.i.) or replication (24h p.i). Columns represent the fold increase of the % of infected cells or MFI of siArl8b- or siArl14-treated samples relative to siControl-treated samples for Arl8b and Arl14 (A, B) and Arl3 and Arl16 (C, D). Graphs are representative of 2 experiments. No statistical significance test was performed since the number of samples for such was not sufficient.

By flow cytometry we evaluated two parameters: percentage (%) of infected cells and amount of bacteria internalized by each cell (given by Mean Fluorescence Intensity – MFI). We used two time-points: 2 hours post-infection, to evaluate the effect of silencing in the

internalization/phagocytosis of the bacteria and 24 hours post-infection, to evaluate the replication of the bacteria inside the macrophages, always comparing the results for silenced BMDMs with non-silenced control (treated with non-targeting siRNA).

Arl8b and Arl14 seem to affect both phagocytosis and replication of *Salmonella*, since we observed an effect when the infections last for either 2h or 24h: an increase in both % of infected cells and MFI (figure 10 A, B). Arl3 silencing was less efficient (figure S1) and this could explain the reason for the less striking results obtained with this Arl. Nevertheless, its silencing seems to lead to a higher % of infected cells and MFI after 24h of infection (figure 10 C, D), so we expect that with a more efficient silencing, a higher difference would be observed. Silencing of Arl16 did not show any striking effects in any of the parameters tested (figure 10 C, D). Also interesting to note is that the fold increase in the % of infected cells and MFI when Arl8b and Arl14 were silenced, compared with the control, is the same between 2h and 24h (figure 10 A, B). This may indicate that, in fact, there was no change of the replication in the absence of these Arl GTPases, a hypothesis that will be further addressed in the discussion.

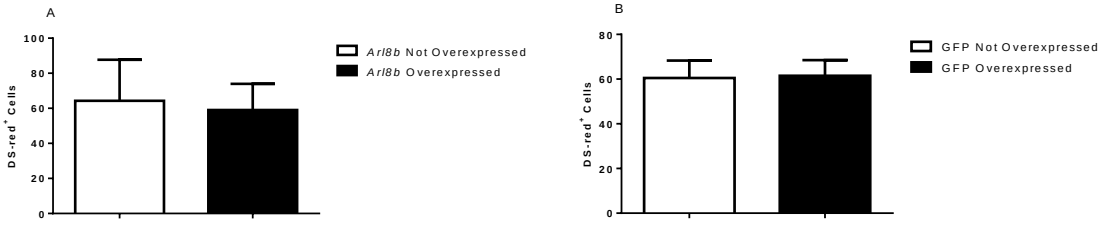

Figure 11: Arl8b overexpression assay in *Salmonella*-infected BMDMs. Macrophages were transfected with Arl8b-GFP or GFP for 8 hours and then infected with *Salmonella* for 24 hours, after which the samples were collected for flow cytometry. Columns represent the percentage of GFP-Arl8b overexpressing cells or cells that do not overexpress Arl8b with internalized *S.* Typhimurium (A) or the percentage of GFP overexpressing cells or cells that do not overexpress GFP with internalized *S*. Typhimurium (B). No statistical significance test was performed since the number of samples for such was not sufficient, as only one experiment was done.

When overexpressing Arl8b, after 24h of infection, we did not observe any difference in infection between the cells that overexpress and the cells that do not overexpress Arl8b, which indicate that there are no differences in replication (figure 11A). Since the overexpression of GFP showed no difference in infection when compared to BMDMs without GFP overexpression, as measured by flow cytometry, we conclude that GFP has no influence in the obtained results (figure 11B).

We then pursued our work with immunofluorescence assays for all the conditions tested by flow cytometry (not all results are shown). The obtained results, by Widefield Fluorescence Microscopy, for Arl8b- and Arl14-silencing and Arl3- and Arl16-silencing, 2 hours post-infection, are presented in figures 13 and S3, respectively. We also quantified the

number of bacteria internalized by 100 randomly selected infected cells upon silencing, for the 2 hours post-infection time-point (figure 12). The obtained results confirm the data obtained by flow cytometry. At 2 hours post-infection Arl8b- and Arl14-silenced BMDMs have a higher number of internalized bacteria when compared to the BMDMs treated with non-targeting siRNA (figure 12A, figure 13), which would be due to a higher phagocytosis. Arl3 or Arl16 silencing does not affects phagocytosis, as the number of internalized bacteria is similar between silenced and control samples (figure 12B, figure S3). So, immunofluorescence assays confirm the higher phagocytosis upon Arl8b- or Arl14-silencing.

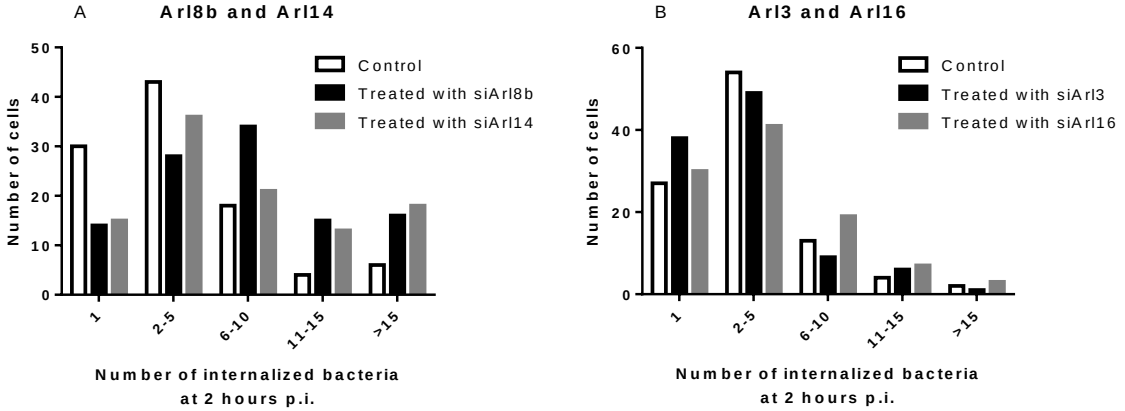

Figure 12: Quantification of the number of bacteria internalized by infected cells, upon silencing with Arl8b, Arl14, Arl3 or Arl16 specific-siRNAs. After immunofluorescence assay, 100 infected macrophages on each sample (100 infected macrophages for each silenced Arl) where analyzed for the number of bacteria internalized. This was possible due to fact that *Salmonella* was labeled with DS-red. Columns represent the number o cells with 1, 2-5, 6-10, 11-15 or >15 internalized bacteria. No statistical significance test was performed since the number of samples for such was not sufficient.

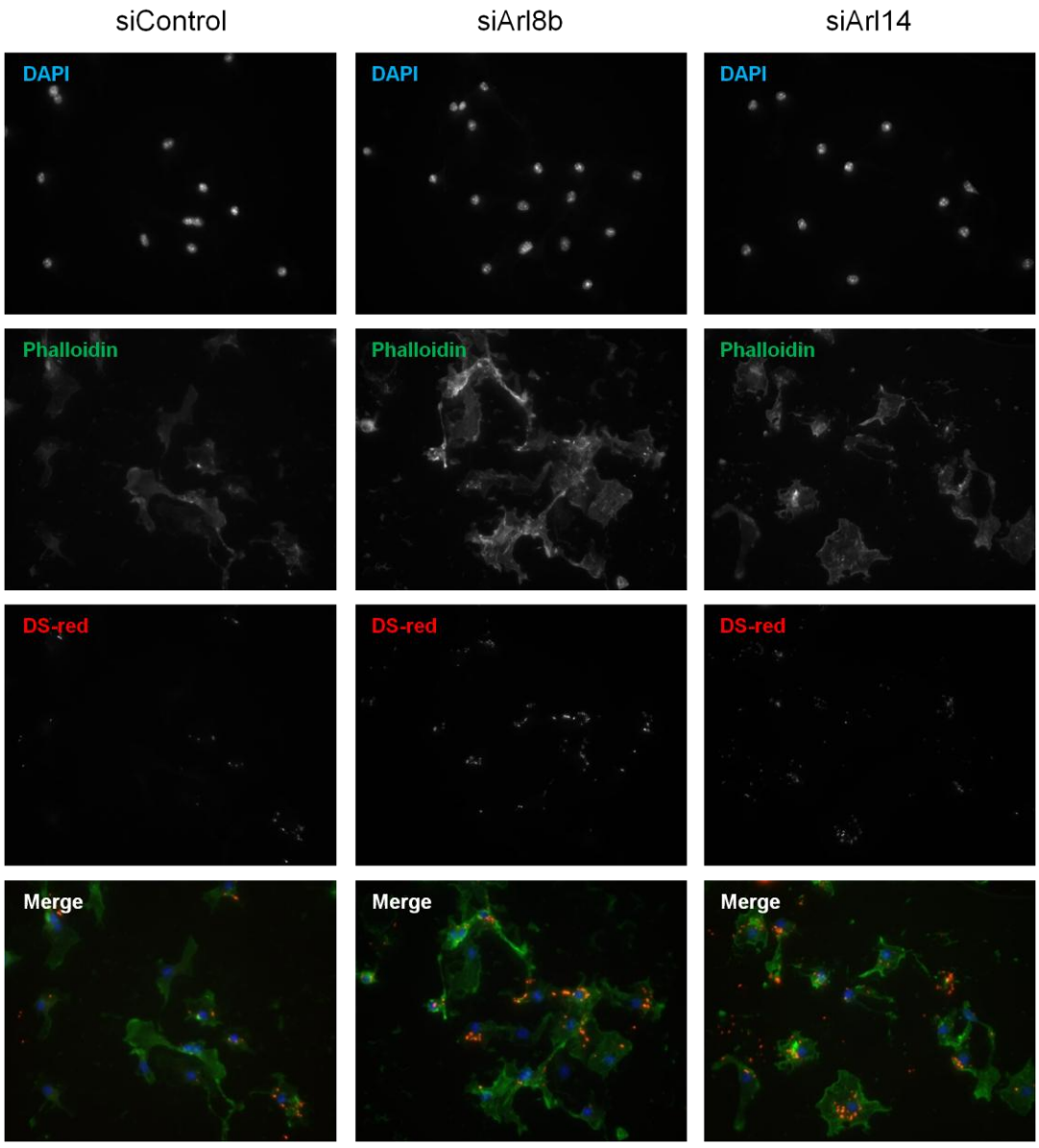

Figure 13: Immunofluorescence assay for Arl8b and Arl14 silencing for 2 hours post-transfection. DAPI (blue) was used to stain the nucleus and phalloidin (green) to stain the actin. *Salmonella* was already labeled with DS-red (red). There are more internalized bacteria in Arl8b- or Arl14-silenced samples relatively to samples treated with non-targeting siRNA (control).

DISCUSSION

By PCR we determined the expression pattern of twenty Arl genes in bone marrow-derived macrophages (BMDMs) and verified that only Arl4c (or Arf7) and Arl9 are not normally expressed in these cells. However, other Arl GTPases have already been described, such as Arl17, which is expressed in human cells but not in murine cells [2]. We then performed qRT-PCR and verified that *S.* Typhimurium expressing either SPI1- or SPI2-T3SS, *E. coli* and *P. berghei* up-regulated the levels of specific Arl GTPases, which suggests that, somehow, these microbes subvert these molecules to their own benefit. To study if this is the case we analyzed more thoroughly Arl3, Arl8b, Arl14 and Arl16, to observe if they are involved in the infection by *Salmonella*, using *Salmonella*-infected macrophages.

S. Typhimurium infections

qRT-PCR results showed that SPI1-expressing bacteria had less striking effects leading to up-regulation of less Arls, than SPI2-expressing bacteria (table 1). This can be due to the fact that SPI1 effectors are needed for the bacteria to induce their own internalization and, consequently, SPI2-expressing bacteria will not promote their phagocytosis, since they don't express SPI1. The fact that at 24 hours post-infection the up-regulation of Arl GTPases is more remarkable than at 4 hours (table 1) can simply be explained by the fact that the bacteria had more time to induce effects in the host defense mechanisms. The up-regulation of Arl8b levels was expected, as this Arl is known to be essential for SCV maturation and, consequently, for replication of the bacteria.

Silencing of Arl8b and Arl14 led to an increase in phagocytosis (figure 10 A, B; figure 12; figure 13) and, apparently, also to an increase in replication (figure 10 A, B), when compared to cells treated with non-targeting siRNA. Moreover, we have shown that the bacteria up-regulate the expression levels of these Arls (table 1). Beyond that, overexpression of *Arl8b* did not seem to cause any changes in replication (figure 11 A). Altogether these results came out as surprising since:

- One of the main reasons for the success of *Salmonella* infection is its ability to survive inside macrophages, in the SCV, where it can replicate avoiding the fusion of the phagocytic and endolysosomal pathways. Thus, *Salmonella* is a particular microbe to whom the earlier steps of phagocytosis are necessary for its survival, as they will enable the bacteria to enter the cell. So why would the bacteria up-regulate the levels of Arl8b and Arl14 if the silencing of these Arls increases phagocytosis?

- Arl8b is essential for the maturation of the SCV (and consequently to replication itself) and is also present in lysosomes. So, how could the silencing of Arl8b lead to the apparent increase in replication and its overexpression to no effects?

We propose the following model which is based in two points:

1. **The apparent effects in the replication are, in fact, a reflection of the previous increase in phagocytosis:**
   The silencing of Arl8b and Arl14 leads to an increase in phagocytosis, which means that more bacteria are internalized. As referred earlier, the fold increase of infection when Arl8b and Arl14 are silenced, compared with the control, is the same at 2h and 24h (figure 10 A, B). This means that, in that time frame, the phagocytosed bacteria replicated equally between silenced and control conditions, and thus the higher infection observed at 24 hours post-infection for Arl8b and Arl14 silencing would be due to the higher phagocytosis at 2 hours post-infection. In fact, we suggest that replication was not affected. If the silencing of these Arls increased replication, than at 24 hours we would observe a higher fold increase in the Arl8b- or Arl14-silenced samples relative to control samples, and that was not verified. Furthermore, this would explain the lack of effect in replication when we overexpressed Arl8b. Following the same argument, Arl8b overexpression would decrease phagocytosis and, at 24 hours, the fewer internalized bacteria would not have replicated enough to be many more when compared to the control samples. Nevertheless, the presence of Arl8b would allow the replication.

2. **The existence of replication in the absence of Arl8b would be explained by the existence of a compensation mechanism by Arl8a:**
   In the absence of Arl8b, its isoform Arl8a could compensate and therefore enable the maturation of the SCV which would allow the survival of the internalized bacteria. In fact, for several small GTPases, it is known that when one isoform is absent, the other usually can assume the functions. Such type of mechanism has been already described for Rab1a and Rab1b GTPase isoforms [7]. Furthermore, as Arl8b, Arl8a is also known to be present in lysosomes [4].

Thus we propose that the differences observed at 24 hours post-infection reflect the ones observed at 2 hours post-infection when phagocytosis is occuring. Arl8b and Arl14 silencing increases phagocytosis, a result that is supported by flow cytometry and immunofluorescence analyses. So, we speculate that these Arls prevent phagocytosis. As

for replication, this parameter is not affected by Arl14. The absence of Arl8b would be compensated by the presence of Arl8a which would allow a normal replication.

However, we still fail to explain why the cell would up-regulate Arl8b (and Arl14) if these Arls prevent phagocytosis, since this is a favorable event for the bacteria. It is possible that this occurs because the benefits for replication of Arl8b up-regulation would overcome the disadvantages.

We also observed an up-regulation of Arl16 upon qRT-PCR but no differences in phagocytosis or replication of the bacteria between Arl16-silenced samples and control were detected (figure 10 C, D; figure S3). This means that the bacterium needs Arl16, but not directly to influence its own phagocytosis or replication. It is possible that this Arl GTPase interferes with other mechanisms of the infection cycle of *Salmonella*.

As to Arl3 we verified that its silencing also led to an increase in the infection, at least, at 24 hours post-infection (figure 10 C, D). This could mean that this Arl, contrary to the Arl8b or Arl14, has a specific effect in replication. However, this increase was not as significant as for Arl8b and Arl14 silencing (figure S1). We expect that, had the silencing been better, the results would be more striking (a better silencing could be achieved doing shorter time points than 24h hours post-transfection).

E. coli and _P. berghei_ infections

Previously, Seixas *et al.* found that *S.* Typhimurium and *E. coli* up-regulate the levels of the same set of Rab GTPases. Therefore, we were expecting that both bacteria strains would have modulated equally the same set of Arls. However, this was not the case, since *E. coli* and *Salmonella* did not show the same pattern of up-regulation of Arl expression (table 1, figure 2). All the Arls that had their levels up-regulated by *E. coli* were also up-regulated by *Salmonella.* This can be because the MOI used for the infections with *E. coli* was not high enough – a higher MOI could possibly lead to more striking effects. Yet, we also tested two ratios of infection (10:1 and 30:1) and they had similar patterns, with the exception of Arl13a that was only up-regulated when the bacteria were used in a MOI of 10:1 (both live and heat-killed) and Arl4a which was surprisingly only up-regulated by heat-killed *E. coli* at a MOI of 30:1. This indicates that the bacteria are only capable of modulating the levels of these Arl proteins when specific ratios are used. So, we propose that the fact that *E. coli* is a non-pathogenic bacterium may explain the lower effects of this bacterium in Arl GTPase levels. Among the Arls up-regulated by *E. coli* were Arl14 and Arl16, but not Arl8b. As *E. coli* does not replicate intracellulary and does not need the presence of a vacuole, it would also not need Arl8b for survival. Moreover, as Arl8b contributes to the maturation of lysosomes, *E.*

coli would not benefit from its up-regulation. Also, Garg *et al.* showed that, without Arl8b, there was less killing of *E. coli,* so the presence of Arl8b would not be beneficial [3].

As for *P. berghei* infections we observed an up-regulation of Arl14, as well as Arl1, Arl4d, Arl5a, Arl5c and Arl13a which means that these Arls might be important for the parasite infection. Moreover, heat-killed parasite up-regulated the expression of Arl13a. Again, as in the case of *E. coli* infections*,* Arl8b was not up-regulated.

Contrary to *Salmonella*, phagocytosis is a deleterious event for *E. coli* and *P. berghei*, as these microbes are not able to avoid phagolysosomal fusion [1, 6]. So, these microbes take no benefit from the increase in phagocytosis. For the malaria parasite, macrophage-mediated phagocytosis of *Plasmodium berghei*-infected RBC is one of the main mechanisms for clearance of the parasite and thus host defence against malaria [4]**.** Knowing what happens with Rab GTPases we expect that, the silencing of the up-regulated Arl GTPases in *E. coli*- or *P. berghei* infected macrophages would lead to an increase in phagocytosis. Therefore the microbes could up-regulate the levels of specific Arls as a way to avoid phagocytosis. Since *E. coli* and *P. berghei* do not replicate inside macrophages they do not need a vacuole, as *Salmonella* needs. Also, since Arl8b is involved in the maturation of the vacuole [5] and in phagosome-lysosome fusion [3] there would be no advantage for these microbes for its up-regulation. Maybe the host will even use Arl8b against these two microbes once they are successfully phagocytosed by macrophages as this Arl is also present in the endolysosomal pathway that interacts with the phagocytosis pathway. Since we used *P. berghei* in its blood-stage of the infection, we hypothesize that the up-regulated Arls by this parasite, such as Arl14, will play a role in this phase. Such role could be to avoid the phagocytosis of the parasite-infected RBCs which would prevent the replication of the parasite, since when phagocytosed by macrophages, the parasite will not replicate inside RBC. However, since we did not try silencing and overexpression assays with *E. coli* or *P. berghei*-infected macrophages, these hypotheses are merely speculative. What this work provides is that these bacteria and parasite modulate the expression of Arls, as was shown by qRT-PCR.

CONCLUSION AND FUTURE PERSPECTIVES

This work provides evidence that different microbes modulate differentially the expression of Arl GTPases, as was already verified, by our group, for Rab GTPases. This different modulation seems to arise from the nature of the microbes (bacteria vs parasites) or even their pathogenicity (a fact that, for the Rab GTPases seemed irrelevant, since pathogenic and non-pathogenic bacteria modulated Rab GTPases equally). Like Rab GTPases, Arl GTPases seem to be important for phagocytosis.

It also confirms that Arl8b is indeed very important for the outcome of *S.* Typhimurium infection. Importantly, we describe a new role for this GTPase in phagocytosis avoidance by the bacteria. Moreover, this is, to our knowledge, the first study to reveal a role for Arl14 in *Salmonella* infection and, possibly, also Arl3 (albeit with different roles, since Arl3, despite its weaker silencing efficiency, seemed to only affect replication and not phagocytosis).

We also propose a model in which the silencing of, at least, Arl8b and Arl14 increases phagocytosis but has no effect in replication of *Salmonella*. The absence of Arl8b would be compensated by the presence of its isoform Arl8a which would allow the replication of the bacteria inside the SCV at normal rates.

Overall, this work also provides evidence that, despite their similarities, Rab and Arl proteins have distinct roles and so they are targeted differently by the microbes.

This study leaves several questions that still have to be answered. Thus, as to future perspectives to this work, we propose:

- Study more in detail the role of Arl8b and Arl14 in phagocytosis and confirm that the main reason for the up-regulation of these Arls is to avoid phagocytosis. This could be done by testing shorter time-points like 10 minutes, 15 minutes or 20 minutes post-infection which would enable the following of the internalization process of the bacteria. If this is confirmed, then the next step would be to find out why the bacteria would want to avoid phagocytosis;
- Study the Arl8a/Arl8b compensation hypothesis doing a double knockdown;
- Study the interaction of effectors of these Arls with *Salmonella;*
- Obtain better silencing results for Arl3 and study if this Arl indeed affects replication, but not phagocytosis;
- Perform silencing and overexpression assays with *E. coli* and *P. berghei* infections*;*

In conclusion, since the function of most Arls is mainly unknown, they are a very promising field of research, and this work convincingly shows how complex their interactions with microbes can be and how vast their roles/functions in infection are.

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SUPPLEMENTARY DATA

Protocol S1 – cDNA production protocol

1 – Distribute, per PCR tube, 10µl of 1:10 diluted RNA (use 1µg) and add 2µl of Mix 1 per PCR tube

2 – Run on PCR machine 65ºC for 5 minutes

3 – Add 7µl of Mix 2 per PCR tube and leave 2 minutes at room-temperature

4 – Add 1µl of Mix 3 per PCR tube

5 – Run on PCR machine (25ºC 10 min, 42ºC 50 min and 70ºC 15 min)

Mix 1 (amount per PCR tube)	Mix 2 (amount per PCR tube)	Mix 3 (amount per PCR tube)
dNTP (1µl)	5x Buffer (4µl)	SuperScriptII RNase H-reverse transcriptase (0.25 µl)
Random Hexamer Primers (0.1µl)	DTT (2µl)	H_2O (0.75µl)
H_2O (0.9µl)	RNaseOUT (1µl)	

Protocol S2 – PCR Protocol

1 – Distribute 23µl of Master Mix 23µl per PCR tube

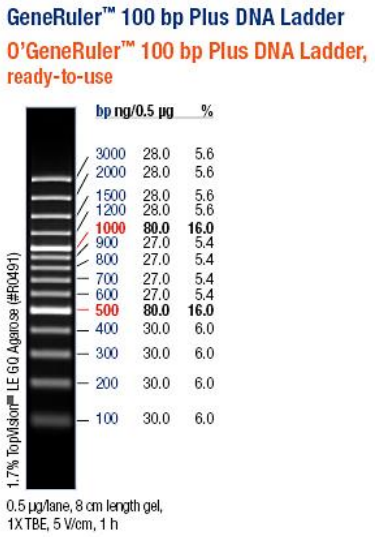
2 – Add 2µl of cDNA per PCR tube

3 – Add 2µl of Arl-specific Primers per PCR tube

4 – Run on PCR machine for 3 minutes at 94ºC and then for 32 cycles of 94ºC for 40 seconds, 58ºC for 40 seconds and 72ºC for 1 minutes

5 – Resolve PCR products, as previously indicated in Materials and Methods section, using a GeneRuler™ 100bp DNA ladder, ready-to-use

Master Mix	Amount per PCR tube
Taq (1:10 diluted)	1.25µl
dNTP	0.5µl
Buffer	5µl
$MgCl_2$	1µl
H_2O	13.25µl



Protocol S3 – Flow Cytometry protocol (After silencing/overexpression assays and infection with *Salmonella*):

1. Detach the macrophages from the 24 wells plate with a syringe plunger and put the content in eppendorfs
2. Centrifuge 5 minutes at 5000rpm and discard the supernatant
3. Add 150µL of FACS buffer *(the protocol for the overexpression assays ends here. For those assays, samples are added to FACS tubes and are ready for analysis by Flow Cytometry)*
4. Distribute the content in a new 24 wells plate, centrifuge 5 minutes at 1200rpm and discard the supernatant
5. Dilute the anti-CD11b antibody (1:100) with PBS (containing 2% FBS and 0.01% NaN_3), and add 25µL to each well
6. Wait 20 to 30 minutes and add 150µL of FACS buffer
7. Centrifuge 5 minutes at 5000rpm and discard supernatant
8. Add 150µL of FACS buffer to each well and put the content in tubes for FACS

Protocol S4 – Immunofluorescence protocol (for silencing assays – coverslips were previously placed in 24 well plates, when transfection with the siRNA and infections were performed):

1. Wash 1X with PBS
2. Add 500µL of PFA for 15 minutes (for fixation)
3. Wash 3X with PBS (put at -20ºC if the rest of the protocol is not done right away)
4. In vacuum, retire the PBS and add 400µL of perBlock buffer. Put the plate at 37ºC for 30 minutes
5. Wash 2X with PBS
6. Staining with phalloidin: Put the coverslips in parafilm and add 50µL of 1:200 diluted phalloidin. Put at 37ºC for 1 hour
7. Staining with DAPI: Put coverslips in PBS and wash 3X. Add 500µL of DAPI for 15min. Wash again 3X with PBS.
8. Mount coverslips with Mowiol.

Table S1: PCR and qRT-PCR primer sequences

Arl gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
1	GGCGCCCAGGCTCAACTAGG	AAGCCACCCATGATGAACCGCC
2	TGGAGCACCGCGGATTCAAGC	CCCAGATGAGGCCATCCGTGC
3	AGACCAGGAGGTGCGAATC	GGTGTGATGTGGCTGATGTC
4a	ATTCTGGGTTTGGACTGTGC	TGTGCATCGGGTGTATGA
4c	CCTGAAACGCAGGAAGTCTC	AATTCGAATCATCGCTTTGG
4d	CCTGCTAGGGGGTCAGTACA	GGCCGAGGTAGGAGGTAGAG
5a	AAGAAAAGCTGGGTTGCTGA	CATCTGATCTTCAGCCGTGA
5b	GGCTGGTTTTCGTGTTTGTT	CACGTGTCCCCTTTTCAACT
5c	CACTTCCTCATGTGGGACCT	TGCTTGTTGGCAAAGATCAG
6	AACAAGCTGAAGCCTTCCAA	CTTCTTTGGCCACAACCATT
8a	ACCATCAAGCTCTGGGACATTGGG	CCTGATCCGCAGCATCCACCA
8b	AATTGGATGGGTGGTTTGAA	CTTATGCAGTCCCCGCTTAG
9 (old)*	ATGAAGTGTGACCGGGTAGC	CCAGGAACTCCATTTGCCTA
9 (new)*	GTCCAGCACAGCACAGCTCCT	TGGCTCACTGCCACCAATCTCCA
9 (last)*	TGATGAAGTGTGACCGGGTA	CCAGGAACTCCATTTGCCTA
10	GAGGGAGGTTTTCCTCTTGG	AGCGGTGCTATGATGCTCCT
11	TCCTGCTCTTGGCAGATGGCG	GCTCCTACCCTCTGTGGTTGCCT
13a	AAGCAGCAGAAACACCCAGT	CGTTCAGGTGGAAAGTCCAT
13b	GTGAGAGGGCTGAACGAGTC	TTCTCCACGGTCTGCTTCTT
14	CTCCTCGGACTTGACTCAGC	GTCTTCCAGTCGCTTTTTGC
15	GTTGCTGGCTTTTTCAGGAG	AAGCGCTCGAAAACACAGAT
16	GCAGTTGACAGCAAGATGGA	TGTCTTGGGACCATTGTTCA
GAPDH	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA

*for Arl9 we tested three different sets of primers, named by us *old*, *new* and *last*

Table S2: siRNA sequences

Arl gene	Target Sequence
Arl3	UGAAGCAACUCGCAUCUGA
	GGUCAGGAACUAACGGAAU
	GGCUAAACCUCCACACCAU
	GCAUGAACUGGGUCUGCAA
Arl8b	GAUGAAGCCUCUGCAAAU
	UAAAGGCAACGUCACAAUA
	GAUAGAUGCUGCAGAUCGA
	UAGAGAAAUUUGCUGCUAU
Arl14	GGAAAUCUACUCUGCUUUA
	CAAGAGAGACCUUAGCAAU
	GAUGACGGGUUCAGGAAAU
	GGACUGUUCCGAAGGCAAA
Arl16	GACCUACCCUGUUACAUGA
	GAAAUAAGUGCCAGGAAUG
	UUACAGACAUUGUGGCCCA
	CAGAAUCAACACAGGCACA

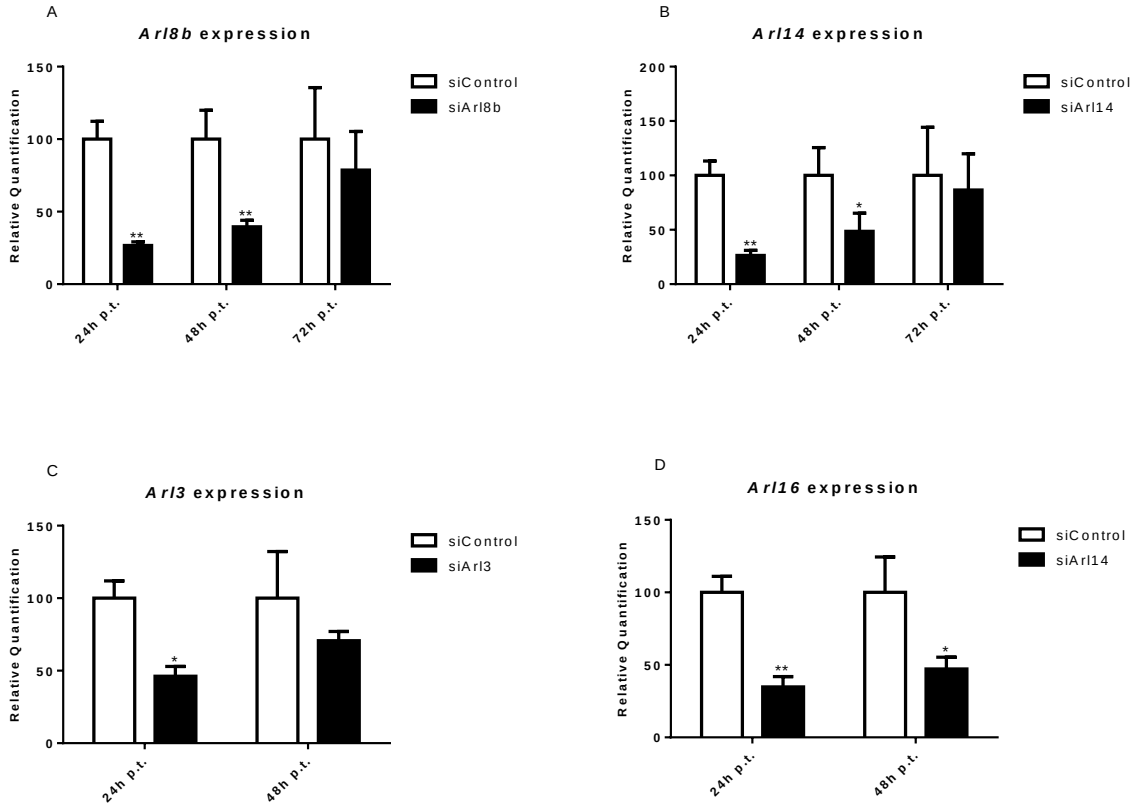


Figure S1: Efficiency tests of Arl3, Arl8b, Arl14 and Arl16 silencing in macrophages treated with 80pmol of specific siRNAs for those Arls relative to macrophages treated with siRNA control. For Arl8b and Arl14 we tested two concentrations of siRNA (40pmol and 80pmol) and qRT-PCR was performed on samples frozen 24h, 48h, 72h and 96h post-transfection. The better results for Arl8b and Arl14 were obtained with 80pmol for 24h and 48h post-transfection (data for 40pmol and 96h are not shown). The same happened with Arl3 and Arl16. However, the silencing of Arl3 is less efficient. These results are representative of all the silencing assays realized.

	Identity (%)	Nucleotide ID
Arl3	87%	*Homo sapiens*: NM_004311.3
		Mus musculus: NM_019718.2
Arl8b	83%	*Homo sapiens*: NM_018184.2
		Mus musculus: NM_026011.3
Arl14	79%	*Homo sapiens*: NM_025047.2
		Mus musculus: NM_027843.1
Arl16	87%	*Homo sapiens*: NM_001040025.1
		Mus musculus: NM_197995.2

Table S3: Percentage of identity between *Homo sapiens* and *Mus musculus Arl3*, *Arl8b*, *Arl14* and *Arl16* gene sequences obtained by BLAST analysis

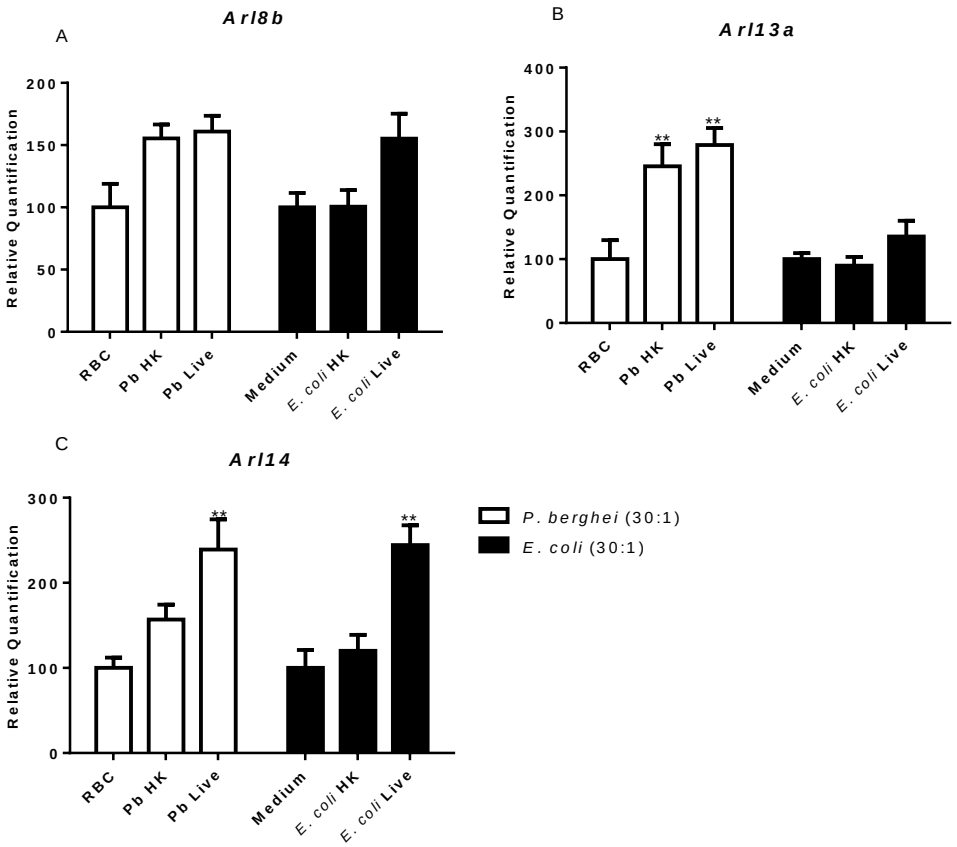


Figure S2: Expression of Arl8b, Arl13a and Arl14 genes on BMDMs after infection with *P. berghei* and *E. coli*. Columns represent the relative quantification of the cDNA levels of each Arl GTPase, analyzed by real-time quantitative PCR and normalized against GAPDH gene expression levels. Error bars indicate the standard error of the mean of 2 independent assays. Statistical significance (*p<0.05, **p<0.01) refers to the difference between macrophages incubated with infected RBC (*Pb* Live) or heat-killed parasites (*Pb* HK*)* and macrophages incubated with uninfected RBC.

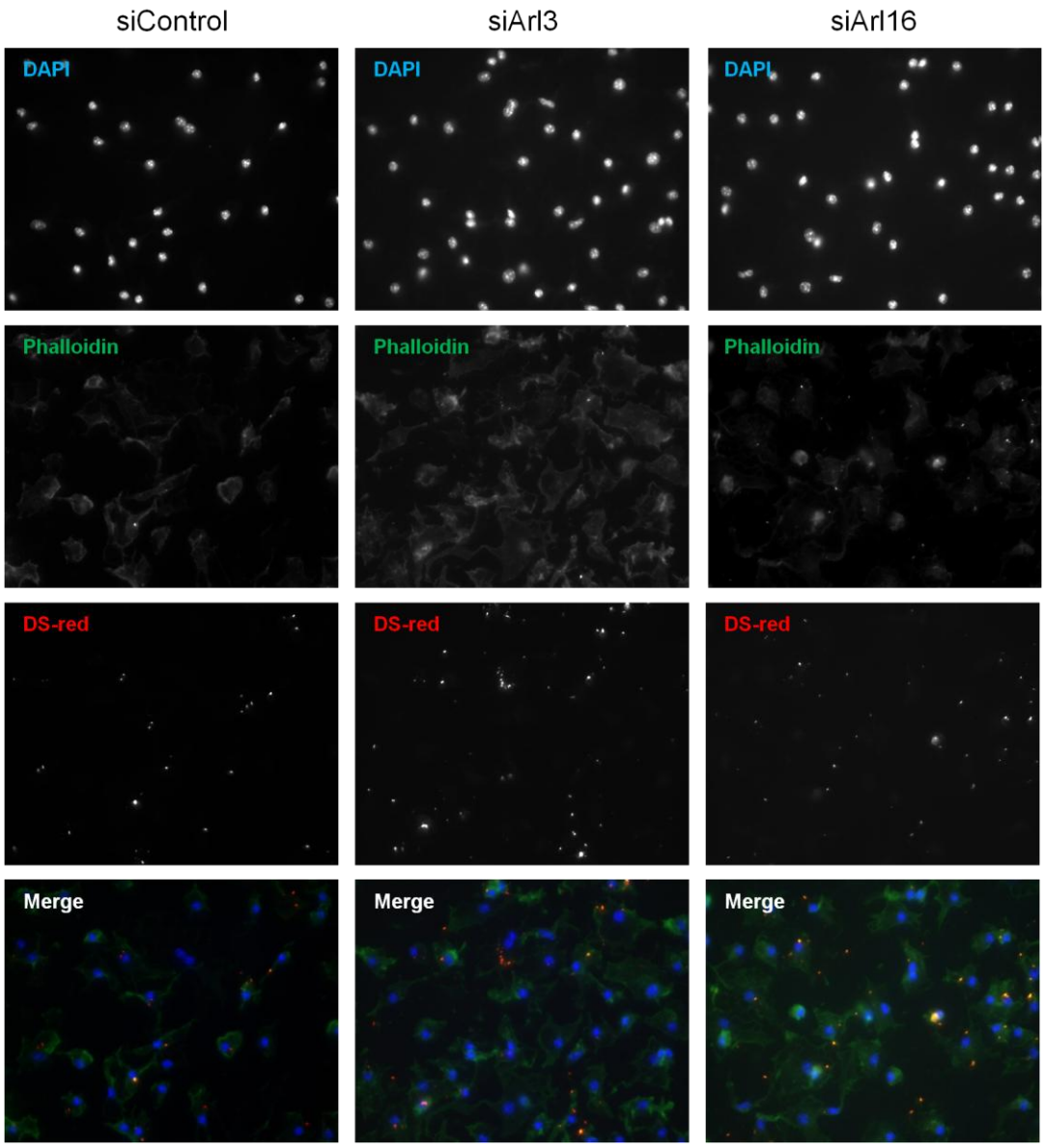


Figure S3: Immunofluorescence assay for Arl3 and Arl16 silencing for 2 hours post-infection. DAPI (blue) was used to stain the nucleus and phalloidin (green) to stain the actin. *Salmonella* was already labeled with DS-red (red). No differences in the internalization were detected between silenced samples and samples treated with non-targeting siRNA (control).