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# Infection des cellules placentaires par *Brucella*: rôle de la protéine multifonctionnelle CD98hc

Sonia Vection

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# THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE L'UNIVERSITÉ DE MONTPELLIER

En Microbiologie, Interactions Hôte-Pathogène

École doctorale 168 : Sciences Chimiques et Biologiques pour la Santé

Unité de recherche INSERM U1047 : Virulence Bactérienne et Infections Chroniques

## Infection of human placental cells by *Brucella*: Role of the multifunctional host protein CD98hc

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Le 07 Avril 2022

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UNIVERSITÉ  
DE MONTPELLIER







*La rigueur vient toujours à bout de l'obstacle.*

Léonard De Vinci



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## Abbreviations

<b>AA</b>	Amino acid
<b>aBCV</b>	Autophagic BCV
<b>BCH</b>	2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid
<b>BCV</b>	<i>Brucella</i> -containing vacuole
<b>BMDM</b>	Bone marrow derived macrophage
<b>Cas</b>	CRISPR-associated
<b>CASK</b>	Calcium/Calmoduline-dependent serine protein kinase
<b>CASK</b>	Calcium/Calmoduline-dependent serine protein kinase
<b>CAT</b>	Cationic AA transporter
<b>CFU</b>	Colony forming units
<b>CMV</b>	Cytomegalovirus
<b>CNF</b>	Cytotoxic necrotizing factor
<b>COG</b>	Conserved oligomeric Golgi complex
<b>CRISPR</b>	Clustered Regularly Interspaced Short Palindromic Repeats
<b>CTB</b>	Cytotrophoblast
<b>DAPI</b>	4,6-diamidino- 2-phenylindole
<b>DC</b>	Dendritic cell
<b>ECM</b>	Extracellular matrix
<b>EEA-1</b>	Early endosomal antigen 1
<b>EGF</b>	Epidermal growth factor
<b>EGFR</b>	Epidermal growth factor receptor
<b>ELISA</b>	Enzyme-linked immunosorbent assay
<b>ER</b>	Endoplasmic reticulum
<b>ERES</b>	Endoplasmic reticulum exit sites
<b>ES</b>	Embryonic stem
<b>EVT</b>	Extravillous trophoblasts
<b>FAK inhibitor-14</b>	1,2,4,5-Benzenetetraamine tetrahydrochloride
<b>FBS</b>	Fetal bovine serum
<b>FPP</b>	Farnesylpyrophosphate
<b>FPR2</b>	Formyl peptide receptor 2
<b>FSGS</b>	Focal and Segmental Glomerulo Sclerosis
<b>Fsp1</b>	Fibroblast specific promoter 1
<b>GGPP</b>	Geranylgeranylpyrophosphate
<b>GM-CSF</b>	Granulocyte-macrophage colony-stimulating factor
<b>GSH</b>	Glutathione
<b>HAT</b>	Heterodimeric AA transporter
<b>HDT</b>	Host-directed therapy
<b>HMG-CoA</b>	3-hydroxy-3-methylglutaryl-coenzyme A
<b>IP</b>	Intra-peritoneal
<b>KO</b>	Knock out

## ABBREVIATIONS

<b>LAMP-1</b>	Lysosomal-associated membrane protein 1
<b>MAGUK</b>	Membrane-Associated Guanylate Kinases
<b>MAPK</b>	Mitogen-activated protein kinases
<b>mDF</b>	Murine derm fibroblasts
<b>MEF</b>	Murine embryonic fibroblasts
<b>M<math>\phi</math></b>	Macrophages
<b>NSCLC</b>	Non-small cell lung cancer
<b>ON</b>	Overnight
<b>PBS</b>	Phosphate-buffered saline
<b>PCR</b>	Polymerase chain reaction
<b>PI3-K</b>	Phosphatidylinositol 3-kinase
<b>PMN</b>	Polymorphonuclear leukocytes
<b>PRR</b>	Pattern recognition receptor
<b>RNAi</b>	RNA interference
<b>sCASK</b>	Soluble calcium/calmoduline-dependent serine protein kinase
<b>sgRNA</b>	Small guide RNA
<b>SLC3A2</b>	Solute carrier family 3 member 2
<b>STB</b>	Syncytiotrophoblast
<b>T4SS</b>	Type 4 secretion system
<b>TA</b>	Tannic acid
<b>TORCH</b>	<i>Toxoplasma gondii</i> , Others, Rubella virus, Cytomegalovirus, Herpes simplex virus
<b>UPR</b>	Unfolded protein response
<b>Y2H</b>	Yeast two-hybrid
<b>ZF</b>	Zinc-finger
<b>ZNF</b>	Zinc-finger nuclease





# Introduction



## Introduction

### 1. Brucellosis

#### 1.1. Origin

Brucellosis is an infectious disease caused by bacteria of the *Brucella* genus. The disease, previously called “Malta fever” was discovered in the 1850s by a British army doctor, David Bruce, from a soldier that succumbed to the infection. Later, the work of Dr Themistocles Zammit highlighted the zoonotic nature of the disease. Indeed, he was able to isolate the bacterium responsible for the disease from milk, urine, and blood of infected native goats.

In the late 19<sup>th</sup> Century, epidemics of brucellosis were observed in ships in which the crew had no contact with goat’s milk. Given the humidity and proximity inside the ships, it was shown that *Brucella* was able to spread by inhalation. This observation was also supported by the fact that most of the scientists working on brucellosis at the time fell ill. A common practice was indeed to identify bacterial colonies by smell and so scientists would sniff cultures and get infected. Thanks to pasteurization, animal vaccination and testing of the goats, Malta was declared free of brucellosis in 2005 (Wyatt, 2013).

#### 1.2. Epidemiology

Brucellosis is not limited to the island of Malta but is distributed worldwide. However, geographical distribution varies depending on the species of *Brucella* considered. Bovine brucellosis, caused by *Brucella abortus*, has been eradicated in several countries: the island of Cyprus, New Zealand, Finland, Australia, Denmark, Canada, The Netherlands, Norway, Sweden, and the United Kingdom. A disease is considered eradicated when no cases have been observed in 5 years. However, brucellosis is still endemic in Africa, central and south America, Mexico, central Asia, India, and the Mediterranean rim. Depending on the country, surveillance and preventive measures are taken to eradicate brucellosis (Goodwin & Pascual, 2016; Seleem et al., 2010).

In many countries surveillance of brucellosis is based on the mandatory report of animal and human cases to the authorities. Unfortunately, this is not always done making the determination of the real incidence of the disease very difficult and likely

underestimated. Approximately, more than half a million of new human cases are reported each year worldwide (Pappas et al., 2005). Also, it is supposed that over 300 million of the 1.4 billion cattle population is infected (de Figueiredo et al., 2015). These numbers place brucellosis in the top 10 infectious diseases worldwide.

### 1.3. Brucellosis in animals

Animal brucellosis occurs in livestock as well as domestic animals and for a long time, brucellosis was considered to affect only mammals. The most described zoonotic reservoirs for *Brucella* are cattle, sheep and goats, swine, and dogs in which *Brucella* is mainly found in reproductive organs including placenta and epididymis. Infection occurs horizontally, by ingestion of infected placenta left in the wild, milk or genital secretions containing bacteria (Atluri et al., 2011). Consequences of infection differ from male (infertility and epididymitis) to female in which infection is more obvious as the main symptoms are abortion, stillbirth, birth of weak offspring and a decreased milk production.

Unfortunately, there is no available treatment for animals. Animal vaccines exist but the cost is too expensive to correctly protect herds. The type of vaccine differs depending on the animal host. To protect cattle, a vaccine was developed from a spontaneously attenuated strain of *B. abortus* (strain 19). It is the most effective vaccine to date with an efficiency around 70%. One drawback is that vaccinated animals are difficult to discriminate from naturally infected ones using common serological tests and this is due to the smooth phenotype of the S19 strain. Even though it is not currently used in the USA, this vaccine is still employed in India and Argentina in cattle (Yang et al., 2013). To minimize downsides, another vaccine was later developed. The RB51 vaccine is a spontaneous rough mutant selected after repeated *in vitro* passages of *B. abortus* 2308 strains on media containing rifampicin and penicillin. In the US, RB51 replaced the S19 vaccine in prevention of bovine brucellosis (Yang et al., 2013). It is to note that this vaccine strain caused a milk borne outbreak in the USA in 2019 from bacteria shed by immunized animals (Negrón et al., 2019). Furthermore, as this strain lacks O-LPS, positive serological response in vaccinated animals is not detectable using common tests. A different vaccine is used to protect sheep and goats from *B. melitensis* infection. In this case it is the Rev 1 *B. melitensis* attenuated strain. One downside is that this vaccine cannot be used in

## 1. BRUCELLOSIS

pregnant animals as it causes abortions. Therefore, only young animals are vaccinated with this strain. It was shown to be very efficient in the vaccination programs of Tajikistan and Portugal (Yang et al., 2013).

All the vaccines described above need to be used carefully as they are pathogenic to humans. Furthermore, the RB51 strain is resistant to rifampicin, one antibiotic normally used in the treatment of human brucellosis (Goodwin & Pascual, 2016). As animal treatments are not available and vaccines are expensive, brucellosis leads to serious economic losses for farmers as most infected animals are slaughtered to prevent epidemics (Byndloss & Tsolis, 2016).

### 1.4. Human brucellosis

Humans are accidental hosts of *Brucella* and infection can occur by consumption of unpasteurized milk products from infected animals (milk, cheese, or ice cream), direct contact with infected tissues (placenta for example) or by inhalation of aerosols. Symptoms are nonspecific and influenza-like: fatigue, undulant fever, articular and muscular pains (Byndloss & Tsolis, 2016). Contrary to animals, a treatment is available for human infection. The World Health Organization recommends an oral regimen of 200 mg of doxycycline plus 600 – 900 mg of rifampicin daily for a minimum of 6 weeks. For pregnant women rifampicin in combination with trimethoprim-sulfamethoxazole (TMP-SMX) for 6 to 8 weeks is recommended. Treatment is not void of side effects and relapses are observed. Moreover, recommendations have not been updated in the last 25 years which raises the question of alternative courses of treatment (Franco et al., 2007). If left untreated, brucellosis can turn into a debilitating chronic disease with manifestations ranging from osteoarticular disease to hepatitis, epididymo-orchitis, encephalitis and endocarditis.

Absolute diagnosis of brucellosis requires isolation of the bacterium from biological samples (Yagupsky et al., 2019). This is not always possible, and several techniques are used for diagnostics. Serologies are blood tests that can be performed to detect *Brucella*-specific antibodies. Different tests are available such as tube agglutination (S.A. Wright), slide agglutination (Rose Bengal antigen) or enzyme-linked immunosorbent assay (ELISA). Another option is polymerase chain reaction (PCR) to detect traces of bacterial DNA. PCR analysis can be performed on tissues or blood samples.

### 1.5. Brucellosis and pregnancy

The link between abortion and *Brucella* infection is clear in animals, but in the last 10 years epidemiological studies also reported a connection between *Brucella* infection of pregnant women and adverse obstetrical outcomes. The first human abortion due to *Brucella* infection occurred in France in 1905, quickly followed by the description of an abortion in a pregnant farmer a year later. Data around whether *Brucella* infection is linked to increased obstetric complications were controversial. However, recent reports argue that brucellosis has a significant impact on adverse pregnancy outcomes in humans. Indeed, the rate of adverse outcomes in *Brucella*-infected pregnant women ranges from 14 to 56%, which is more than in healthy women. Obstetric complications can translate into spontaneous abortions, *in utero* fetal death and preterm delivery (Bosilkovski et al., 2020; Vilchez et al., 2015).

*Brucella* can also be transplacentally transmitted to the fetus during pregnancy (congenital brucellosis), upon contact of newborns with maternal fluids secreted during delivery or by breast feeding (neonatal brucellosis). Brucellosis in babies can lead to poor feeding, respiratory distress syndrome, fever, sepsis, and multiple organ failure in the worst cases. Fortunately, early detection and treatment (rifampicin and TMP-SMX for 6 to 8 weeks) of infection in pregnant women drastically reduces the risks of adverse pregnancy outcomes. In endemic regions, women in the age of bearing a child should be aware of the risks brucellosis represents and informed on how the disease is transmitted (Bosilkovski et al., 2020).

Pregnancy being a particular time in life with numerous physiological changes to ensure the fetus survival, efforts are needed to diagnose and educate about brucellosis, especially in pregnant women living in endemic regions.

## 2. *Brucella*

In part 1, we described the epidemiology and manifestations of brucellosis in animals and humans and focused on the particular case of pregnant women. We will now target the causative agent of the disease: the bacteria of the *Brucella* genus and how they enter and develop inside hosts cells.

## 2. BRUCELLA

### 2.1. Description of the different species

*Brucellae* are Gram-negative, facultative intracellular coccobacilli that can infect a wide range of hosts including mammals. Hosts vary between the 10 recognized species of *Brucella*. Historically, *Brucella* species were classified by host preferences, microbiological characteristics, and pathogenicity. Therefore, “classical” species include *Brucella melitensis* (causes brucellosis in small ruminants and humans), *Brucella abortus* (cattle), *Brucella suis* (swine), *Brucella ovis* (sheep), *Brucella canis* (dogs) and *Brucella neotomae* (desert rats). More recently, new species of *Brucella* have been identified: *Brucella ceti* (cetaceae), *Brucella pinnipedialis* (pinnipeds), *Brucella microti* (voles) and *Brucella papionis* (baboons). Lastly, “atypical” *Brucella* species were described: *Brucella vulpis* (foxes), *Brucella inopinata* (human breast implant) and *Brucella inopinata*-like strains isolated from amphibians, particularly frogs (González-Espinoza et al., 2021). These new species also have a zoonotic potential as an amphibian-type *Brucella* isolate recently caused brucellosis in human (Rouzic et al., 2021). It is important to note that most of the *Brucella* species described in the table below have a zoonotic potential.

<b><i>Brucella</i> species</b>	<b>Main host</b>	<b>Human cases</b>	<b>References</b>
<i>Brucella melitensis</i>	Small ruminants	Yes +++	Díaz Aparicio, 2013
<i>Brucella abortus</i>	Cattle	Yes ++	Díaz Aparicio, 2013
<i>Brucella suis</i>	Pigs	Yes ++	Díaz Aparicio, 2013
<i>Brucella canis</i>	Dogs	Yes +	Wanke, 2004
<i>Brucella ovis</i>	Ram	No	Burgess, 1982
<i>Brucella neotomae</i>	Desert rats	Yes, but rare	Suárez-Esquivel et al., 2017
<i>Brucella ceti</i>	Cetaceans	Yes, but very rare	McDonald et al., 2006; Sohn et al., 2003
<i>Brucella pinnipedialis</i>	Pinnipeds	No	Foster et al., 2007
<i>Brucella microti</i>	Voles	No	Audic et al., 2009
<i>Brucella vulpis</i>	Foxes	No	Scholz et al., 2016
<i>Brucella papionis</i>	Baboon	No but can infect human trophoblasts <i>in vitro</i>	Whatmore et al., 2014; García-Méndez et al., 2019a
<i>Brucella inopinata</i>	Human	-	Scholz et al., 2010
<i>Brucella inopinata</i> -like strain (BO2)	Human	-	Tiller et al., 2010
“Amphibian-type” isolates	Frogs / Ray / Chameleon	Yes, but very rare	Soler-Lloréns et al., 2016 Fischer et al., 2012 Eisenberg et al., 2012 Whatmore et al., 2015 Eisenberg et al., 2017, 2020

**Table 1 : Overview of the different *Brucella* species described to date.**

Even in countries where eradication programs are in place and measures are taken to monitor domestic animals, it is very difficult to control brucellosis in wildlife reservoirs. For example, re-emergence of *B. melitensis* occurred in a French dairy cattle farm recently.



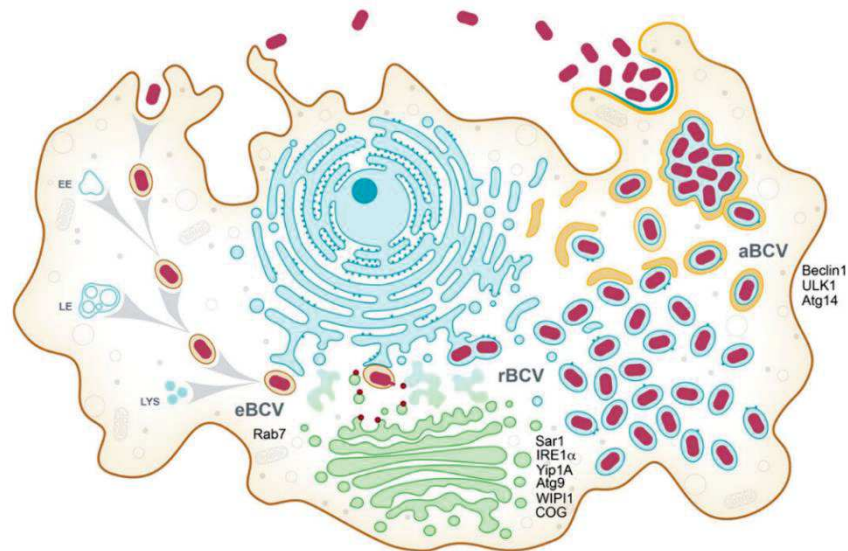
The possible cause of this infection is a population of infected *Alpine ibex* in the Bargy massif (Lambert et al., 2018). Also, more and more “atypical” species of *Brucella* are discovered in new hosts among wildlife. Unfortunately, due to the emergence of new animal reservoirs and new potentially zoonotic species, eradication is difficult. Therefore, research on the *Brucella* genus is critical to understand more about this expanding bacterial family.

## 2.2. Cellular microbiology of infection

In hosts, upon mucosal infection (digestive or respiratory), *Brucella* can enter into epithelial cells and translocate across the mucosal epithelium. In the submucosa, bacteria encounter resident macrophages (M $\phi$ ), dendritic cells (DCs) and polymorphonuclear leukocytes (PMNs). Macrophages and DCs are the primary hosts cells in which these bacteria replicate. Infected professional phagocytes then move to the closest lymph node leading to dissemination of infection in different organs of the reticuloendothelial system such as spleen, lungs, and bone marrow. Brucellosis is thus a systemic infection and virtually any organ can be infected (de Figueiredo et al., 2015).

Once *Brucella* has entered host cells, bacteria reside in a specific compartment that follows the endosomal pathway. This vacuole interacts with different intracellular compartments to mature, allowing bacterial intracellular replication. Once the cycle is complete bacteria egress from the cell to disseminate and infect nearby cells (Figure 1).

## 2. BRUCELLA



**Figure 1: Model of *Brucella* intracellular life cycle in macrophages.** After entry by phagocytosis, the BCV interact with early endosomes (EE), late endosomes (LE) and lysosomes (LYS) to mature into the eBCV. The pH of the eBCV acidifies and this triggers expression of the T4SS and secretion of bacterial effectors in the host cell. After prolonged interactions with the ER and host proteins, the eBCV converts into the rBCV allowing replication of *Brucella*. Finally, the rBCV interact with autophagosomes giving rise to aBCV. The replication cycle is complete, and bacteria egress from the cell. From Celli, 2019.

### 2.2.1. Entry

Free *Brucella* enter epithelial and phagocytic cells by phagocytosis (de Figueiredo et al., 2015a). However, opsonized bacteria are ingested by professional phagocytes through Fc and complement receptors at the cell surface. Also, this mode of entry leads to enhanced bacterial killing (Gorvel & Moreno, 2002; Moreno & Barquero-Calvo, 2020). Exact mechanisms of *Brucella* entry are yet to be described but according to Watarai and colleagues, *Brucella* would enter at specific regions on the cell membrane, enriched in sphingolipids and cholesterol, called lipids rafts (Watarai et al., 2002).

Once in the host cell, *Brucella* resides in a special membrane-bound compartment called the *Brucella*-containing vacuole (BCV). The BCV first traffics through the endosomal pathway and interacts with compartments of this pathway. This leads to the quick acquisition of endosomal markers such as the small GTPase Rab5 and the early endosomal antigen EEA-1. At this point, the BCV is called eBCV for endosomal *Brucella*-containing vacuole. As it continues its trafficking through the endosomal pathway, the vacuole acquires markers of late endosomes such as Rab7, CD63 and the lysosomal-associated membrane protein LAMP-1. This is followed by acidification of the vacuole to a pH of 4.5-5.0. This step is critical for the induction of the VirB type 4 secretion system (T4SS), a major virulence factor of *Brucella* allowing intracellular replication and secretion of

bacterial effectors in the host cell (Boschiroli et al., 2002). Low pH and recruitment of Rab7 are key parameters in the transition from eBCV to the endoplasmic reticulum (ER)-derived rBCV (replicative BCV) (Celli, 2019). This new compartment is the replicative niche of *Brucella*, the host-derived intracellular compartment remodeled by the pathogen in order to multiply (Martinez et al., 2018).

### 2.2.2. Replicative niche

Several intracellular bacteria choose the ER as their replicative niche (Celli & Tsolis, 2015). The ER is critical as it synthesizes important molecules (proteins, lipids, etc.) and regulates transport of newly synthesized proteins. It thus provides *Brucella* nutrients and new membranes for BCV maturation. In the case of *Brucella* infection, expression of the T4SS is necessary for establishment of the rBCV and interaction with the ER. This system secretes bacterial effectors in the host cell that will hijack its functions to the benefit of bacterial survival and replication. One effector, BspB, was shown to be necessary for rBCV biogenesis and efficient replication (Myeni et al., 2013). In fact, this effector can physically interact with the conserved oligomeric Golgi complex (COG) of the host cell and impair ER-to-Golgi trafficking by recruiting COG-containing vesicles to rBCV (Miller et al., 2017). The COG complex is a membrane trafficking machinery facilitating vesicle transport between Golgi cisternae. It is composed of 8 subunits (COG1-8) and can interact with proteins on the vesicle and target membrane to mediate vesicle fusion (Blackburn et al., 2019).

Moreover, it was shown that the unfolded protein response (UPR), which is triggered by ER stress, was also necessary for rBCV formation. In the cell the ER is in charge of protein synthesis. However, unfolded or misfolded proteins can accumulate in this compartment inducing cellular stress (Hetz et al., 2020). Surveillance of protein folding is mediated by the UPR, a signal transduction pathway which senses correct folding and triggers adaptive mechanisms to relieve the load of unfolded or misfolded proteins. During this process eBCV interacts with ER exit sites (ERES), a region in the ER where COPII-mediated vesicular transport takes place. This interaction triggers the activation of IRE1, one of the 3 detection proteins that can induce UPR. This results in up-regulation of the expression of the components of the inner-layer coat of the COPII complex (Sec23 and Sec24) as well as the small GTPase Sar1 responsible for the control of COPII assembly.

## 2. BRUCELLA

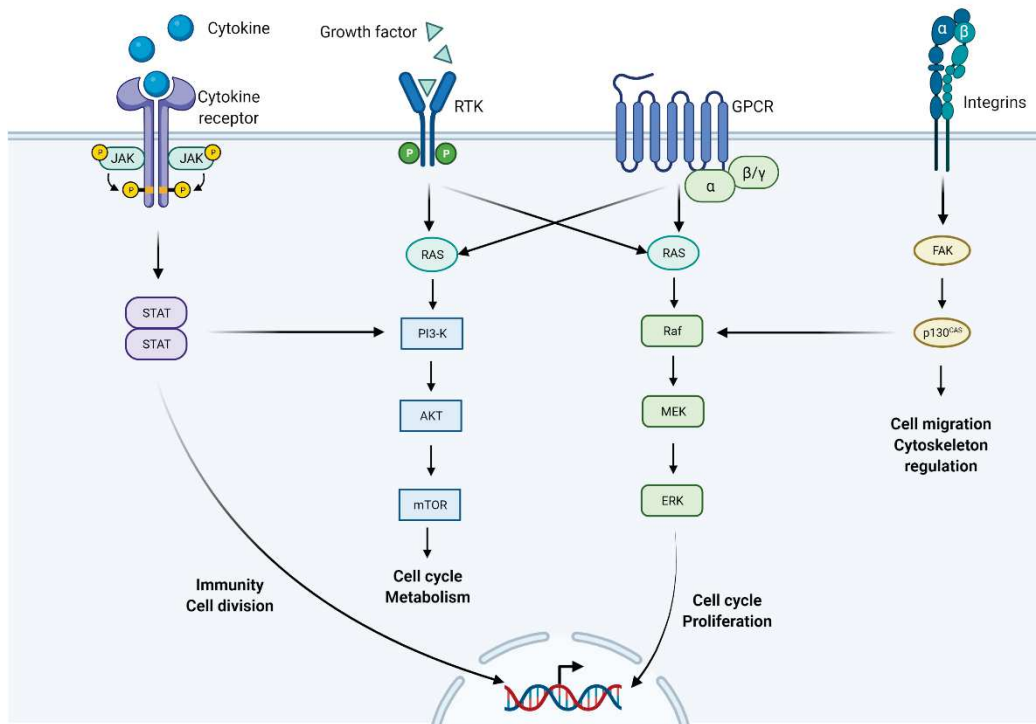
COPII vesicles then fuse with eBCVs allowing acquisition of ER markers and maturation into rBCVs (Taguchi et al., 2015).

### 2.2.3. Late stage of infection

After replication in the ER is complete, the rBCV will transition to a new compartment associated with autophagy: the aBCV (autophagic BCV). Autophagy is a cellular mechanism involved in adaptation to starvation and stress and is crucial for cell growth and differentiation. The basis of this process is the degradation of cytosolic components in double-membrane vesicles called autophagosomes. After an initiation phase, the target cargo is packed, the membrane forming the autophagosome elongates and closes. This vesicle will then fuse with lysosomes to destruct its content (Rashid et al., 2015). Therefore, the aBCV is characterized by multiple membranes, an acidic pH and an accumulation of LAMP-1. Formation of the aBCV is dependent on the autophagic initiator proteins Beclin1, UKL-1 and ATG14L. This step completes *Brucella* intracellular cycle and leads to subsequent infections events (Starr et al., 2012).

### 2.3. Signalization triggered by *Brucella* in infected cells

In order to interact with each other and their environment, cells rely on signal transduction. This process allows the cell to respond to a chemical or physical signal after its transmission as a series of molecular events inside the cell. This series of event is called a signaling pathway. Multiple signaling pathways are described ranging from interaction with the extracellular matrix, cell cycle and proliferation, cell survival and metabolism, cell death signals and response to pathogens (Figure 2). Signaling pathways can also interact with each other, by sharing effectors, forming a network allowing coordination of cellular responses.



**Figure 2: Simplified representation of the main intracellular signalling pathways.** Is represented on this scheme the JAK/STAT pathway, activated after detection of cytokines; the PI3-K/AKT/mTOR pathway and the Ras/MEK/ERK pathway, both activated following stimulation by growth factors or activation of G protein coupled receptors; and the integrin pathway, activated after activation of integrins. JAK: Janus kinase, STAT: Signal Transducers and Activators of Transcription, RTK: Receptor Tyrosine kinase, PI3-K: phosphatidylinositol 3-kinase, mTOR: mammalian target of rapamycin, MEK: Mitogen-activated protein kinase, ERK: Extracellular signal-regulated kinase, FAK: Focal Adhesion kinase. Created on BioRender.com.

Some work has been carried out on the signal transduction pathways activated during *Brucella* infection and several kinase pathways were highlighted in this context. Pre-treatment of HeLa with protein tyrosine kinases, mitogen-activated protein kinases (MAPK) or phosphatidylinositol 3-kinases (PI3-K) inhibitors decreased *B. abortus* internalization. So did several clostridial toxins and some glucotransferases targeting different members of the Rho and Ras subfamilies of GTPases (Guzmán-Verri et al., 2001). Conversely, treatment of HeLa cells with *Escherichia coli* cytotoxic necrotizing factor (CNF) increased *B. abortus* invasion and replication. CNF permanently activate several small GTPases of the Rho subfamily (Chaves-Olarte et al., 2002). Implication of PI3-K was confirmed by another study in which inhibition by wortmannin and downregulation of PI3-K by RNA interference resulted in inhibition of entry of *B. melitensis* and *B. abortus* in drosophila cells and murine macrophages (Qin et al., 2008). Implication of the phagocytic receptor TREM-2 was shown to be important for entry and replication of *B. abortus* in murine BMDMs (Wei et al., 2015). Other molecules such as p38 and JNK were found to be important for intracellular replication of *B. melitensis* (Dimitrakopoulos et al., 2013).

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Stimulation of formyl peptide receptor 2 (FPR2), a G protein coupled receptor considered as a pattern recognition receptor (PRR), resulted in higher entry of *B. abortus* 544 but had no impact on its intracellular growth. This higher uptake was followed by an increased ERK phosphorylation (Reyes et al., 2021). Use of tannic acid (TA), a specific type of tannin shown to restrict growth of several pathogens, reduced invasion of *B. abortus* in RAW 264.7 cells. This reduction was due to a deficit in F-actin polymerization and the suppression of ERK and p38 phosphorylation in treated cells (Reyes et al., 2018). Similarly, emodin, an active ingredient in herbs used in Chinese medicine, decreased adhesion and entry of *B. abortus* in RAW 264.7 cells. No effect was observed on growth. As for TA, treated cells showed inhibition of F-actin polymerization and reduced ERK phosphorylation. In this study they also show that treatment with a MEK agonist increases *Brucella* entry (Huy et al., 2018). Infection by *Brucella* also induced activation of the MAPK pathway. It is known that infection of murine macrophages J774 by *B. neotomae* induces phosphorylation of p38 in a T4SS-dependent manner (Kang & Kirby, 2019). Similarly, infection by *B. abortus* triggered the phosphorylation of p38, ERK and JNK in human trophoblasts HPT-8 cells (Zhang et al., 2017).

## 3. Placenta

Now that we have defined the pathogenesis of *Brucellae*, how they enter in the host, their replication cycle inside the infected cells and the intracellular signaling events caused by infection, we will focus on one organ for which *Brucella* has a known tropism: the placenta.

### 3.1. The human placenta

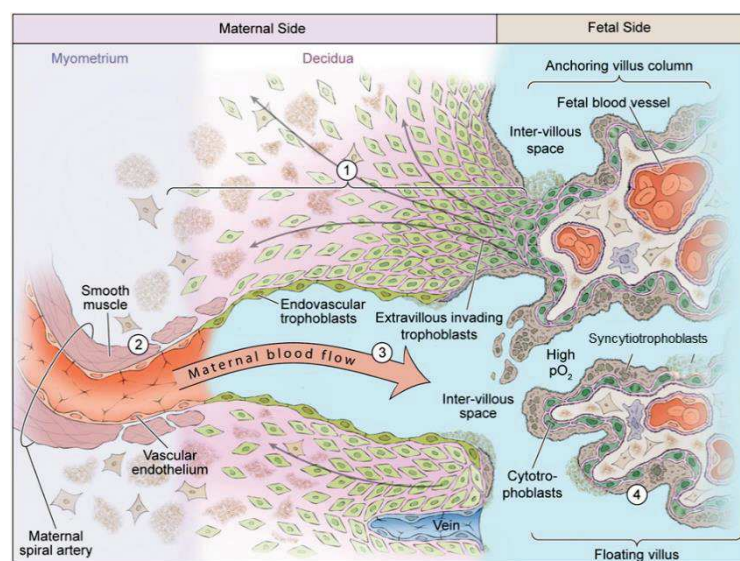
The placenta is the first fetal organ to develop during pregnancy. Even though it is transient, it ensures a multitude of functions normally carried out by several different organs such as oxygen and nutrient transport, protection against xenobiotics and pathogens, hormone secretion and waste removal. It also remodels maternal arteries to establish blood circulation and regulates maternal metabolism to support pregnancy.

Around 5 days post fertilization the blastocyst is composed of 2 lineages: the inner cell mass from which the embryo will develop and the trophectoderm which will give rise to the placenta. Rapidly, the blastocyst will embed into the maternal decidua, this step is known as implantation. After this point, cells from the trophectoderm generate the first

trophoblast cells, cytotrophoblasts (CTBs) and the multinuclear primitive syncytium. The latter will later give rise to the multinuclear syncytiotrophoblast (STB) layer by fusion with differentiated villous cytotrophoblasts. This STB layer forms a protective barrier and provide an interface for fetal-maternal interactions. Cytotrophoblasts proliferate and breach through the STB layer to form villous trees (made of a cytotrophoblast core surrounded by STB layer on the outside). At the same time, CTB will proliferate and fuse to create the cytotrophoblast shell which encircles the embryo between the villi and the maternal decidua. At the regions where the cytotrophoblast shell is in contact with maternal decidua individual cells leave the shell to migrate and invade the decidua, there are called extravillous trophoblasts (EVTs) (Burton & Fowden, 2015; Turco & Moffett, 2019).

### 3.2. The specialized cells of the placenta: the trophoblasts

As described above, several types of specialized placental cells exist (Figure 3).



**Figure 3: The different types of trophoblasts in human placenta.** Cytotrophoblasts (CTBs, in dark green) are located in the villi and will fuse to give rise to the layer on top of it the syncytiotrophoblasts (STBs, in grey). At the sites of contact between anchoring villi and the maternal decidua, some CTBs will migrate and differentiate in extravillous trophoblasts (EVT, in light green) that will remodel maternal arteries and endometrium. Modified from Kingdom et al., 2010.

The first one to arise are CTBs. These cells are located beneath the STB layer and ensure the cellular turnover of the latter as when the STBs die of apoptosis, underlying CTB fuse to create a new STB layer (Turco & Moffett, 2019). One *in vitro* model representing these cells is the BeWo cell line. It was developed by isolation of cells from a choriocarcinoma

### 3. PLACENTA

that metastasized to the brain. These cells were then cultured by passing in hamster cheek pouch for over 304 passages in an 8-year period (Pattillo & Gey, 1968a).

STBs are highly polarized, multinucleated cells. They line the placental villi and are in direct contact with the maternal blood contained in the intervillous space. Therefore, they are the main interface for feto-maternal interactions notably nutrient and oxygen exchanges. Thanks to their microvilli, the surface area with the blood is increased up to 7-folds. The STB layer is also responsible for the endocrine activity of the placenta. Placental hormones are key for the development of the placenta but also have a great impact on maternal metabolism and behavior such as increased food intake (Burton & Fowden, 2015).

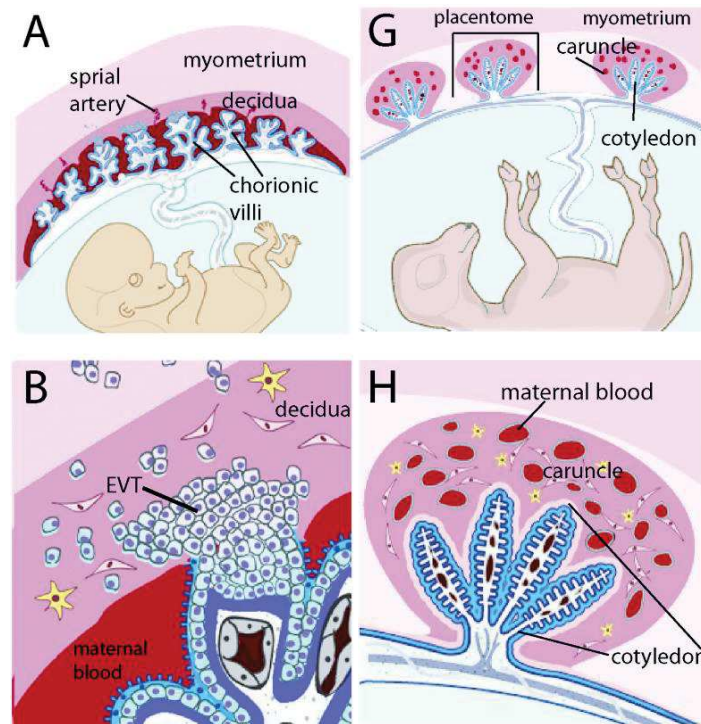
The last type of trophoblasts are EVT. These cells migrate from the villous trees to the maternal decidua towards maternal arteries. During the first trimester they ensure hypoxic conditions by blocking maternal arteries which is critical for placental development. Later, they are responsible for the remodeling of maternal arteries, turning them into vessels of high conductance and low pressure allowing maternal blood to fill the intravillous space (Maltepe & Fisher, 2015).

#### 3.3. Comparison with bovine placenta

As the first observations of placental infections by *Brucella* were done in cattle it seems interesting to compare bovine and human placenta to understand their differences (Smith, 1919). Several criteria allow classification of placental type among placental mammals (Figure 4).

The first criterium is morphology and is based on the distribution of the chorionic villi. Human placenta is characterized as discoid whereas bovine placenta is multicotyledonary (Peter, 2013; Turco & Moffett, 2019). This term refers to the presence of localized areas of chorionic villi in the placenta named cotyledons. Around 100 cotyledons develop in cattle during pregnancy and each of them is associated with a maternal caruncle to form placentomes. In the caruncles, the fetal villi associate with maternal crypts. The placentome is the functional unit of this type of placenta and form the interface for exchanges between the maternal and fetal tissues (Furukawa et al., 2014).





**Figure 4: Structural comparison between human and bovine placenta.** **A.** Haemochorial human placenta is composed of villous trees anchored in maternal decidua bathing in maternal blood. **B.** The uterine-trophoblast interface is composed of EVTs migrating in the maternal decidua at the tip of anchoring villi, STBs at the outer lining of villi directly in contact with maternal blood and of maternal cells in the decidua. **G.** Epitheliochorial placenta is composed of many placentomes in the uterus. **H.** A placentome is composed of a maternal caruncle, containing maternal blood vessels, and fetal cotyledons. Modified from Robbins and Bakardjiev, 2012.

Another classification is based on the histological structure of the placenta *i.e.*, the number of layers that separates maternal tissue from fetal blood. Human placenta is said to be hemochorial. In this type of placenta, which is the most invasive, differentiated trophoblasts invade through the maternal epithelium to come in direct contact with maternal blood (Turco & Moffett, 2019). By doing so, the maternal tissue layers are lost due to erosion. On the opposite, bovine placenta is defined as synepitheliochorial (Peter, 2013). In this case, the placenta is superficial, and no significant invasion of the uterine tissue is observed. However, contrary to the strict epitheliochorial definition, some specialized trophoblasts migrate and modify the uterine epithelium. So, in this system, the maternal epithelium is not lost but modified (Furukawa et al., 2014).

### 3.4. Placental pathogens and route of infection

One of the roles of the placenta is to protect the developing fetus from pathogens. This is possible because of the placental architecture and maternal immunity. However, some pathogens are able to breach the placental fortress and establish infection.

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Infection can occur at two different contact sites between the mother and the fetus. Let's consider first the blood-STB interface. As described above, STBs constitute the outer lining of chorial villi and are in direct contact with maternal blood. This layer is made of fused multinucleated trophoblasts and this unique structure seems to be key in the protective role of the placenta. Furthermore, STBs can resist infection by several pathogens such as cytomegalovirus (CMV), the bacterium *Listeria monocytogenes* and the protozoan parasite *Toxoplasma gondii* (Arora et al., 2017). One hypothesis for how pathogens would enter via this interface is that they might take advantage of the shedding of old STB that died from apoptosis in hemochorial placentas. At this specific time, the underlying layer of CTBs that will fuse to give a new STB is exposed and not yet differentiated. As the structure of the CTB layer is similar to any epithelium with junctions between the cells, this might be a route of infection for placental pathogens (Costa et al., 2020).

The second site of contact between mother and fetus is the uterus-trophoblast interface. This is the site where EVT invade the maternal decidua. EVTs have innate defense mechanisms against pathogens (Costa et al., 2020). Indeed, they can restrict growth of *L. monocytogenes* and some strains of *T. gondii*. Several studies in pregnant mice have shown that multiple pathogens colonize this region among them are *L. monocytogenes*, *T. gondii*, *Coxiella burnetii* and *Brucella abortus*. Also, *Salmonella enterica* and CMV were found within the decidua in pregnant mice and human placenta respectively. The maternal decidua and EVTs are not directly accessible to pathogen so one hypothesis for infection is cell-to-cell infection. Pathogens might infect maternal leucocytes that will then be recruited in the maternal decidua where they will be in proximity of EVTs.

Classically, placental pathogens are known as "TORCH" (*Toxoplasma gondii*, Others, Rubella virus, Cytomegalovirus, Herpes simplex virus). Even though this list has been revised and pathogens included such as Zika virus, more pathogens should be added to the list such as *Brucella* and *Salmonella* (Costa et al., 2020).

Pathogen	Type	Lifestyle	Recognized adverse pregnancy outcomes
<i>Brucella</i> spp.	Bacterial	Facultative intracellular	1 <sup>st</sup> and 2 <sup>nd</sup> trimester fetal death, stillbirth, preterm labor, intrauterine growth restriction
<i>Coxiella burnetii</i>	Bacterial	Obligate intracellular	Stillbirth, intrauterine growth restriction, increased severity of maternal disease
<i>Listeria monocytogenes</i>	Bacterial	Intracellular/extracellular	1 <sup>st</sup> and 2 <sup>nd</sup> trimester fetal death, stillbirth, preterm labor, severe neonatal infection, increased severity of maternal disease
<i>Mycobacterium tuberculosis</i>	Bacterial	Primarily intracellular	Preterm labor, several neonatal infection
<i>Treponema pallidum</i>	Bacterial	Primarily extracellular	2 <sup>nd</sup> trimester fetal death, stillbirth, fetal hydrops, several neonatal infection
<i>Leishmania</i> spp.	Protozoan	Intracellular/extracellular	Increased severity of maternal disease
<i>Plasmodium falciparum</i>	Protozoan	Obligate intracellular	2 <sup>nd</sup> trimester fetal death, stillbirth, intrauterine growth restriction, several neonatal infection, increased severity of maternal disease
<i>Toxoplasma gondii</i>	Protozoan	Obligate intracellular	1 <sup>st</sup> and 2 <sup>nd</sup> trimester fetal death, preterm labor, severe neonatal infection
<i>Trypanosoma</i> spp.	Protozoan	Intracellular/extracellular	Intrauterine growth restriction, severe neonatal infection
<i>Cytomegalovirus</i>	Viral	Obligate intracellular	1 <sup>st</sup> and 2 <sup>nd</sup> trimester fetal death, preterm labor, severe neonatal infection
<i>Lymphocytic choriomeningitis virus</i>	Viral	Obligate intracellular	1 <sup>st</sup> and 2 <sup>nd</sup> trimester fetal death, fetal hydrops, severe neonatal infection
<i>Parvovirus B19</i>	Viral	Obligate intracellular	1 <sup>st</sup> and 2 <sup>nd</sup> trimester fetal death, stillbirth, preterm labor, fetal hydrops
<i>Rubella virus</i>	Viral	Obligate intracellular	Severe neonatal infection
<i>Varicella zoster virus</i>	Viral	Obligate intracellular	Severe neonatal infection, increased severity of maternal disease
<i>Zika virus</i>	Viral	Obligate intracellular	Intrauterine fetal growth restriction, stillbirth, perinatal death

**Table 2: Pathogens capable of transplacental infection.** Adapted from Robbins and Bakardjiev, 2012.

### 3.5. Trophoblast infection by *Brucella*

In cattle the tropism of *Brucella* for trophoblasts is well recognized. Indeed, in experimentally infected animals, over 90% of the total *Brucella* organisms are found within the cotyledons, fetal fluids, and membranes (Samartino & Enright, 1993a). And even though case reports mentioned a link between infection by *Brucella* and adverse pregnancy outcomes and *Brucella* was isolated from human placenta, most of the studies on *Brucella* intracellular lifestyle were done in HeLa cells, murine BMMs (Starr et al., 2008) (Celli et al., 2005), as well as murine and human macrophages (J774, THP-1) (Boschiroli et al., 2002). The first work in human trophoblasts was done in 2013 by Salcedo and colleagues (Salcedo et al., 2013). It was shown that pathogenic *Brucella* species can infect and replicate inside the different types of human trophoblasts. However, some differences were highlighted between the different species. In EVT, *B. melitensis* was able to replicate in the classic ER-derived compartment which was not the

#### 4. THE EUKARYOTIC PROTEIN CD98HC

case for *B. abortus* or *B. suis*. Indeed, these species were found replicating inside LAMP-1 positive inclusions. Furthermore, infection by *B. melitensis* impaired migration capacity of EVT. This could lead to severe placental deficiencies if the EVTs are not able to properly invade the maternal decidua and arteries in infected pregnant women.

Later work focused on the consequences of *Brucella* infection on trophoblastic functions. The ability of *B. melitensis* to infect all 3 types of trophoblasts was confirmed. This species was able to alter hormone production and migration of EVTs. However, there was no effect on the fusion capacity of CTBs. It was also shown that *B. melitensis* infection can be transmitted from EVTs to CTBs. This gives first insights into cell-to-cell spreading of infection inside the placenta. This study also focused on *B. papionis*, a species isolated from stillbirth in primate. It is important to note that this is the only species with a direct link between infection and abortion in primates. Moreover, infection of human CTBs by *B. papionis* impaired fusion activity (García-Méndez et al., 2019b).

#### 4. The eukaryotic protein CD98hc

From what we have discussed regarding the placenta and trophoblasts, it is obvious that cellular migration and fusion are key processes in this organ. Without them there would be no anchoring of the placenta in the maternal decidua and no remodeling of the maternal arteries to supply blood to the fetus. Also, the fetus would be totally vulnerable to infections if not protected by the layer of syncytial trophoblasts. One fundamental protein involved in both processes is CD98hc.

##### 4.1. Role of CD98hc in placenta

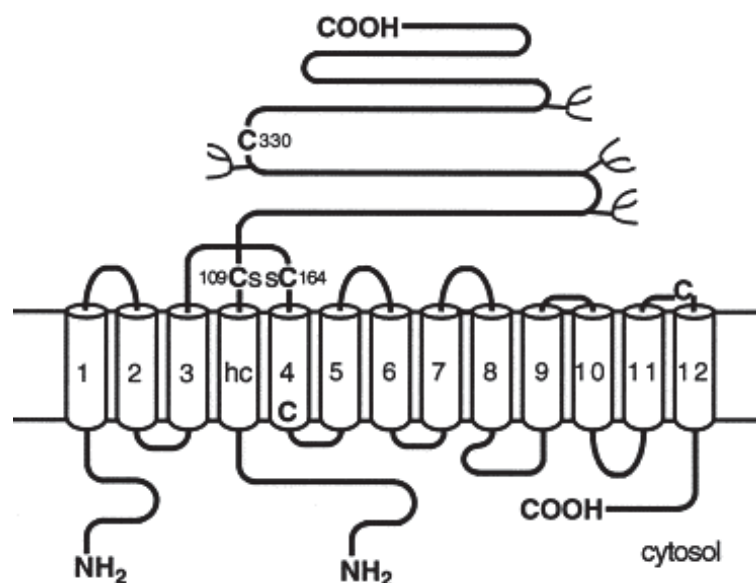
Studies performed in BeWo cells (CTBs) showed the importance of CD98hc in cellular fusion. Silencing CD98hc using transfection of antisense oligonucleotides or RNA interference (RNAi) results in inhibition of cell fusion by as much as 57% (Kudo et al., 2003; Kudo & Boyd, 2004). BeWo cells do not spontaneously fuse *in vitro*. To induce cellular fusion and formation of STB, forskolin is added to the cells. In the studies mentioned above, it was observed that expression of CD98hc increased in a time-dependent manner during syncytialization. CD98hc also seems to be involved in hormone secretion as hCG levels were lower in forskolin-treated BeWo cells transfected with antisense CD98hc oligonucleotide or with RNAi (Kudo et al., 2003; Kudo & Boyd, 2004).

Finally, crosslinking of CD98hc with monoclonal antibodies in forskolin-treated cells increased even more the rate of cell fusion (Dalton et al., 2007).

CD98hc was also shown to be involved in the migration activity of EVT<sub>s</sub>. To prove its implication, several constructs with the different parts of CD98hc were designed and inserted inside FLC4 cells which do not express any endogenous CD98hc. The transmembrane domain of the protein was shown to be important for the migration capacity of EVT<sub>s</sub> (Kabir-Salmani et al., 2008).

#### 4.2. Structure of CD98hc

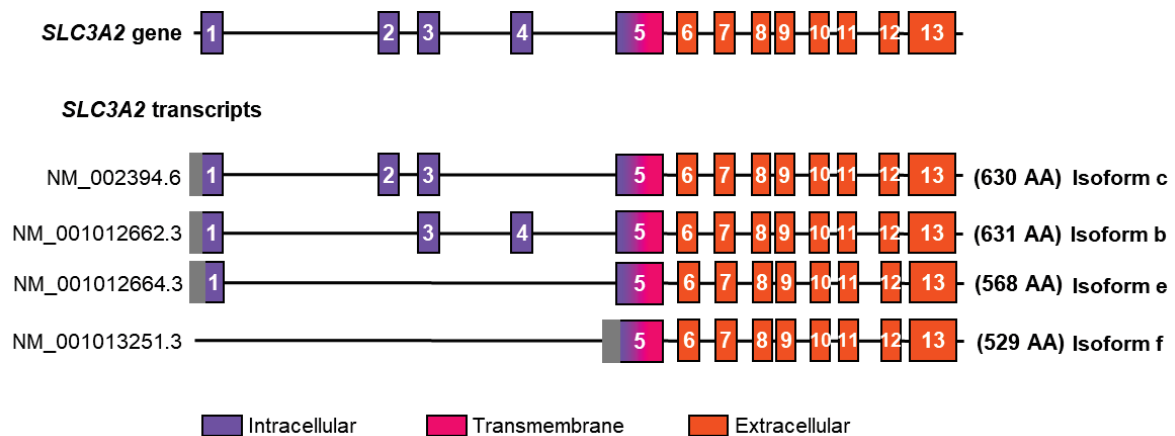
CD98hc is part of a heterodimer, sometimes named 4F2, which was originally discovered as a surface antigen in lymphocytes (Haynes et al., 1981). The complex is composed of 2 subunits, a glycosylated 80kDa heavy chain (CD98hc) and a 40kDa light chain, which are linked by a disulfide bridge between a conserved extracellular cysteine of CD98hc (position 109 of the human protein isoform f) and a matching cysteine in the light chain sequence (Figure 5).



**Figure 5: Schematic representation of human CD98hc protein, associated with a light chain (12 transmembrane domains protein) at the cell surface to form an AA transporter. Two conserved cysteine residues are shown: C109S, involved in the formation of the disulfide bridge between the chains, and C330S. From Pfeiffer et al., 1998.**

#### 4. THE EUKARYOTIC PROTEIN CD98HC

CD98hc is encoded by the solute carrier family 3 member 2 (*SLC3A2*) gene which is highly conserved in mammals and the protein is expressed by all cell types except platelets. Four different isoforms of CD98hc are described (Figure 6). The main difference between the transcripts are changes in the first four exons of the sequence. According to GeneCards, the isoform c is considered as the canonical sequence and our analysis were thus based on this transcript sequence. However, it is important to mention that almost all published studies have been performed on the shorter protein (isoform f), which was the first to be identified and characterized.



**Figure 6: Schematic representation of human *SLC3A2* gene and transcripts (according to RCh38.p7 genome assembly).** Exonic sequences are depicted as boxes, with a color code for sequences encoding the intracellular, transmembrane, or extracellular domains. The different transcripts are shown, and the Genbank accession number is indicated for each variant. UTR sequence is indicated as a grey box. The size (in AA) and the name of the isoforms encoded by each variant is indicated in bold. Adapted from Scalise et al., 2018.

CD98hc is considered essential as disruption of its gene results in embryonic lethality in mouse (Tsumura et al., 2005). CD98hc is classified as a type II membrane glycoprotein with an extracellular carboxylic terminus, a single-spanning transmembrane domain, and an intracellular amino end. Four arginine residues (potential sites for glycosylation) are present on the extracellular domain of the protein. CD98hc expression was detected in most tested human cell lines as well as malignant human cells (Devés & Boyd, 2000).

#### 4.3. Cellular functions of CD98hc

The cellular functions of CD98hc, in cellular migration or fusion, rely on its roles in two essential molecular mechanisms: the modulation of integrin signaling and the transport of amino acids (AA) which are mediated by different domains of the protein (Fenczik et al., 2000).

## 4.3.1. Amino acid transport

Cells are defined by a membrane that separates them from their environment. Consequently, intracellular and extracellular fluids have a different composition. This difference is also seen in AA levels which tend to be higher inside the cell. AA are crucial for cell physiology including protein synthesis, cell survival and are used as fuel by the cell (Bröer & Bröer, 2017). To allow the acquisition of essential AA that are only present in the environment, extracellular AA must be transported inside the cell. This is the role of AA transporters, membrane-bound proteins that mediate transport of AA into and out of cells or intracellular organelles. AA transporters can be found at the cell plasma membrane (to mediate import or export of AA between the cell and its environment) or in intracellular compartments such as the Golgi apparatus, endosomes and mitochondria (Nicolàs-Aragó et al., 2021). Multiple pathways are involved in AA transport and regulation of intracellular AA levels as imbalances can have serious consequences (Figure 7) (Kandasamy et al., 2018). Defects in AA transporters are associated with tumor progression, diabetes, neurodegenerative diseases, and pancreatic pathologies.

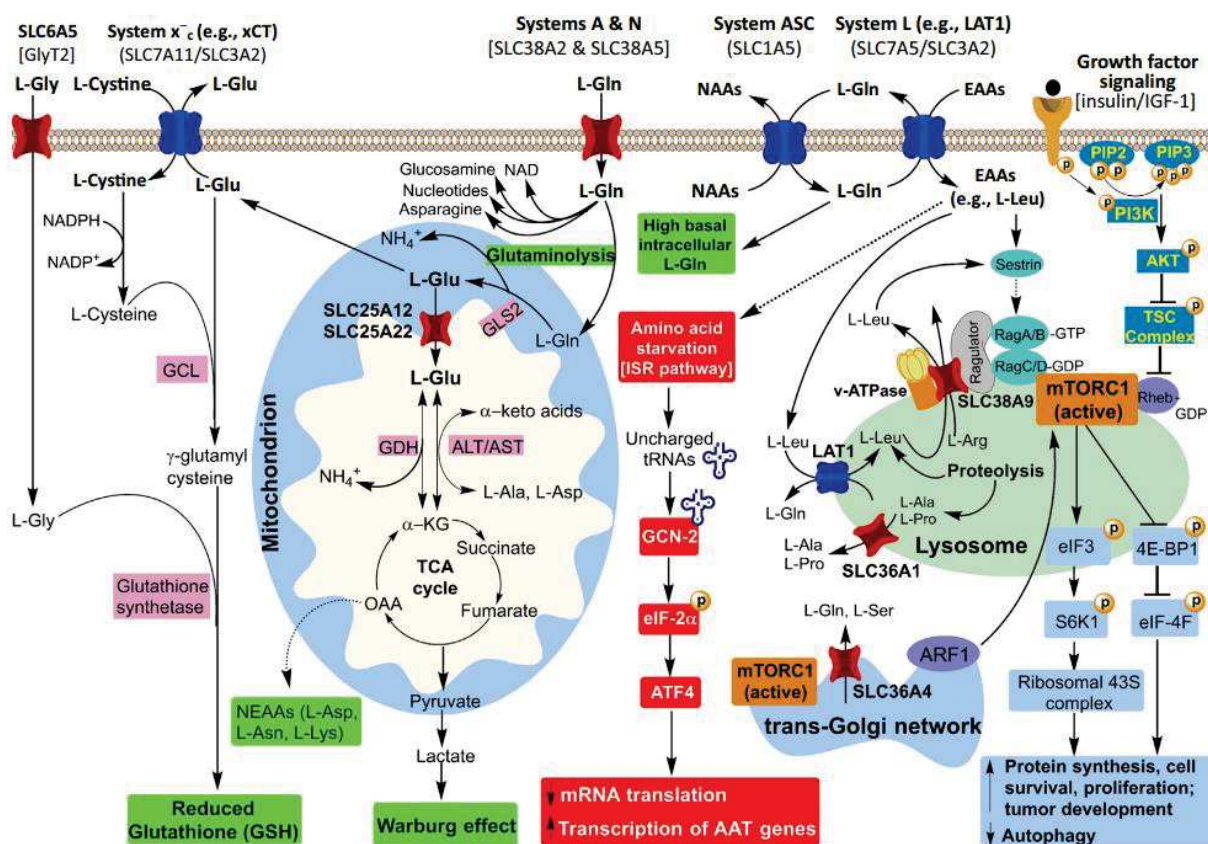


Figure 7: Role of AA transporters in mTOR activation, energy metabolism, nutritional stress and tumor progression. From Kandasamy, 2018.

#### 4. THE EUKARYOTIC PROTEIN CD98HC

Several families of AA transporters are described including the SLC7 family of AA transporters of which CD98hc is a member. This family is divided in 2 subgroups: the cationic AA transporters (CATs) and the glycoprotein-associated AA transporters also called light chains of the heterodimeric AA transporters (HATs). The associated heavy chains of HATs are CD98hc and rBAT. The CAT proteins do not associate with CD98hc and are mainly involved in the Na<sup>+</sup>-independent transport of cationic amino acids. On the contrary, HAT proteins form heterodimers with either CD98hc or rBAT and are obligatory exchangers with a wide substrate selectivity. In fact, the specificity of the substrate transported depends on the light chain (Table 3).

Family	Heavy chain	Light chain	System	Characteristics	Substrates	Expression pattern	Not detected or lowest expression
HETERODIMERIC AA TRANSPORTERS (HAT)	CD98hc (SLC3A2)	LAT1 (SLC7A5)	L	Na <sup>+</sup> -independent	Large neutral AA (Leu, Ile, Phe, Met, Tyr, His, Trp, Val)	Abundant in lung, liver, brain, skeletal muscle, <b>placenta</b> (expression in the placenta is stronger at full-term), bone marrow, testis, ovary, lymphocytes, monocytes (especially activated), fetal liver.	Weaker expression in thymus, cornea, retina, peripheral leukocytes, spleen, kidney, colon lymph node. Not expressed in intestine
		LAT2 (SLC7A8)		Na <sup>+</sup> -independent	Smaller neutral AA (Gly, Ala, Ser, Thr, Asn, Gln, Met, Leu, Ile, Val, Phe, Tyr, Trp, His)	Strongest expression in kidney and <b>moderate expression in placenta</b> and brain, liver, prostate, testis, ovary, lymph node, thymus, spleen, skeletal muscle, heart and small intestine. Also expressed in fetal liver.	
		xCT (SLC7A11)	x <sup>c</sup>	Na <sup>+</sup> -independent	Cys/L-glutamate exchanger (antiporter)	Kidney, brain, stomach, liver, macrophages, retinal pigment cells	
		y <sup>+</sup> LAT1 (SLC7A7)	y <sup>+</sup> L	Na <sup>+</sup> -dependent & -independent	Uptake of arginine, leucine and glutamine (Na-dependent); uptake of dibasic AA (Na-independent)	Highest expression in kidney and peripheral blood leukocytes	Weaker expression in lung, heart, <b>placenta</b> , spleen, testis and small intestine
		y <sup>+</sup> LAT2 (SLC7A6)		Na <sup>+</sup> -dependent & -independent	Uptake of arginine, leucine and glutamine (Na-dependent); uptake of dibasic AA (Na-independent); arginine/glutamine exchanger (antiporter)	Brain, lung, small intestine, testis, heart, kidney, liver	
		Asc-1 (SLC7A10)	asc	Na <sup>+</sup> -independent	Small neutral L- and D-AA	Brain, heart, kidney, liver, lung, pancreas, <b>placenta</b> and skeletal muscle	
		rBAT (SLC3A1)	b <sup>0,+</sup> AT1 (SLC7A9)	b(0,+)	Na <sup>+</sup> -independent	Cysteine and neutral and dibasic AA	Kidney, intestine, brain
CATIONIC AA TRANSPORTERS	CAT-1 (SLC7A1)			Na <sup>+</sup> -independent	Cationic amino acids	Ubiquitous	Not expressed in liver, plasma and intracellular membranes, in epithelial cells
	CAT-2 (SLC7A2)			Na <sup>+</sup> -independent	Uptake of arginine, lysine and ornithine	High levels in skeletal muscle, <b>placenta</b> and ovary. Intermediate levels in liver and pancreas.	Low levels in kidney and heart
	CAT-3 (SLC7A3)			Na <sup>+</sup> -independent	Cationic amino acids (arginine, lysine and ornithine)	Highly expressed in thymus, <b>uterus</b> and testis. In brain, highest expression in thalamus, hippocampus and amygdala	Low levels in brain, mammary gland, prostate, salivary gland and fetal spleen
	CAT-4 (SLC7A5)				Unknown	Brain, testis, placenta/plasma and intracellular membranes	
L SYSTEM OF AA TRANSPORTERS	LAT3 (SLC43A1)		L	Na <sup>+</sup> -independent	Large neutral amino acids (mainly branched-chain amino acids and phenylalanine)	All tissues tested (highest expression in pancreas). In fetus, highest expression in liver and lower levels in kidney, and lung. High levels in prostate cancer cells	
	LAT4 (SLC43A2)			Na <sup>+</sup> Cl <sup>-</sup> and pH-independent	Neutral L-AA (Leu, phenylalanine, valine and methionine)	Higher expression in <b>placenta</b> , kidney and peripheral blood leukocytes	

**Table 3: The SLC7 family of amino acid transporters.** Adapted from Verrey et al., 2004 and Nguyen and Merlin, 2012.



CD98hc function in AA transport relies on its capacity to associate with any of the following light chains: LAT1, LAT-2, y<sup>+</sup>LAT1, y<sup>+</sup>LAT-2, xCT and asc-1. Once inserted in the plasma membrane, the light chain can act as the catalytic protein mediating AA transport *per se*. CD98hc was shown to be necessary for addressing the light chains to the plasma membrane as without CD98hc LAT1 was stuck in the Golgi apparatus; CD98hc therefore acts as a chaperone for the complex (Nakamura et al., 1999). The conserved cysteine at position 109 (C109) of CD98hc is a key residue for this function, as the mutation C109S results in intracellular retention of light chains and a drastic reduction of AA transport (Estévez et al., 1998). Implication of C109 was later confirmed by site directed mutagenesis (Pfeiffer et al., 1998). To the opposite, mutation of the other conserved cysteine residue C330S, located on the extracellular domain of CD98hc, had no effect on AA transport. To determine if the whole protein or only a domain of CD98hc was important for AA transport, chimeras of CD98hc and CD69 (another type II transmembrane protein) were constructed. Using these tools, it was shown that the extracellular domain, but not the intracellular or transmembrane domain, of CD98hc was required for AA transport (Fenczik et al., 2000).

A defect in CD98hc-dependent AA transport would have severe consequences for cell survival and proliferation. Indeed, embryonic stem cells knock out (KO) for CD98hc die of ferroptosis (Ballina et al., 2016). However, cell death was preventable by addition of  $\beta$ -mercaptoethanol ( $\beta$ -ME) in the culture media.  $\beta$ -ME reduces extracellular cystine into cysteine, which can then be imported inside the cell by CD98hc-independent transporters, thus allowing cell survival. However, CD98hc KO cells showed imbalances in intracellular AA content, high levels of oxygen reactive species and, as mentioned above, the cells developed adaptations of CD98hc-independent transporters to survive.

#### 4.3.2. Modulation of integrin signaling

As we discussed above, the different functions of CD98hc are mediated by different domains of the protein (Fenczik et al., 2000). While the extracellular domain is involved in AA transport, the transmembrane and intracellular domains of the protein are responsible for the modulation of integrin signaling.

Integrins are a superfamily of adhesion receptors located on the cell surface that bind to cell-surface ligands, soluble ligands, and the extracellular matrix (Kechagia et al., 2019).

#### 4. THE EUKARYOTIC PROTEIN CD98HC

They link the extracellular matrix and the intracellular cytoskeleton and relay signals in both directions. Integrins are heterodimers composed of an  $\alpha$  (18 types) and a  $\beta$  subunit (8 types) that associate in a noncovalent manner to create unique 24 complexes. Both subunits are type I transmembrane proteins with a large extracellular domain, a single-pass transmembrane domain and a cytoplasmic tail. They associate in the ER before being addressed to the membrane as a heterodimer. Integrins are involved in a variety of biological processes including migration, survival, leucocyte traffic, development, and host defense (Harburger & Calderwood, 2009).

Integrin activation can be triggered by cytoplasmic signals from within the cell (inside-out signaling) or by ligand binding to the extracellular domain of the heterodimer (outside-in signaling) (Kechagia et al., 2019). After ligand binding, integrins cluster and experience major conformational changes leading to intracellular transduction of the signal. To do so, adaptor proteins will accumulate forming signaling proteins hubs regulating integrin downstream signaling pathways. The central protein in this process is focal adhesion kinase (FAK). After binding to the cytoplasmic tail of integrins FAK dimerizes, and this induces autophosphorylation of FAK at tyrosine 397 (Katoh, 2020). This creates a motif that enables the binding and activation of proteins with a SH2 domain such as Src which in turn phosphorylate the FAK-associated proteins. Among them, p130 Cas and paxillin regulate the Rho family GTPases involved in actin reorganization and thus cell motility.

The first hint about the interaction of CD98hc with integrins was provided in a study where CD98hc was isolated as an effector complementing the suppression of  $\beta$  integrin activation occurring in the presence of free  $\beta 1$  tails (Fenczik et al., 1997). Later, CD98hc was shown to bind specifically to  $\beta 1$  and  $\beta 3$  integrins but not  $\beta 1D$  or  $\beta 7$  (Zent et al., 2000). Also, functional  $\beta 1$  integrin is necessary for CD98hc signaling (Rintoul et al., 2002). Construction of chimeras of CD98hc and CD69 lead to the identification of the transmembrane and intracellular domain of CD98hc as being necessary and sufficient for integrin binding (Fenczik et al., 2000). CD98hc-null cells were generated to gain insight in the role of CD98hc in integrin function. Using this tool CD98hc was shown to be involved in integrin-dependent cell spreading, cell migration and protection from anchorage deprivation-induced apoptosis (anoikis). These defects were associated with suppression of p130<sup>CAS</sup>, FAK and Akt phosphorylation, which are critical in early steps of integrin

signaling. These defects were rescued by a chimera made of intracellular and transmembrane domains of CD98hc and extracellular domain of CD69 (Feral et al., 2005). Moreover, mutation of AA residues on integrin involved in the interaction with CD98hc impaired cell spreading (Prager et al., 2007).

#### 4.4. CD98hc in infection

While initially identified as central in tumorigenesis, a growing number of studies have revealed a key role of CD98hc in host-pathogen interactions in the past years. The ubiquity and multiple functions of CD98hc and its associated light chains have indeed allowed many pathogens to exploit them in different phases of their infectious cycle.

This chapter is presented as a manuscript that reviews the current knowledge about the involvement of CD98hc and its associated light chains in host-pathogen interactions. It summarizes the molecular and cellular mechanisms by which these host proteins are hijacked by viruses, bacteria or parasites (manuscript ready for submission).

**CD98hc in host-pathogen interactions:  
roles of the multifunctional host protein during infections**

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## **Abstract**

The eukaryotic protein CD98hc (also known as 4F2, FRP-1 or SLC3A2) is a membrane glycoprotein and one of the heavy chains of the family of heterodimeric amino acid transporters. It can associate with any of 6 different light chains to form distinct amino acid transporters. CD98hc is also involved in mediation of intracellular integrin signalling. Besides its physiological roles in the development of the placenta and the immune system, CD98hc is important during pathological processes such as tumorigenesis and host-pathogen interaction. Since its first identification as Fusion Regulatory Protein 1 regulating cell fusion in cells infected by the Newcastle disease virus, CD9hc has been reported to be mediating many viral, apicomplexan, and bacterial infectious processes. In this review we describe the role of CD98hc and its associated light chains in bacterial, apicomplexan, and viral pathogenesis. We also discuss the consequences of infection on the expression and localisation of these proteins. The identification of processes in which CD98hc is important could provide new therapeutic targets in infectious diseases.

## Introduction

The eukaryotic protein CD98hc (also known as 4F2, FRP-1 or SLC3A2) is a type II transmembrane glycoprotein of 80kDa encoded by the gene *SLC3A2*, whose inactivation in mice is embryonically lethal (1). It is one of the two heavy chains of the family of heterodimeric amino-acids transporters (HAT) and can associate with any of the following 6 light chains: xCT, LAT1, LAT2,  $\gamma^+$ LAT1,  $\gamma^+$ LAT2 or Asc-1 (Table 1). HAT are specialized transporters that mediate the transfer of amino acids (AA) across plasma membranes. They are mostly exchangers with a broad spectrum of substrates, the specificity of the substrate depending on the light chain. Addressing of the light chains to the cell surface depends on expression of the heavy chain, which can be expressed on its own (2).

CD98hc also functions as a potentiator of outside-in integrin signalling (3). The protein contains a short N-terminal intracellular domain, a transmembrane domain and a large extracellular C-terminal ectodomain. The intracellular and transmembrane domains mediate the modulation of integrin signalling (3) whereas the extracellular domain is necessary for AA transport (4). Distribution of CD98hc at the host cell surface is not homogeneous. This glycoprotein accumulates in lipid rafts, some microdomains of the plasma membrane enriched in sphingolipids and that modulate membrane fluidity (5). Lipid rafts serve as functional platforms for the interaction of cells with the “outside world” and host most of the plasma membrane signalling, as well as exo- and endocytosis machineries.

CD98hc was initially discovered in an immunological context, as a marker of monocytes and activated B and T cells (6). It is now established that CD98hc is required for clonal expansion of B cells (7) and T cells (8) during adaptive immunity. CD98hc is, however, ubiquitously expressed in different cell types (9), with high levels in certain tissues including the placenta. CD98hc has essential roles in several physiological processes that are important for the development of this organ. The protein is required for the migration of extravillous trophoblasts (10), cells that invade the maternal decidua to anchor the placenta and remodel maternal arteries to supply blood to the foetus. CD98hc is also essential for the fusion of cytotrophoblasts that leads to the formation of the outermost layer of foetal-derived tissue in direct contact with maternal blood (11, 12).

CD98hc is also highly expressed by numerous cancer cells and this has been directly linked to transformation and tumour growth (3, 13). Even though the exact molecular mechanisms are not known, several hints lead to think that the role of CD98hc in lymphocyte proliferation relies on its function as a mediator of integrin signalling (8). The light chain xCT is also highly expressed by a variety of malignant tumours such as lymphoma, glioma, breast carcinoma and prostate cancer, and this has been linked to a high requirement of cystine uptake from the microenvironment for growth and viability of cancer cells (14).

Besides its essential role in AA uptake, immunity, placental biology and tumorigenesis, an increasing number of studies also highlighted a role of CD98hc in host-pathogen interactions. In this review we will describe the role of CD98hc, as well as that of its associated light chains, in viral, bacterial and apicomplexan infectious processes. We will also explain the impact of infection on the expression or localization of these host proteins. The nomenclature for these proteins is complex, and has changed over time, for simplicity, we will use CD98hc with the name used in the publication in parenthesis.

## **1. Viral infections**

Viruses are acellular organisms that obligatory replicate inside host cells. Upon infection, they hijack cellular mechanisms to generate new viral particles, egress from the infected cell important factors in the infection by a wide range of viral pathogens, playing a role at different stages of the infectious cycle.

### **1.1. Attachment and Entry**

As a ubiquitous cell surface protein, CD98hc is exploited by several pathogens as a receptor or co-receptor. These include Noroviruses, the main cause of epidemic gastroenteritis worldwide. Murine norovirus 1 (MNV-1), frequently used as a model for human viruses, uses CD98hc during viral attachment. CD98hc was identified as a putative MNV-1 interacting protein using a viral overlay protein binding assay (15). A 33% decrease in MNV-1 binding was observed in RAW 264.7 cells transfected with anti-CD98hc siRNA, suggesting that CD98hc is involved in MNV-1 binding to these cells. This seems to be cell-type dependent as no effect

was observed in SRDC (murine dendritic cells). Recombinant CD98hc was also found to directly interact with VP1, the viral capsid protein that mediates receptor binding. These results suggested that CD98hc would act as a receptor for MNV-1; however, incubation of MNV-1 with recombinant CD98hc did not inhibit infection but enhanced MNV-1 infectivity with a significantly increased number of viral particles binding. This suggests that CD98hc does act not as a receptor, but rather as a co-receptor in MNV-1 infection.

Vaccinia virus (VACV) is part of the poxviruse family. This enveloped virus transits through different infectious forms during its replication cycle (16). The cytoplasmic mature virion (MV), surrounded by a single lipid membrane, can acquire an additional membrane derived from the Golgi or endosomes to form a wrapped virion (WV). Upon egress, the WV outer membrane fuses with the host cell plasma membrane forming extracellular virions (EV). CD98hc was found to be enriched in lipid rafts of VACV infected cells and seems to be involved in viral entry rather than binding to target cells (17). Knockdown or knockout of CD98hc resulted in a decreased infection by vaccinia MV without affecting the viral attachment to the surface of HeLa or activated T cells. Multiple strains of VV were used to rule out a possible strain-specific result; CD98hc was found to be required for all vaccinia strains known to enter into cells via endocytosis, but not for those that enter by membrane fusion. Moreover, CD98hc co-localized with viruses in endocytic vesicles very rapidly after entry. Chimeric proteins lacking one of the three domains of CD98hc were unable to restore sensitivity to VACV infection to CD98hc<sup>-/-</sup> cells, showing that the full-length protein is required. The role of CD98hc in VACV infection therefore appears to depend on its involvement in endocytosis, rather than being a receptor for viral attachment. This conclusion was confirmed in a later study on primary human leukocytes (18).

Hepatitis C virus (HCV) is one of the major pathogens causing chronic liver diseases. This enveloped virus targets hepatic cells and can remain dormant for quite some time before symptoms manifest. CD98hc (SLC3A2) is specifically required for the entry step of infection by HCV in infected hepatocytes (Nguyen et al., 2018). Knock down of CD98hc by siRNA significantly decreased HCV entry, but not attachment, replication, translation or virion production. Moreover, co-immunoprecipitation experiments revealed a direct interaction between CD98hc and the viral envelope protein E2, suggesting that HCV may co-opt CD98hc to facilitate viral entry.



The human cytomegalovirus (HCMV) is the cause of very common viral infections, with a prevalence of more than 80% in developing countries. Infection is mainly asymptomatic but can lead to severe consequences in immunocompromised patients or for the foetal development. In a proteomic study aiming to determine the consequences of HCMV infection on host membrane proteins, a decrease in CD98hc abundance was observed in infected human foreskin primary fibroblasts both at the cell surface and in whole cell lysates (20). These results were confirmed by flow cytometry, cell surface biotinylation and immunofluorescence microscopy. This effect seems to be the result of protein internalization and degradation rather than regulation at the transcriptional level because a previous study showed that the transcription of CD98hc is not affected by this virus (21). Downregulating surface expression of receptors after entering into cells can be observed upon viral infections. This is beneficial for their replication because it either blocks re-infection of already infected cells or prevents an interaction between viral glycoproteins and the receptor at the surface of infected cells, which could interfere with virus egress (22). Viswanathan *et al.* thus hypothesized that CD98hc may be part of a complex that contains receptors for HCMV entry (20).

Adenoviruses of the D subgroup are causative agents of epidemic keratoconjunctivitis and genital tract infections. Previous research showed that adenovirus Ad37 interacted with a 50kDa protein on the surface of human ocular cells. To identify this protein, proteins from conjunctival cell membranes were purified and mass spectrometry was used (23). Four membrane proteins, including the CD98hc-dependent light chain LAT1, were identified. However, LAT1 was not found as directly interacting with the virus. Therefore, even though LAT1 is not in direct interaction with Ad37, these results suggest that LAT1 is part of a protein complex involved in the entry of the virus.

## **1.2. Membrane Fusion**

Internalization of most enveloped involves membrane fusion. This relies on the interaction between viral envelope glycoproteins and proteins expressed on the host cell surface (which can be distinct from the virus receptor). Co-expression of viral and host proteins with fusogenic properties at the surface of host cells can also result in cell-to-cell fusion and formation of multinucleated syncytial cells. Syncytium formation is one of the major virus-

induced cytopathic effects, however inducing fusion of infected cells with neighbouring cells can also be a strategy for viral propagation (24).

CD98hc was initially named Fusion Regulatory Protein-1 (FRP-1) after its identification as a regulator of fusion in cells infected by a member of the paramyxovirus family: the Newcastle disease virus (NDV) (25). HeLa and FL cells spontaneously form multinucleated cells 24h after infection with NVD. Several monoclonal antibodies were found to enhance formation of giant multinucleated cells in NVD infected HeLa cells. These antibodies immunoprecipitated two HeLa surface proteins: gp80 and gp135. Due to their role in NDV-induced cell fusion these proteins were respectively named FRP-1 (later named CD98hc) and FRP-2. NVD-induced fusions were more numerous and occurred earlier in cells incubated with an anti-CD98hc antibody targeting the extracellular domain of the protein. However, in cells expressing a dominant negative mutant of CD98hc in which the cytoplasmic domain is replaced by the cytoplasmic domain of human parainfluenza virus type 2 haemagglutinin-neuraminidase, no cell fusion was observed even in the presence the antibody HBJ127, which targets CD98hc extracellular domain (26). This observation was true with other paramyxoviruses such as mumps virus, SV5 and SV31. Suppression of cell fusion was not due to a difference in virus penetration or replication since there was no difference in production of virus-specific polypeptides. Interestingly, substitution of the conserved cysteine at position 330 of CD98hc (C330) with a serine is sufficient to suppress virus-induced cell fusion in HeLa cells. Thus, CD98hc is required for NDV-induced cell fusion, and this function is mediated by its cytoplasmic domain.

Spontaneous cell fusion is also observed during human parainfluenza virus 2 (HPIV-2) infection. The majority of HPIV-2 infected HeLa cells form multinucleated giant cells after infection (27). Two viral proteins are required for this phenomenon: the hemagglutinin-neuraminidase (HN) and the fusion glycoprotein (F). Upon natural HPIV-2 infection, HN mediates attachment of the virus to sialic acid-containing cell receptors, thus triggering a conformational change allowing F to induce fusion between the envelope of the virus and the plasma membrane. Transient expression of HN and F in HeLa cells also results in cell-to-cell fusion, showing that these viral proteins are sufficient for inducing this phenomenon. The authors also observed that the anti-CD98hc antibody HBJ127 suppresses infection-induced fusion which, intriguingly, is the opposite to the effect seen with NVD infection. Cell-to-cell

fusion is also induced by Human Immunodeficiency Virus (HIV), the etiological agent of acquired immune deficiency syndrome (AIDS). HIV is an enveloped virus that mainly infects CD4<sup>+</sup> positive T lymphocytes. The first hint for a role of CD98hc in HIV-1-induced cell fusion appeared in a study published in 1994 in which an antibody targeting CD98hc (FRP-1) induced syncytium formation in cells overexpressing the HIV gp160 protein or in cells persistently infected with HIV (28). Later, the same group found that the HIV gp120 protein induces expression of CD98hc in CD4<sup>+</sup> T cells, suggesting that CD98hc plays an important role in the formation of syncytium upon HIV infection (29).

The Kaposi's sarcoma-associated herpes virus (KSHV, or HHV-8) is an oncovirus responsible for several cancers arising in immunocompromised (mainly AIDS) patients, notably the Kaposi's sarcoma. With this virus, productive infection is not the rule and KSHV rather establishes a latent infection with periodic reactivation. KSHV has a very broad tissue tropism and can infect many different cell types, indicating a broad variety of receptors. Several host molecules can serve as receptor for KSHV, notably heparan sulfate (HS) proteoglycan and several integrins ( $\alpha3\beta1$ ,  $\alphaV\beta3$ ,  $\alphaV\beta5$  and  $\alpha5\beta1$ ) (30), probably depending on the target cell type. While primary studies suggested that KSHV could enter by direct fusion of its envelope with the plasma membrane (31), it is now accepted that this enveloped virus rather uses an endocytosis-related mechanism to enter cells (30, 32). The viral envelope then fuses with the endosomal membrane releasing the capsid into the cytoplasm. In a study aiming to characterize the attachment sites for KSHV on HT1080 cells, 70% of KSHV particles were found to be bound to CD98hc-enriched sites (33). However, human salivary gland epithelial cells, which are resistant to KSHV infection, express all putative KSHV receptors except  $\alphaV\beta3$  integrin, suggesting that CD98hc alone is not sufficient to allow infection and is likely part of a virus-induced multi-receptor complex. In this respect, the CD98hc-associated light chain xCT was identified as an essential host factor for KSHV-mediated fusion in cells (34). This discovery was based on a screen using a cDNA library established from a cell line permissive to KSHV infection. The rationale was that a cDNA library established from a cell line permissive to infection should contain the sequences encoding for the host proteins that would render non-permissive cells permissive to KSHV-mediated fusion. This finding was confirmed by overexpressing xCT in several non-permissive cells which rendered all of them permissive to KSHV-mediated fusion. Reversely, KSHV-mediated fusion in permissive cells was inhibited by

antisera against different extracellular regions of xCT. Also, high endogenous xCT expression was detected in all permissive cell lines and only weakly in cells with low KSHV fusion activity. The role of xCT and CD98hc in KSHV infection was confirmed in a later study where Veettil *et al.* found that a multi-molecular complex containing CD98hc/xCT and integrins ( $\alpha V\beta 5$ ,  $\alpha V\beta 3$ , and  $\alpha 3\beta 1$ ) is formed on human dermal microvascular endothelial (HMVEC-d) cells very early after infection (35). As no colocalization was observed between KSHV and LAT1, another CD98hc-dependent light chain, infection specifically involves the CD98hc/xCT heterodimer. The study also showed that initial attachment of KSHV to HS and  $\alpha 3\beta 1$  integrin was essential for subsequent interaction with CD98hc/xCT to  $\alpha 3\beta 1$ , highlighting that a precise chronology of temporal interactions within this complex is important for the virus to enter target cells. Mono- and polyclonal antibodies targeting CD98hc or xCT only moderately inhibited KSHV binding to and entry into HMVEC-d cells, suggesting that CD98hc/xCT does not play major roles in these steps of infection. These antibodies did, however, dramatically inhibit KSHV gene expression without blocking the delivery of viral DNA. Thus, the CD98hc/xCT heterodimer does not seem to act as a binding receptor for KSHV, but its presence in the complex may be critical for clustering together the different receptors that are directly involved in viral binding and entry. This study also revealed a role for CD98hc/xCT in controlling viral-gene expression, probably by regulating intracellular signalling cascades. Finally, sulfasalazine, a selective inhibitor of the CD98hc/xCT (xc<sup>-</sup> system), was proven to be effective in the prevention of KSHV-induced tumour progression in an immune-deficient xenograft mouse model, suggesting that targeting this system could be a complementary antiviral therapy (36).

### 1.3. Nuclear Egress

Herpes simplex virus 1 (HSV-1), another member of the *Herpesviridae*, is best known for causing oral and genital mucosal lesions. In the replication cycle of this enveloped virus, the nucleocapsids of the newly formed virions are assembled in the nucleus of the infected cell. Nucleocapsids must thus cross the nuclear membranes to acquire their final envelope in the cytoplasm. This requires two steps: budding of the nucleocapsids through the inner nuclear membrane and then fusion with the outer nuclear membrane to release de-enveloped nucleocapsids into the cytoplasm. CD98hc was found to be essential for the second step. HSV-

1 infection induced a massive recruitment of both CD98hc and  $\beta$ 1 integrin (normally localized at the plasma membrane) to the nuclear membrane of Hep-2 cells, where they colocalized with the viral envelope glycoproteins gB and gH and with the HSV-1 egress factor UL31 (37). A direct interaction between CD98hc and the viral proteins gB, gH, UL31, UL34, Us3 was demonstrated by co-immunoprecipitation. Knocking down CD98hc or  $\beta$ 1 integrin expression in infected cells resulted in reduced intra- and extracellular levels of progeny virus. This was due to a disorganization of the viral nuclear egress complex that resulted in most of the enveloped virions being trapped in membranous invaginations in the perinuclear space. This study thus showed that CD98hc and  $\beta$ 1 integrin are required for nuclear egress of nascent HSV-1 particles, probably by interacting with several viral proteins and regulating development fusion between HSV-1 envelopes and the outer nuclear membrane, a process that is essential for efficient viral replication.

#### **1.4. Oxidative Stress**

Oxidative stress is a key cellular defence mechanism used by cells to inhibit infections. The CD98hc/xCT heterodimer (xc<sup>-</sup> system) is a key player in the maintenance of intracellular redox balance. Production of ROS, which then diffuse into intracellular organelles such as phagosomes, is a mechanism used by some cells for controlling intracellular pathogen (38). While these compounds are microbicidal, they can also cause damage to cellular membranes, proteins and nucleic acids. To prevent this, cells utilize a cytosolic redox buffering system: the antioxidant molecule glutathione (GSH). The heterodimer CD98hc/xCT exchanges intracellular glutamate for extracellular cystine, and the latter is rapidly reduced in the intracellular space into cysteine that is used for protein synthesis but also serves as a rate-limiting precursor of GSH (14). Ensuring an appropriate balance between ROS and GSH allows fine tuning of the oxidative stress, to allow antimicrobial activity while maintaining a redox equilibrium appropriate for cell viability. This is exploited by a range of viral pathogens either for survival or to enhance pathogenesis.

xCT (SLC7A11) was shown to participate in the intrinsic protection of macrophages against HIV infection. Macrophages can only be moderately infected by HIV-1, and the cells that do become infected serve as reservoirs to disseminate HIV-1 to CD4<sup>+</sup> T cells. The molecular

mechanism of this intrinsic antiviral defence was recently shown to involve xCT, as both depletion of xCT mRNA and chemical inhibition of its transport activity boosted HIV infection in macrophages (39). xCT was found to act as a downstream mediator of the transcription factor Nrf2, which is a master regulator of the antioxidant response in cells. xCT impedes the infection cycle after reverse transcription but immediately before 2-LTR circle formation, which is a hallmark of viral cDNA entry into the cell nucleus. The molecular mechanism of the blockage is not fully understood, but the authors propose a model in which xCT-mediated control of intracellular cysteine levels could prime a downstream restriction involving either protein synthesis or antioxidative response. This study thus showed a role for xCT in the permissiveness of some cell types to HIV infection.

Qin et al found that KSHV encodes several microRNAs that upregulate xCT expression in infected macrophages and endothelial cells (40). This increased the susceptibility of cells to KSHV infection (higher intracellular viral load and viral transcript expression) and protects infected cells from death induced by oxidative stress. This finding has clinical relevance since increased xCT expression was also observed in more advanced human KS tumours, which contain a larger number of KSHV-infected cells. A similar situation is seen with the Japanese encephalitis virus where upregulation of both CD98hc (SLC3A2) and xCT (SLC7A11) expression was observed upon infection of SH-SY5Y neuroblastoma cells (41), again suggesting that stimulation of the antioxidant response occurs to counteract the oxidative stress elicited by infection.

The CD98hc/xCT heterodimer (xc<sup>-</sup> system) plays a role in HIV pathogenesis in the retina. Ectopic expression of the HIV-1 transactivator protein Tat in ARPE-19 cells (human retinal pigment epithelia) downregulates the expression of two enzymes involved in GSH synthesis, thus decreasing GSH levels in these cells (42). Because of the function of CD98hc/xCT in controlling oxidative stress, Bridges and colleagues evaluated its role in this phenomenon. Unexpectedly, they observed a Tat-mediated up-regulation of CD98hc and xCT expression, both at the mRNA and proteins levels, and increased transport function of this heterodimer. These findings were confirmed in the retina of Tat-transgenic mice. The authors thus propose a model in which Tat reduces the endogenous synthesis of GSH, thus causing oxidative stress. As a consequence, expression of CD98hc/xCT is upregulated, which enhances the release of

glutamate into the extracellular space. Elevated levels of glutamate would in turn cause apoptotic cell death, responsible for the excitotoxicity in the retina of AIDS patients.

CD98hc expression is highly up-regulated upon HCV infection, both at the mRNA and protein level (Nguyen *et al.*, 2018). This up-regulation is due to oxidative stress induced by the viral protease NS3/4A. HCV infection also increased expression of the LAT1 light chain. Increased expression of the CD98hc/LAT1 heterodimer on infected hepatocytes was shown to facilitate AA transport, thus promoting cell proliferation. This study thus showed that CD98hc is exploited not just for HCV entry into host cells, but also to enhancing AA transport into infected cells, a process that might enhance viral replication. This study did not address a possible up-regulation of the xCT light chain, in HCV-infected cells. Increasing CD98hc/xCT levels in HCV-infected cells could also constitute a positive feedback mechanism to protect them against infection-induced oxidative stress.

## **2. The role of CD98hc in bacterial and protozoal infections**

As with viral infections, several facultative intracellular bacterial and protozoal pathogens exploit CD98hc and its light chains at different stages of their infection cycles.

### **2.1. Attachment and Entry into Host Cells**

Direct interaction with CD98hc at the cell surface can be exploited for both binding and entry. This is the case for enteropathogenic *Escherichia coli* (EPEC). These bacteria infect human intestines and are important diarrheal pathogen. Adherence of EPEC to the intestinal mucosa causes rearrangement of actin cytoskeleton in host cells, causing deformations named 'pedestals', a key step in the induction of diarrhoea. Shames and colleagues found that CD98hc accumulates on these pedestals (43). In another study by Charania and colleagues, surface plasmon resonance was used to demonstrate binding of recombinant human CD98hc to EPEC (as well as its murine counterpart *Citrobacter rodentium*) via the first 103 residues of its extracellular C-terminal domain (44). Knockdown of CD98hc resulted in a strong decrease in EPEC adherence to Caco2-BBE intestinal cells and its overexpression in transgenic mice increased EPEC adherence to colon tissue *ex vivo*.

The importance of CD98hc for *Brucella* infection was demonstrated by our group. These facultative intracellular pathogens are responsible for brucellosis, a zoonosis causing huge economic losses worldwide. CD98hc was initially found to interact with the *Brucella* VirB2 protein which is the major component of the pilus of its type 4 secretion system (45). Confocal microscopy showed that CD98hc was associated with the bacteria at early time points of infection, suggesting a role in adhesion and/or internalization. Knocking down CD98hc expression by RNAi significantly lowered the infection rate in HeLa cells. In murine derm fibroblasts (mDFs), the numbers of intracellular *Brucella* were also drastically lower in cells with a knockout (KO) for CD98hc compared to wild type mDFs.

A recent study reported that CD98hc is a receptor for the malarial pathogen *Plasmodium vivax*. Different *Plasmodium* species and strains use a number of combinations of parasite ligands and host receptors to enter cells. The host protein CD71 (transferrin receptor 1) is a receptor for *P. vivax* binding to reticulocytes (immature red blood cells). This binding is trypsin sensitive, however some strains exhibit trypsin resistant binding, suggesting that there is another receptor; Malleret *et al.* have shown that it is CD98hc (46). CD98hc is expressed on CD71<sup>+</sup> reticulocytes and, as for CD71, *P. vivax* invasion can be blocked with both polyclonal and monoclonal (UM7F8) anti-CD98hc antibodies. The *P. vivax* cognate ligand for CD98hc was identified as PvRBP2a. This was found by measuring the binding of HEK-2 expressing *P. vivax* proteins at their surface to reticulocytes. This interaction was also confirmed biochemically. It is interesting that both CD71 and Basigin (CD147), another recently reported cellular receptor for *Plasmodium*, can be found in lipid rafts and interact with CD98hc. Further, as discussed above, CD98hc and CD147 work together to regulate cell fusion events in both osteoclast formation and HIV infection (47).

## **2.2. Intracellular Survival and Replication**

In addition to its role in entry of *Brucella* into cells, we also found that CD98hc is important for intracellular survival and replication. The number of intracellular bacteria in CD98hc KO mDF was significantly lower at 24 and 48 hpi, indicating an essential role of CD98hc in *Brucella* intracellular replication (45). In a more recent study using human trophoblast cell lines, we found that *Brucella* infection differently affects CD98hc expression depending on the type of trophoblasts. Expression of CD98hc was increased after infection of extravillous trophoblasts,



whereas it was decreased in cytotrophoblasts (48). The exact role of CD98hc in *Brucella* infection is still not clear but these results gave the first insight into the requirement of CD98hc for *Brucella* infection, which is currently still under investigation.

*Salmonella* are enterobacteria causing salmonellosis and typhoid fever. These bacteria colonise animal and human intestines and are transmitted via the faeco-oral route. Internalization of *Salmonella enterica* serovar Typhimurium in cells induces host membrane damage leading to AA starvation (49). This starvation induces relocalisation of mammalian target of rapamycin (mTOR) to the *Salmonella*-containing vacuole (SCV) where it becomes activated and blocks autophagy, thus allowing *Salmonella* escaping from this anti-microbial cellular machinery. Recruitment of mTOR to the SCV requires the activity of AA transporters including the CD98hc/LAT1 heterodimer. Indeed, treatment of infected cells with LAT1 inhibitors such as D-phenylalanine or BCH blocked this phenomenon. Reversely, knockdown of *SLC7A5* (encoding LAT1) resulted in an increased early colocalization between intracellular *Salmonella* and the autophagy marker LC3. Phosphorylation of the p70 ribosomal S6 kinase 1 (S6K1), a serine threonine kinase downstream of PI3 kinase, is another hallmark of mTOR activation. In this study, the authors also showed that knocking down *SLC7A5* or *SLC3A2* decreases S6K1 phosphorylation in infected cells, thus showing a direct role of CD98hc/LAT1 in mTOR activation. *Salmonella* infection also induces relocalisation of LAT1 from the Golgi apparatus to intracellular vesicles in HeLa cells. Finally, replenishment of AA content was also shown to be due to active uptake of extracellular AA through the CD98hc/LAT1. A role of the CD98hc/LAT1 heterodimer in *Salmonella* infection was also demonstrated in the pathological context of ulcerative colitis (UC). UC is an inflammatory bowel disease characterized by defects in colonic epithelial barrier defences, causing abdominal pain and haemorrhagic diarrhoea. The initial cause of UC is unknown but genetic, environmental and immune factors play important roles in the development of the disease. Bacteria also contribute to its etiology: in UC, bacteria of the microbiome can come in close contact with colonic epithelial cells, leading to inflammation. Moreover, prior enteric infections are a risk factor for developing UC. CD98hc/LAT1 seems involved in the altered bacterial defence against bacterial infection in UC patients through regulation of expression of both genes by the chromosome-associated protein D3 (CAP-D3). CAP-D3 regulates the expression of several genes involved in innate immunity and was previously shown to be essential for intestinal bacterial clearance in the

*Drosophila* model (50). A recent study also found that CAP-D3 levels are reduced in patients suffering from active UC compared to healthy patients or patients with inactive UC (51). The authors also showed that knocking down CAP-D3 expression in human intestinal cell lines results in impaired autophagy-mediated clearance of two important enteric pathogens: *Salmonella* and adherent-invasive *E. coli* (AIEC). Interestingly, the activity of LAT1 is involved in the defect in anti-bacterial defence in CAP-D3 deficient cells. Moreover, LAT1 was found to be targeted to the SCV very rapidly after *Salmonella* infection, and this relocalisation takes place earlier in CAP-D3 deficient cells. Earlier localization of LAT1 to SCV was correlated with an increased activation of mTOR and repression of autophagy in these cells. Finally, expression of CD98hc (SLC3A2) and LAT1 (SLC7A5) were significantly increased in UC patient, as well as in cells in which CAP-D3 was knocked-down. Therefore, CAP-D3 is a repressor of CD98hc and LAT1 expression, and reduced expression of CAP-D3 in UC patients results in an increased expression of these proteins, inhibition of autophagy and defect in responses to bacterial infection in colon epithelial cells. Altogether, these studies showed that CD98hc/LAT1 is a key element in *Salmonella* interactions with host cells through its enrolment in the innate immunity through the autophagy pathway and adaptation of cells to infection-induced AA starvation.

### **2.3. Cytoprotection of Infected Cells**

In the study by Shames and colleagues on EPEC infections, the authors also demonstrated an interaction between CD98hc and the bacterial protein EspZ, an effector of EPEC type III secretion system (43). To gain insight into the role of such an interaction in EPEC virulence, they first infected epithelial cells with a  $\Delta$ EspZ mutant and observed higher cell death. This suggested that EspZ is involved in the protection of cells upon EPEC infection, which was confirmed in a later study (52). Using RNAi silencing, Shames and colleagues also showed that CD98hc contributes to EspZ-mediated protection from EPEC-induced cytotoxicity. In addition, CD98hc was found to play a role in host defense mechanisms (through the modulation of cytokine expression) as well as in the rapid turnover of mucosal epithelial cells during EPEC infection (44). Therefore, the host protein CD98hc seems to play several roles during EPEC infections: adherence of bacteria to intestinal cells, modulation of host immune responses and

survival of infected cells, all of which would provide the pathogen with valuable time to colonize efficiently prior to dissemination.

In the case of the causative agent of tuberculosis, *Mycobacterium tuberculosis* (Mtb), expression of xCT increases host susceptibility to infection (53). In a recent study, Cai and colleagues assessed the role of the CD98hc/xCT heterodimer. Expression of xCT at the mRNA and protein levels was significantly higher in peripheral blood monocytes of patients with active Mtb infection compared to healthy controls or patients with latent Mtb infection. Increased xCT expression was also observed after incubation of macrophages with bacterial lysates indicating that bacterial components, rather than active infection, are causing this effect. Finally, increased xCT expression was found to be mainly due to activation of the Toll-like receptor 2 (TLR2) and the p38/PI3K signalling pathway. The importance of the xc<sup>-</sup> system (CD98hc/xCT) in Mtb infection was also studied in a mouse model. Both bacterial loads in the lungs and pulmonary inflammation were significantly lower in xCT KO mice than in wild-type animals, suggesting that xCT increases host susceptibility to tuberculosis. Chemical inhibition of xCT using sulfasalazine also provided protection against Mtb infection both *in vivo* and *in vitro*. As noted above, this molecule also was also beneficial in KSHV-induced tumours (36). The authors finally found that inactivating xCT by gene disruption or chemical inhibition decreases the production of GSH, thus probably increasing ROS/GSH ratios, in macrophages. In these experimental conditions, the oxidation state of intracellular Mtb was also increased, causing death of bacteria. Enhancement of the antimicrobial activity of macrophages against Mtb upon xCT disruption thus relies on increased oxidative stress in intracellular mycobacteria. Altogether, this study revealed an essential role of xCT in TB pathogenesis. This host protein, whose increased expression is clinically associated with development of active TB, increases inflammation and host susceptibility to Mtb infection by inhibiting the antimicrobial functions of infected cells.

## 2.4. Cell Fusion

Internalization of *Burkholderia pseudomallei* by host cells can induce cell fusion resulting in the formation of multinucleated giant cell (MNGC). The underlying mechanisms of MNGC formation are not completely understood but one hypothesis is the ability of intracellular

bacteria to induce actin polymerization. Suparak and colleagues described the use of monoclonal antibodies to identify host surface proteins involved in this phenomenon (54). They found that the anti-CD98hc antibody MEM-108 (whose epitope covers C-ter residues 410–413, 547–549 and 571–575 (55)) suppresses the formation of infection-induced MNGC in a dose dependent manner in the macrophage-like U937 cells. Increased surface expression of CD98hc was also observed upon infection in these cells, suggesting that *B. pseudomallei* may up-regulate surface expression of CD98hc to promote cell-to-cell fusion. Importantly, the MEM-108 antibody had no effect on the intracellular replication of *B. pseudomallei*, suggesting that CD98hc has no role in the multiplication of bacteria in these cells.

## 2.5. Other Mechanisms

Upregulation of CD98hc expression is a common feature in several bacterial infections. In a transcriptional analysis used to identify target host proteins during with *Neisseria meningitidis* infection, infected HBMEC (human brain microvascular endothelial cells) were found to express higher levels of CD98hc at 4 and 8 h post infection (56). In the same line, infection of human fallopian epithelial cell cultures by the obligate intracellular pathogen *Chlamydia trachomatis* resulted in increased release of several host proteins including CD98hc (SLC3A2) and the glutamate transporter SLC1A5 (57). Interestingly, transport of tryptophan depends on CD98hc (Table 1) and tryptophan starvation was shown to block *Chlamydia* intracellular replication (58). The exact impact or role of CD98hc in *Neisseria* or *Chlamydia* infections are still unknown, but these observations suggest that both pathogens could exploit amino acid transporters to obtain nutrients within infected cells.

## 3. Conclusions

The ubiquity and multiple functions of CD98hc (and its associated light chains) have allowed many viral, bacterial and protozoal pathogens to exploit it in different phases of their infectious cycle. During viral infections, several heterodimers containing CD98hc serve for virus attachment or internalization into host cells by fusion or endocytosis. The fusogenic properties of CD98hc can also be involved in cell-to-cell fusion of infected cells. During

bacterial infections, the host proteins can also participate in pathogen adherence and infection-induced cell-to-cell fusion. In many instances, expression of the genes encoding CD98hc or its associated light chains is modified upon infection. Increasing expression of HAT transporters at the surface of infected cells leads to increased AA import. This can result in enhanced proliferation of infected cells, but also inhibition of the autophagy-mediated antimicrobial defence mechanism via activation of mTOR signalling. In addition, modulating the function of the antiporter CD98hc/xCT, which controls GSH levels, affects the cellular antioxidant response. For pathogens, controlling oxidative stress is a powerful mechanism for promoting their replication by maintaining infected cells viability. Alternatively, infection-induced changes in GSH levels can be responsible for the cytopathic effect of some infections.

The involvement of CD98hc-containing heterodimers in a growing number of infectious processes draws new attention to these molecules, which are promising therapeutic targets in the context of infection.

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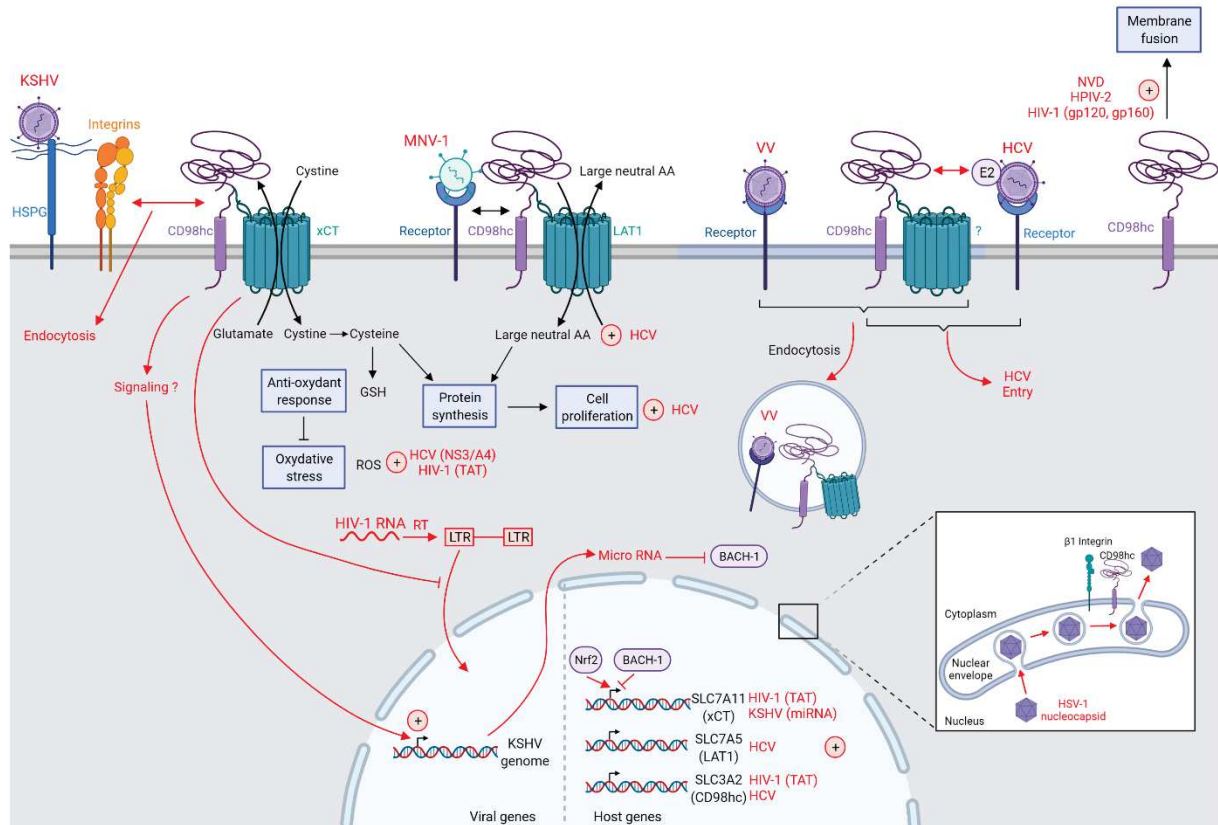
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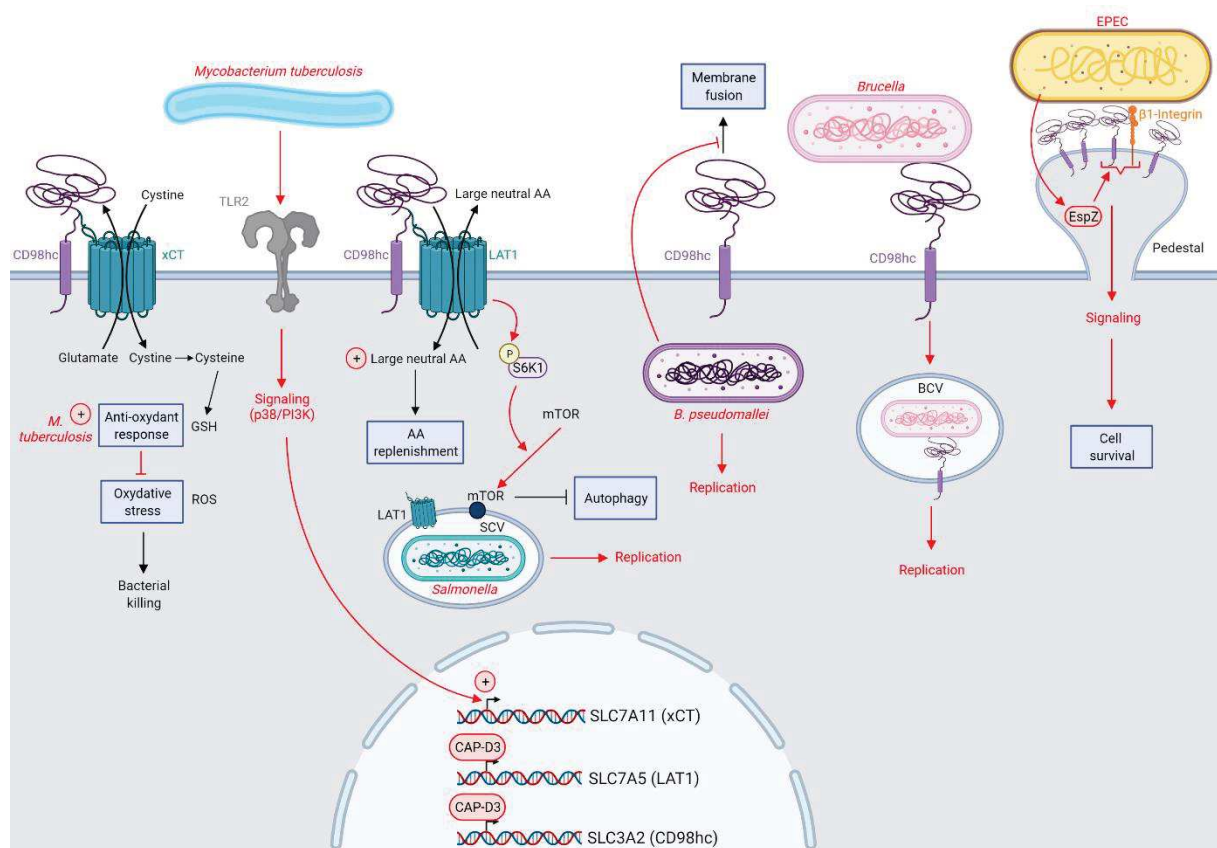
**Table 1: The HAT family of amino acid transporters.** Adapted from Nguyen & Merlin, 2012; Verrey et al., 2004.

Heavy chain	Light chain	System	Characteristics	Substrates
<b>CD98hc</b> (SLC3A2)	xCT (SLC7A11)	<b>xc<sup>-</sup></b>	Na <sup>+</sup> -independent	Cystine/L-glutamate exchanger
	LAT1 (SLC7A5)	<b>L</b>	Na <sup>+</sup> -independent	Large neutral AA (Leu, Ile, Phe, Met, Tyr, His, Trp, Val)
	LAT2 (SLC7A8)		Na <sup>+</sup> -independent	Small neutral AA (Gly, Ala, Ser, Thr, Asn, Gln, Met, Leu, Ile, Val, Phe, Tyr, Trp, His)
	y <sup>+</sup> LAT1 (SLC7A7)	<b>y<sup>+</sup>L</b>	Na <sup>+</sup> -dependent & -independent	Uptake of arginine, leucine and glutamine (Na-dependent); uptake of dibasic AA (Na-independent)
	y <sup>+</sup> LAT2 (SLC7A6)		Na <sup>+</sup> -dependent & -independent	Uptake of arginine, leucine and glutamine (Na-dependent); uptake of dibasic AA (Na-independent); arginine/glutamine exchanger (antiporter)
	Asc-1 (SLC7A10)	<b>asc</b>	Na <sup>+</sup> -independent	Small neutral L- and D-AA
rBAT (SLC3A1)	b <sup>0,+</sup> AT1 (SLC7A9)	<b>b(0,+)</b>	Na <sup>+</sup> -independent	Cysteine and neutral and dibasic AA

**Figure 1: Schematical representation of the roles of CD98hc in viral infections.** KSHV: Kaposi's sarcoma-associated herpes virus, HSPG: Heparan sulfate proteoglycan, MNV-1: Murine norovirus 1, VV: vaccinia virus, HCV: hepatitis C virus, NVD: Newcastle disease virus, HPIV-2: human parainfluenza virus 2, HIV-1: Human immunodeficiency virus 1, GSH: glutathione, RT: reverse transcription, HSV-1: herpes simplex virus 1, ROS: Reactive oxygen species.



**Figure 2: Schematical representation of the roles of CD98hc in bacterial infections.** GSH: glutathione, ROS: reactive oxygen species, AA: amino acids, SVC: *Salmonella* containing vacuole, BCV: *Brucella* containing vacuole, EPEC: enteropathogenic *Escherichia coli*, TLR2: Toll-like receptor 2.







# Aims

## Aims

Our group has been interested in *Brucella* infections for a while. Besides the studies on the pathogen itself, our research also focused on the host factors that are important for infection by these intracellular bacteria. Using a yeast two hybrid screening, the eukaryotic protein CD98hc was found to interact with the VirB2 protein, the major component of the VirB pilus, a component of the type 4 secretion system (Keriel et al., 2015). Knocking down CD98hc in Hela cells significantly lowers the infection rate in these cells. The role of CD98hc in *Brucella* infection was confirmed in murine derm fibroblasts, in which the knockout of CD98hc drastically decreased the number of intracellular bacteria compared to WT cells. Furthermore, CD98hc seems to be important for both entry and intracellular multiplication of *Brucella* in these cells.

*Brucella* are able to infect different cell types including placental cells (Salcedo et al., 2013) and we showed that infection affects several trophoblastic functions in which CD98hc is implicated (García-Méndez et al., 2019b). Preliminary experiments showed that RNAi-mediated knock down of CD98hc in human trophoblastic cell lines (BeWo and JEG-3 cells) inhibits *Brucella* infection in these cells (K. B. García-Méndez, unpublished results). Taken together, these results suggest that, as in epithelial cells or fibroblasts, CD98hc may be important for *Brucella* infection in human trophoblasts.

The aim of my thesis was to evaluate the importance of CD98hc during *Brucella* infection of human trophoblasts. To do so, I first tried inactivating the gene encoding CD98hc in human trophoblasts using the CRISPR/Cas9 system. This part was very challenging since this gene is considered essential because its inactivation is lethal very early during development in mice (Tsumura et al., 2005). I thus developed in parallel a system for conditional inactivation of this gene based on doxycycline-controlled expression of the Cas9 endonuclease.

The other goal of my thesis was to understand which cellular function of CD98hc is necessary for *Brucella* during infection. To answer this question, I used an inhibitor-based approach targeting selectively one of the multiple functions of CD98hc, *i.e.* the transport of AA transport at the plasma membrane or the modulation of integrin-mediated intracellular signaling.

AIMS

Lastly, I used a drug repositioning strategy to evaluate the effect of statins and Gefitinib on *Brucella* infection *in vitro* and *in vivo*.



# Results



Part I.

Importance of CD98hc in  
*Brucella* infection of human  
trophoblasts



## Part I. Importance of CD98hc in *Brucella* infection of human trophoblasts

### 1. Introduction

The first evidence of a link between the eukaryotic protein CD98hc and *Brucella* was discovered upon a yeast two-hybrid (Y2H) screen performed in our group. Y2H is based on the reconstitution of a functional transcription factor when two proteins of interest physically interact. This takes place in genetically engineered yeasts in which the transcription of a reporter gene leads to a specific phenotype. VirB2, the main component of *Brucella* T4SS, was used as a bait. Several host proteins were found to interact with VirB2, including CD98hc. This protein was then shown to be required for optimal *Brucella* infection in HeLa cells and murine derm fibroblasts (Keriel et al., 2015).

CD98hc plays key roles in human placental cells functions, notably in the migration capacity of EVT's and the homotypic fusion of CTBs. We observed that *Brucella* infections affect several functions of these trophoblasts (hormone production, fusion of CTB and invasion of EVT) (García-Méndez et al., 2019b). Moreover, even if RNAi experiments only partially knocked-down CD98hc expression in these trophoblastic cell lines (BeWo and JEG-3 cells), this was sufficient to observe a mild but significant inhibition of *Brucella* infection (K. B. García-Méndez, unpublished results). These observations prompted us to evaluate the importance of CD98hc during *Brucella* infection of human trophoblasts.

The straightest forward strategy to assess the role of a protein in a cellular process is to inactivate its encoding gene. An efficient and specific tool to inactivate genes is the CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas (CRISPR-associated) systems. Present in archaea and most bacteria, these systems provide sequence-specific protection against foreign DNA (Sontheimer & Marraffini, 2010). The finding that one of these systems uses a specific family of endonucleases, Cas9, that can be programmed with single RNA molecules to cleave specific DNA sites revolutionized genetic engineering (Jinek et al., 2012). In this chapter, we used the CRIPR/Cas9 system to try knocking out (KO) expression of CD98hc in human trophoblasts in order to evaluate its importance during *Brucella* infection in these cells. However, because deletion of the gene *SLC3A2* (encoding for CD98hc) is embryonically lethal in mice (Tsumura et al., 2005), this gene is considered as essential for cell viability and its KO was very challenging. We

## 2. RESULTS

therefore launched two strategies in parallel: the constitutive inactivation of *SLC3A2* and its conditional inactivation.

## 2. Results

Part of this work is described in a manuscript in preparation.



**Relocalization of the host protein CD98hc in recycling endosomes  
modulates FAK pathway and is compatible with *Brucella* infection in  
cytotrophoblasts**

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## Abstract

CD98hc is a type II surface glycoprotein with multiple molecular and biological functions, notably in placental trophoblasts. This host protein was also recently involved in the pathogenesis of several viruses and bacteria, including the zoonotic intracellular bacteria *Brucella*. Because *Brucella* shows a tropism for placental cells in both animals and humans, we tried inactivating the gene coding for CD98hc in human cytotrophoblastic (BeWo) cells in order to evaluate its role during *Brucella* infection in these cells. Despite several attempts, we could only obtain a single cellular clone which we believed was negative for CD98hc expression. However, transcripts analyses and the use of several antibodies with different epitopes revealed that these cells express a modified CD98hc. This protein is shorter, has an altered glycosylation pattern and is trapped in recycling endosomes. Such modifications are however compatible with *Brucella* entry and intracellular replication in these cells. Understanding which function(s) is(are) retained by this modified protein could thus provide insights into the role of CD98hc during *Brucella* infection. This study also highlights the importance of a deep molecular characterization when trying to inactivate essential genes.

## Introduction

Bacteria of the *Brucella* genus infect a wide range of mammals and cause brucellosis. In animals, the main symptoms of infection are spontaneous abortion in females and epididymitis and sterility in males. This zoonosis can be transmitted to humans by consumption of unpasteurized milk products, direct contact with infected tissues or inhalation of aerosols (Byndloss & Tsolis, 2016). The disease presents as a flu-like syndrome with a characteristic undulant fever. If left untreated, brucellosis can cause serious complications such as neurobrucellosis or endocarditis. Recommended treatment for human brucellosis is a combination of doxycycline and rifampicin for 6 weeks. With more than 500,000 new human cases reported each year and its impact on animal health, brucellosis is considered as the cause of huge economical losses worldwide (Pappas et al., 2005).

*Brucella* are able to infect a variety of cell types including placental trophoblasts (Salcedo et al., 2013). They replicate inside these cells and alter the fusion capacity of cytotrophoblasts (CTB) and the migration of extravillous trophoblasts (García-Méndez et al., 2019b). One key host protein in these cellular processes is CD98hc (aka 4F2, or FRP-1) (Kudo et al., 2003; Kudo & Boyd, 2004). This type II membrane glycoprotein is part of a heterodimer composed of two subunits: a glycosylated 80kDa heavy chain (CD98hc) and a 40kDa light chain, which are linked by a disulfide bridge (Haynes et al., 1981b). Association of CD98hc with 6 different light chains (LAT1, LAT-2,  $\gamma^+$ LAT1,  $\gamma^+$ LAT-2, xCT and asc-1) results in the formation of amino acid transporters with different substrate specificity. On its own CD98hc, and especially its intracellular and transmembrane domains, modulates integrin signaling (Feral et al., 2005; Fenczik et al., 2000). Also, CD98hc and its light chains are important for different steps of the life cycle of several pathogen (Vection *et al.*, manuscript in preparation).

Previous studies using RNAi in HeLa cells or murine derm fibroblasts with a CD98hc knockout showed that this host protein is important for *Brucella* infection (Keriel et al., 2015). Given the tropism of *Brucella* for placental cells and the importance of CD98hc in trophoblasts functions, we wanted to determine the importance of CD98hc for *Brucella* infection in human CTB. We tried inactivating the gene encoding CD98hc in BeWo cells using the CRISPR/Cas9 technology but could only obtain one cellular clone. These cells express a shorter version of CD98hc with a modified C-terminal extracellular domain and altered glycosylation pattern. This modified protein also has an altered sub-localization pattern and we showed that it is

trapped in recycling endosomes. These modifications are however compatible with *Brucella* infection as bacteria could both enter and replicate efficiently inside these cells. These cells thus constitute a cellular model that could help understanding which function(s) of CD98hc is hijacked for *Brucella* infection. This study also highlights the importance of a detailed molecular characterization when trying to inactivate essential genes.

## Results

### **CD98hc gene knock-out is lethal for human cytotrophoblasts**

The CD98hc protein is encoded by the gene *SLC3A2* and exist as four different isoforms, which differ in their first four exons. To evaluate the importance of CD98hc in *Brucella* infection in human trophoblasts, we tried inactivating *SLC3A2* using the CRISPR/Cas9 technology in the cytotrophoblast-like BeWo cells. We used two different sgRNA molecules, which target the exon 7 or 10 respectively (Figure S1) and that had been previously validated experimentally (S. Wang et al., 2017). The sequences encoding Cas9 and the sgRNA molecule(s) (sgRNA2 alone, sgRNA3 alone or both sgRNA) were introduced in BeWo cells by lentiviral transduction (Figure S1). Importantly, the cell culture media was complemented with  $\beta$ -mercaptoethanol ( $\beta$ -ME) because CD98hc-deficient cells do not survive under standard culture conditions (Feral et al., 2005). Such cells cannot import cystine and have an intracellular AA imbalance (Ballina et al., 2016). Supplementation with  $\beta$ -ME allows reduction of extracellular cystine into free cysteine, which can thus be imported by CD98hc-independent transporters thus guaranteeing cell survival.

After selection with puromycin, three different heterogenous populations of resistant cells were obtained. However, immunolabelling using the monoclonal anti-CD98hc antibody MEM-108 (whose epitope involves the C-ter residues 410–413, 547–549 and 571–575 (Lee et al., 2019) revealed that only a minority (<12%) of these cells were negative for CD98hc (data not shown). After labelling with the same antibody, CD98hc-negative cells were then sorted by flow cytometry and cellular cloning was performed to try isolating CD98hc KO BeWo cells. Several attempts were unsuccessful, suggesting that knocking out the *SLC3A2* gene is lethal for human cytotrophoblasts.

### **A cellular clone with modifications in the *SLC3A2* gene expresses a CD98hc protein with altered sub-cellular localization**

A single cellular clone, obtained after transduction of BeWo cells with the sgRNA2 (targeting exon 7), could however survive and be amplified. These cells, thereafter named “clone 3 cells”, were further characterized. They had no apparent morphological difference compared to wild-type (WT) BeWo cells but presented a growth delay (Figure S2). Moreover,



these cells are not viable if their culture media is not complemented with  $\beta$ -ME (data not shown).

Clone 3 cells seemed negative for CD98hc expression when using the MEM-108 antibody (Figure 1). However, the anti-CD98hc antibodies HBJ126 (whose epitope encompasses the C-ter residues 476–483 and 497–501 (Lee et al., 2019) and C290970 (whose epitope is in the N-ter residues of the longest isoforms) revealed that clone 3 cells do express CD98hc. This suggested that clone 3 cells express a CD98hc protein with modifications in its C-ter domain that alter or mask the epitope of the antibody MEM-108. Moreover, the sub-cellular localization of this modified protein is altered: CD98hc localizes at the plasma membrane in WT cells while it seems strictly intracellular in clone 3 cells (Figure 1C). This was confirmed by flow cytometry analyses, in which the CD98hc protein expressed in clone 3 cells was detected by the antibodies HBJ126 and C290970 in permeabilized cells but not on live cells (Figure S3).

### **BeWo clone 3 trophoblasts express of C-ter modified CD98hc protein with altered glycosylation pattern**

We then analyzed the genome of clone 3 cells to understand the genetic modifications generated by the CRISPR/Cas9 system in these cells. The genomic region theoretically targeted by sgRNA2 (*i.e.* exon 7 of *SLC3A2*) was amplified by PCR. Two different bands were obtained for clone 3 cells (Figure 2A) suggesting that the two *SLC3A2* alleles are different in these cells. Both PCR fragments were then sub-cloned into a pUC29 vector for sequencing. This revealed that both alleles had indeed been modified by the CRISPR/Cas9 system within the exon 7; this resulted in the deletion of the whole exon in one allele and only few nucleotides modification in the other. These modifications would theoretically result in the expression of a CD98hc protein missing a whole alpha helix in its C-ter domain, or substitutions of 3 residues, respectively. Expression of a modified CD98hc was then confirmed by qRT-PCR, which showed that messenger RNAs encoding all isoforms of CD98hc are expressed in clone 3 cells (Figure 2B). However, the PCR product encompassing the exon 7 (targeted by sgRNA2) seems shorter in these cells. Western Blot confirmed that the CD98hc protein expressed in BeWo-clone 3 cells is smaller (Figure 2C) and proved that all isoforms were affected by CRISPR/Cas-mediated modification in the *SLC3A2* gene. Moreover, this shows that the modified CD98hc proteins expressed in BeWo-clone 3 cells has an altered glycosylation pattern compared to the WT protein.

### **The modified CD98hc protein expressed in clone 3 cells is trapped in recycling endosomes**

The punctuated signal observed in clone 3 cells (Figure 1C) suggested that the modified CD98hc protein could accumulate in intracellular vacuoles. To identify these vacuoles, we performed *in situ* immunofluorescence staining using different antibodies to selectively label several intracellular compartments. We found no colocalization of CD98hc with LAMP1 (lysosomal marker), VPS35 (marker of the VPS retromer), BET1L (Golgi vesicular membrane marker) or Rab4 (recycling endosome marker) (Figure S4). To the opposite, CD98hc associates with the recycling endosome marker Rab11A in clone 3 cells (Figure 3A). We also monitored the sub-cellular localization of Galectin-3 (Gal-3), a physiological ligand of CD98hc (Dalton et al., 2007). Gal-3 is also considered as a marker of recycling endosomes as this lectin re-enters the cell by non-clathrin mediated endocytosis (Hönig et al., 2015). While Gal-3 was localized both at the plasma membrane and in the cytoplasm in WT BeWo cells, as described previously (Dalton et al., 2007b), this glycoprotein did not show any surface expression in clone 3 cells (Figure 3C). Moreover, a larger fraction of CD98hc colocalized with Gal-3 in clone 3 cells compared to WT cells (Figure 3D). Taken together, these results indicate that the modified CD98hc protein expressed in clone 3 cells accumulates in recycling endosomes.

### **BeWo clone 3 trophoblasts have altered AA transport and FAK intracellular signaling**

CD98hc is involved in the transport of AA at the plasma membrane through its C-ter ectodomain, whereas its N-ter domain is rather involved in its function as a modulator of integrin-signaling (Feral et al., 2005; Fenczik et al., 2000). We thus hypothesized that the modifications in the C-ter of the CD98hc protein expressed in clone 3 cells could affect the AA transport in these cells. This would be consistent with their slower growth rate (Figure S2) and the absolute requirement of  $\beta$ -ME in their culture media. We thus measured the expression of AA transporters by qRT-PCR in BeWo cells. To have an overview of AA transport in these cells, we measured expression of members of the heterodimeric AA transporters (HAT), *i.e.*, several light chains associating with CD98hc as well as rBAT, the other heavy chain of this family, but also cationic amino acid transporters (CATs). We found that clone 3 cells slightly overexpress three CD98hc-associated light chains (LAT2, xCT and  $\gamma$ +LAT2) (Figure 4), suggesting that they have an altered AA transport.

Since the intracellular localization of CD98hc in clone 3 cells could also affect its ability to modulate integrin mediated signaling in these cells, we first assessed the surface expression of different integrins on BeWo cells and found no difference between WT and clone 3 cells (Figure S5). We next evaluated the effect of selectively inhibiting the FAK kinase, a key molecule in integrin signaling and cytoskeleton remodeling through actin polymerization. Inhibition of FAK normally leads to retraction of cell membrane protrusions, resulting in the formation of actin-based stress fibers (Hsia et al., 2003). BeWo cells were treated with FAK inhibitor 14 and the effect on cytoskeleton was monitored by F-actin labelling. Inhibiting FAK induced the formation of stress fibers in WT cells (Figure 5A). Surprisingly, stress fibers were observed in clone 3 cells even in the absence of FAK inhibitor. In addition, inhibiting FAK decreased adherence to glass coverslips in WT but not in clone 3 cells (data not shown). To further decipher the differences in the integrin pathway between WT and clone 3 cells, we assessed the activation of RhoA, a small GTPase downstream of FAK. BeWo WT and clone 3 cells were incubated with fibronectin coated Dynabeads, stimulated with a magnet and the amount of active RhoA (GTP-RhoA) was monitored by Western Blot. Stimulation of WT cells resulted in increased amount of GTP-RhoA compared to non-stimulated cells (Figure 5B). In clone 3 cells however, the level GTP-RhoA was higher than in WT cells and did not increase further after stimulation, suggesting that RhoA is constitutively activated in these cells.

### **CD98hc-dependant alterations in clone 3 cells are compatible with their infection by *Brucella***

We next evaluated whether the modifications in CD98hc-mediated cellular functions in clone 3 cells affect *Brucella* infection in these cells. Cells were infected with fluorescent *B. melitensis* and the number of intracellular bacteria was quantified by confocal microscopy. Two hours post-infection (hpi), clone 3 cells seemed to contain more bacteria than WT cells (Figure 6) suggesting that *Brucella* entry is more efficient in these cells. *Brucella* could replicate inside both cell lines, as shown by higher numbers of intracellular bacteria 20 hpi compared to 2 hpi in both cases. Clone 3 cells seemed however to contain more bacteria compared to WT cells (Figure 6B). Yet, no major differences in either entry or intracellular replication rates were observed when quantifying live intracellular *Brucella* by CFU counting (Figure 6C).

The FAK protein being a central player in actin cytoskeleton remodeling, which is essential for the entry of most pathogens, we also evaluated its importance in *Brucella* entry.

Vection *et al.*, manuscript in preparation

BeWo cells were thus treated with FAK inhibitor 14 prior to infection. This treatment significantly inhibited *Brucella* entry in BeWo WT (Figure 6C), showing that the FAK pathway is important for *Brucella* entry. On the contrary, FAK inhibition had no effect on *Brucella* infection in clone 3 cells, confirming that the FAK pathway is constitutively activated in these cells.

## Discussion

Inactivation of the gene *SLC3A2* is lethal early during development in mice (Tsumura et al., 2005), demonstrating that CD98hc is an essential protein. We thus anticipated that knocking out this gene in human cytotrophoblasts could be very challenging. Indeed, we have not been able to deplete BeWo cells from CD98hc using a constitutive CRISPR/Cas9 strategy. Several attempts resulted in obtaining only four cellular clones. All of them had no detectable expression of CD98hc using an antibody commonly used to detect this protein (MEM-108) but were positive with other anti-CD98hc antibodies. Molecular characterization of one of these clones (clone 3) showed that these cells express a CD98hc with modifications in its C-ter domain that probably alter the epitope for the MEM-108 antibody. This highlights the importance of an exhaustive molecular characterization when attempting to inactivate essential genes in eukaryotic cells.

Genetic modifications of *SLC3A2* in clone 3 cells resulted in the expression of a shorter CD98hc protein with altered glycosylation pattern and sub-cellular localization (retention in recycling endosomes). The strict dependency of clone 3 cells on the presence of  $\beta$ -ME in their culture media suggest that these cells have a defect in AA transport. CD98hc acts as a chaperone for addressing its associated AA transporter light chains to the plasma membrane (Mastroberardino et al., 1998). It would thus be interesting to verify whether its intracellular retention CD98hc in clone 3 cells also affect the localization of its associated light chains. We also observed that some integrin-mediated signaling pathways are altered in clone 3 cells. Inhibiting FAK had no effect on their morphology and the GTPase RhoA, a downstream mediator of FAK signaling, seems constitutively activated in these cells. RhoA is normally anchored in the plasma and shuttles between its inactive form (GDP-bound) and its active form (GTP-bound). In clone 3 cells we hypothesized that RhoA shuttling could be altered because of a difference in membrane composition.

Despite these phenotypes *Brucella* could efficiently infect clone 3 cells, suggesting that these cells maintain the CD98hc-mediated functions required to sustain intracellular replication of these bacteria in human trophoblasts. In fact, we observed a higher number of *Brucella* inside clone 3 cells early after infection compared to WT cells. This suggests that bacteria could enter these cells using a different or an additional mechanism. However, this did not translate into increased live intracellular bacteria in clone 3 cells, meaning that this

additional entry route would not be compatible with bacterial survival inside cells. We also found that FAK is important for *Brucella* optimal entry in WT cells, but not in clone 3 cells. We thus propose a working hypothesis represented Figure 7. In this model, CD98hc is localized at the cell surface in wild type BeWo cells, together with its partners  $\beta$ 1 integrins and Galectin-3. In this configuration, when integrins are activated their downstream signaling pathways are triggered, notably via the kinase FAK and its downstream mediator RhoA. The complex is then rapidly internalized in endosomes to allow recycling of the different proteins back to the cell surface, therefore ending integrin-mediated signaling. In this context, *Brucella* are able to enter and replicate intracellularly. Whether *Brucella* actively plays a role in the activation of integrin or rather opportunistically takes advantage of this situation remains to be answered. The situation seems different in clone 3 cells, in which the modified CD98hc protein seems to be sequestered in Rab-11 positive recycling endosomes (alongside Galectin-3). One hypothesis is that  $\beta$ 1 integrins could be co-sequestered with CD98hc. The absence of recycling back to the surface could thus result in sustained integrin signaling and RhoA activation. It is also possible that the lipid composition of clone 3 cells plasma membrane is altered, as observed in CD98hc KO fibroblasts (Boulter et al., 2018). Changes in cholesterol and sphingolipid levels could either mechanically modify the stiffness of the membrane or alter the formation of lipids rafts, both resulting in higher *Brucella* entry in clone 3 cells by a mechanism that remains to be elucidated. These working hypotheses need of course to be challenged experimentally, and BeWo clone 3 cells represent a useful tool to evaluate the role of CD98hc in *Brucella* infection in human trophoblasts.

## Materials and Methods

### *Cell culture*

Human trophoblasts (BeWo cells) were cultured in DMEM/F-12, GlutaMAX™ (Gibco) media supplemented with 10% heat inactivated fetal bovine serum (FBS).  $\beta$ -mercaptoethanol (0.1mM) was added in the media for the maintenance of clone 3 cells. Cells were incubated at 37°C and 5% CO<sub>2</sub>.

### *Gene inactivation by CRISPR/Cas*

The lentiviral constructs encoding the CRISPR/Cas9 system that target the human gene *SLC3A2* were purchased from GenScript. These plasmids were co-transfected in HEK293T cells with the packaging plasmids pCSIG (env) and pCMV $\Delta$ R8.91 (gagpol) using Lipofectamine 2000 (Invitrogen). Lentiviral particles were recovered in the supernatant of transfected cells after 48h, filtered (0.44 $\mu$ m filters) and applied to BeWo cells ON. After selection (1.25 $\mu$ g/mL puromycin for 5 weeks) and amplification, live cells were stained with the anti-CD98hc antibody MEM-108 coupled to phycoerythrin (ExBio) and sorted by flow cytometry (BD FACSAria II Flow Cytometer). CD98hc negative cells were collected, subjected to cell cloning by limiting dilution in 96-wells plates and maintained in culture in media supplemented with 20% FBS, 1.25 $\mu$ g/mL of puromycin and 0.1mM  $\beta$ -mercaptoethanol. Clone 3 cells were derived from an individual cellular clone.

### *Flow cytometry*

BeWo cells (1x10<sup>5</sup> cells) were grown over-night (ON), detached using trypsin-EDTA (Gibco), fixed with paraformaldehyde (3% PFA, 25 min, room temperature (RT)) and permeabilized (0.5% Triton X-100, 15 min, RT). Cells were then stained using several anti-CD98hc antibodies (MEM-108 from ExBio, HBJ127 from CliniSciences or C290970 from LS-Bio) and an anti-mouse-TexasRed (Vector Labs) or an anti-rabbit-TexasRed (Vector Labs). Fluorescence was analyzed using the MACS Quant VYB flow cytometer (Miltenyi Biotec).

### *Infection and quantification of intracellular replication*

When stated, BeWo WT and clone-3 cells (1x10<sup>5</sup> cells per well in 24-well plates) were treated ON with 2 $\mu$ M of FAK inhibitor 14 (SML0837, Sigma Aldrich) and washed twice with

PBS before infection with *Brucella melitensis*. BeWo cells were infected by *Brucella melitensis* expressing mCherry with a MOI of 500 (bacteria per cell) using a gentamycin protection assay. After centrifugation (400g, 10 min, RT), infected cells were incubated at 37°C in a 5% CO<sub>2</sub> atmosphere for 30 min before washes with phosphate-buffered saline and addition of 100 µg/mL gentamycin for 1.5h to kill extracellular bacteria. The media was then replaced by fresh media containing 20 µg/mL gentamycin. At 2, 5, 24, or 48h post infection (hpi), intracellular bacterial numbers were determined by lysing the cells with 0.1% Triton X-100. Serial dilutions were plated on TS agar to enumerate colony forming units (CFU).

#### *In situ immunolabelling*

BeWo cells (2x10<sup>5</sup> cells per well) were grown ON in 12-well plates containing sterile coverslips before fixation with PFA. To study the localization of CD98hc cells were fixed with 3% PFA, permeabilized with 0.5% Triton-X100 and stained with several anti-CD98hc antibodies (MEM-108 from ExBio, HBJ127 from CliniSciences or C290970 from LS-Bio) and an anti-mouse-TexasRed (Vector Labs) or an anti-rabbit-TexasRed (Vector Labs). To determine CD98hc subcellular localization cells were treated as mentioned above and stained with anti-CD98hc HBJ127 (CliniSciences) and either anti-Rab11A (sc-166912, SantaCruz) or anti-Galectin-3 (sc-53127, SantaCruz). CD98hc signal was revealed using a secondary antibody anti-rabbit-Alexa Fluor 594 (Invitrogen) and Rab11A as well as Galectin-3 signal was revealed using a secondary antibody anti-mouse-Alexa Fluor 488 (Invitrogen). To assess formation of stress fibers, f-actin was labelled using phalloidin coupled to Alexa 647 (A22287, Invitrogen). Nuclei were stained with 4,6-diamidino- 2-phenylindole (DAPI, 1 µg/ml) and the coverslips mounted using ProLongGold (Life Technologies). Slides were observed with the Olympus FV10C-O confocal microscope and images were treated with the IMARIS software version 9.5.1.

#### *PCR analyses*

To study the modifications induced by the CRISPR/Cas9 system, genomic DNA from BeWo WT or clone 3 cells was extracted using the QiAamp DNA mini kit (Qiagen). The region around exon 7 was then amplified by PCR using the following primers: forward 5'-TTGTAGTGCAGGTGGGAA-3' and reverse 5'-TTAGGCCTAGCACTCCACTG-3'. The amplification was performed over 35 cycles with an annealing temperature of 59°C. The PCR product was cloned into a pUC29 vector and sequenced.



Expression of the different AA transporters was studied using the following primers:

SLC3A1: F-GCTTCTGTGCTGGTGCTCATC; R-ACCAGTCTAGGCACTTTGGAGAGA

SLC7A2: F-ATGTGGGATGGGGTCCTGCC; R-CCCGGGCCATGGCAAACAGAA

SLC7A3: F-AAGCCTGAGCGGCCGAAGCTC; R-TGCTTGCCACGGCATCCTAGC

SLC7A6: F-CTTTCTACTTCATGGGTGTTTACCTG; R-ATCCTGAGTCTCCTATAGCTTACCAA

SLC7A11: F-TGCTGGGCTGATTTATCTTCG; R-GAAAGGGCAACCATGAAGAGG

LAT1: F-AGGAGCCTTCCTTTCTCCTG; R-CTGCAAACCTAAGGCAGAG

LAT2: F-ACCGAAACAACACCGAAAAG; R-GATTCCAGAGCCGATGATGT

LAT3: F-GCTGTGCTGGAGAACCTCTT; R-CTGAGCACGAAGGAACCAAT

LAT4: F-TCTCTCCGTGCTCATCTTCA; R-ATTCTGGAAAGGTGACTGC

SLC3A2: F-GAAGATCAAGGTGGCGGAAGACGAG; R-CCTTCAGAGAGCTCAGGTAATCGAG

SB34: F-GCATCAGTACCCATTCTATCAT; R-GGTGTAATCCGTCTCCACAGA

#### *Signaling assay*

To assess RhoA activation BeWo WT and clone 3 cells were grown to 90% confluence in 100mm plates. Cells were incubated with fibronectin coated Dynabeads for 20min prior to stimulation. Cells were stimulated or not with a magnet for 10min. Cell lysis was performed using RIPA-like buffer (50mM Tris, 200mM NaCl, 0.1% SDS, 1% Triton X-100, 0.5mM MgCl<sub>2</sub>) for 5 min. Lysate was collected and centrifuged at 10000rpm for 10min at 4°C. Samples were incubated with GST-Ras binding domain beads with rotation for 45min at 4°C. After washes with lysis buffer the beads were eluted with SDS-PAGE sample buffer. Bound RhoA was analyzed by Western blotting by using anti-RhoA antibody (26C4, Santa Cruz).

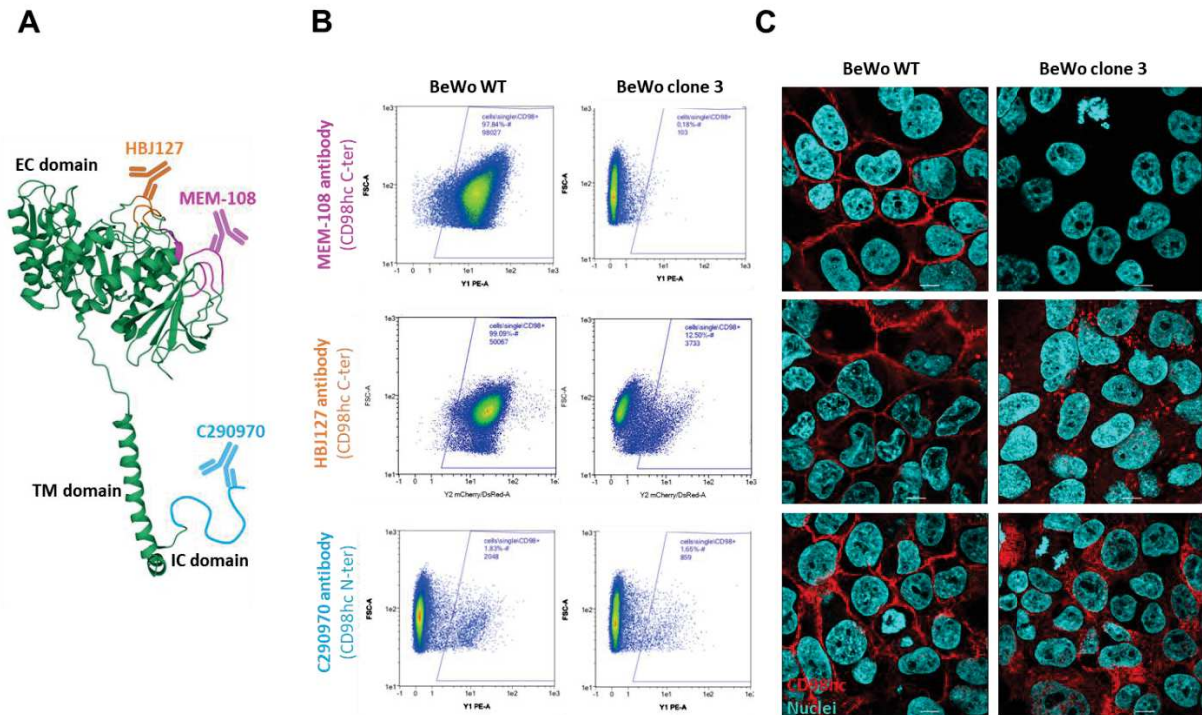
#### *Statistical analysis*

Statistical data analysis was done for CFU counts using a two-way ANOVA test with a Sidak correction for multiple comparisons, using the GraphPad Prism software version 7 or Windows, GraphPad Software, La Jolla California USA, [www.graphpad.com](http://www.graphpad.com).

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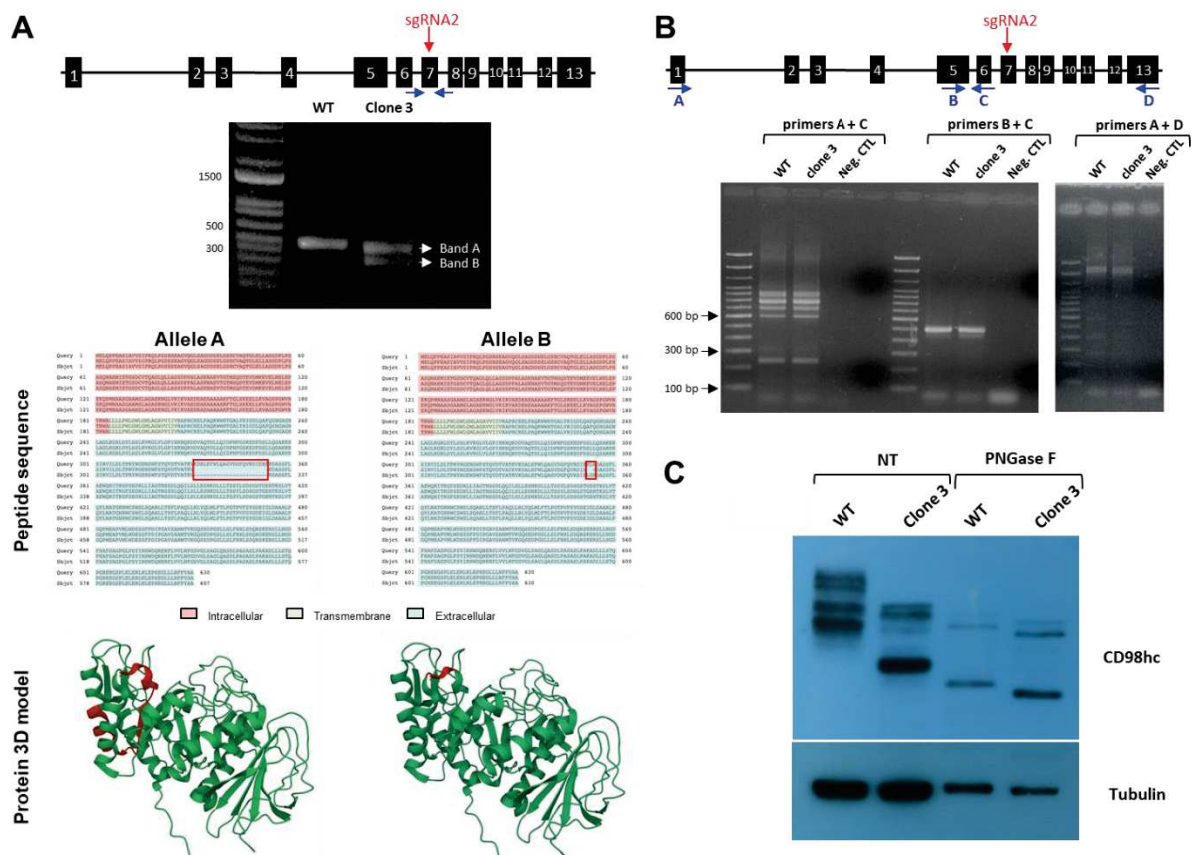
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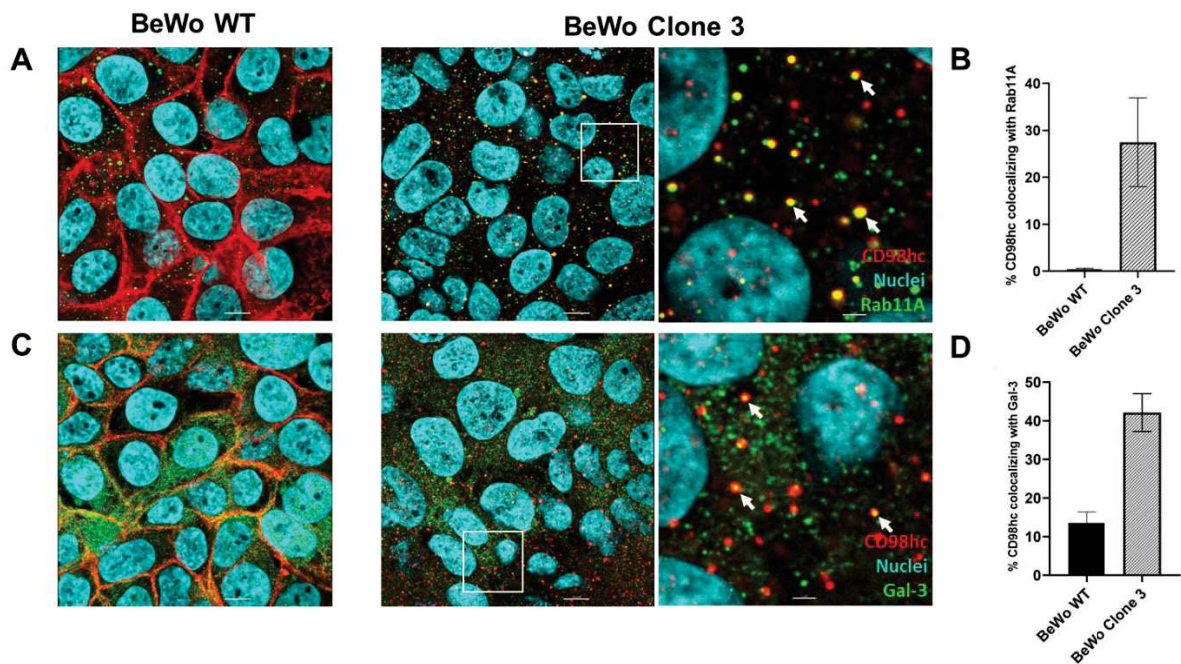
**Figure 1: BeWo clone 3 cells express a CD98hc protein with altered sub-cellular localization.**

**A)** 3D representation of CD98hc showing its extracellular (EC, Nter), transmembrane (TM) and intracellular (IC, Cter) domains. Immunolabelling of CD98hc was performed using the MEM-108 (C-ter, ExBio), the HBJ127 (C-ter, NeoBiotech) or the C290970 (N-ter, LSBio) antibody. The localization of their corresponding epitopes is shown on the 3D view according to a colour coding system. **B)** Live cells (without fixation or permeabilization) were stained with the indicated primary antibody and the corresponding secondary antibodies coupled to phycoerythrin or TexasRed and expression of CD98hc was monitored by flow-cytometry (MACSQuant VYB, Miltenyi). **C)** Cells were grown on glass coverslips, fixed with paraformaldehyde 3% and permeabilized with Triton X-100 before immunolabelling. Cells were then stained with the indicated primary antibodies and a secondary antibody coupled with an Alexa594 (red) and with DAPI (blue). Cells were observed by confocal microscopy and images were treated with the IMARIS software (version 9.5.1).

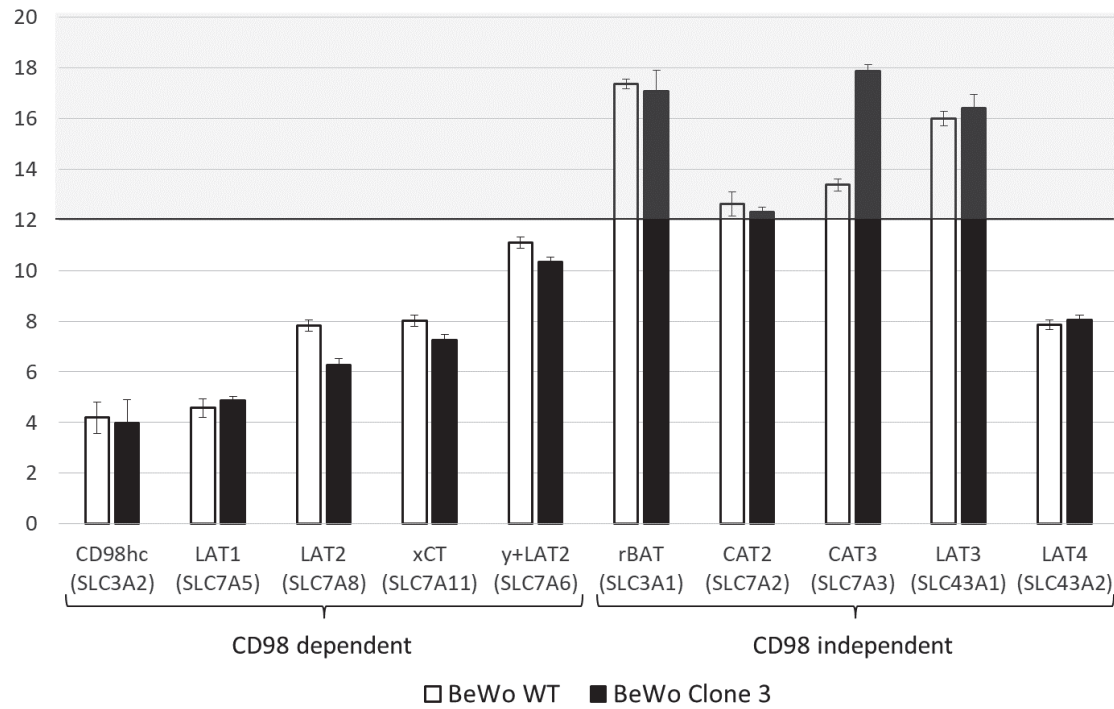


**Figure 2: BeWo clone 3 cells express a CD98hc protein with a modified C-terminal domain.**

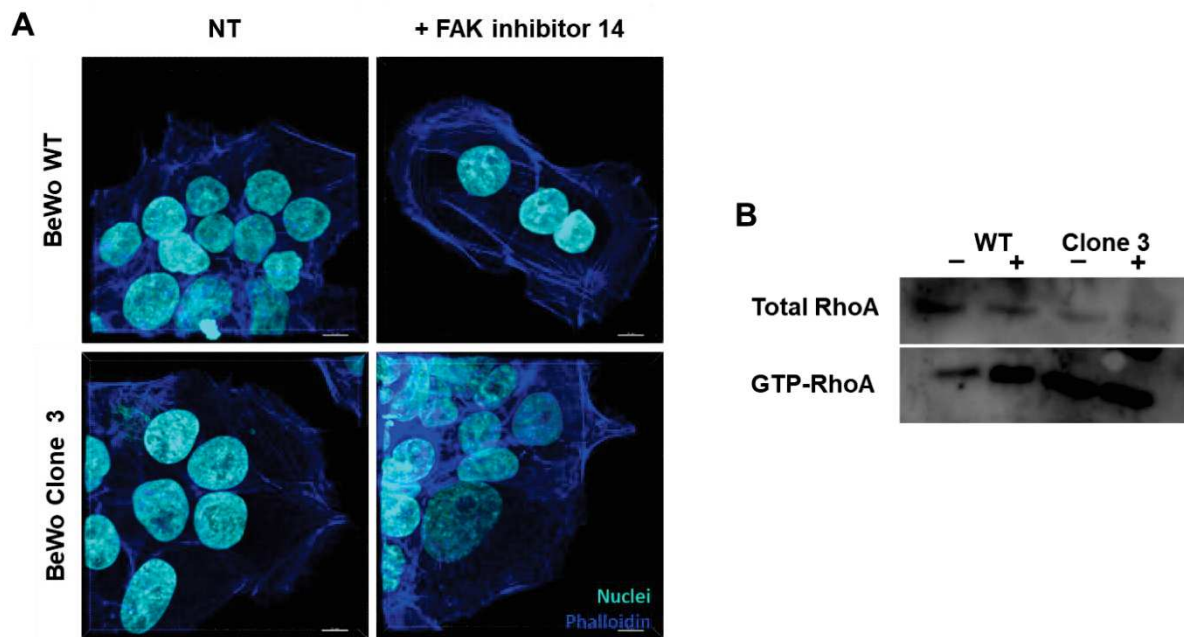
**A)** Exon 7 of *SLC3A2* was amplified by PCR on BeWo wild-type (WT) or clone 3 cells' genomic DNA and sequenced. The theoretical nucleotide sequences of the two transcripts, encoded by each allele, were extrapolated from these sequences, and aligned to the CD98hc reference sequence (NC\_000011.10 from NCBI). The theoretical proteins encoded by each allele were then translated *in silico* using the tool from ExPASy. Differences with the wild-type protein are shown as red boxes on sequences and on the 3D representation of the extracellular domain (PDB, 6JMQ). The residues constituting the intracellular, transmembrane, or extracellular domain are shown with a color code on the peptidic sequence. **B)** Expression of *SLC3A2* transcripts was evaluated by RT-PCR on cells' cDNA using different pairs of specific primers. On top, schematic representation of the human *SLC3A2* locus, showing the position of the primers used for PCR. **C)** Expression of CD98hc protein variants was evaluated by Western blot. Protein samples were submitted to PNGase F enzymatic deglycosylation according to the supplier's instructions. Total protein was resolved by SDS-PAGE on a 7.5% gel followed by transfer to a polyvinylidene difluoride membrane. The blot was immunostained using a rabbit anti-CD98hc antibody targeting the ectodomain (H300, Santa Cruz), or an anti-tubulin as a loading control.



**Figure 3: CD98hc localizes to recycling endosomes in clone 3 cells.** Cells were grown on glass coverslips, fixed with paraformaldehyde 3% and permeabilized with Triton X-100 before immunolabelling with the anti-CD98hc antibody HBJ127 in combination with either an anti-Rab11A (sc-166912, Santa Cruz) **(A)** or an anti-Galectin-3 (sc-53127, Santa Cruz) **(C)** antibody. CD98hc signal was revealed using a secondary antibody coupled to an Alexa 594 (red) whereas Rab11A and Galectin-3 signals were revealed using a secondary antibody coupled to an Alexa 488 (green). Cells were observed by confocal microscopy and images were treated with the IMARIS software (version 9.5.1). White arrows show colocalization of signals. Colocalization signal was quantified using the IMARIS software (version 9.5.1). Here is expressed the percentage of CD98hc signal colocalizing with either Rab11A **(B)** or Galectin-3 **(D)** in clone 3 cells.

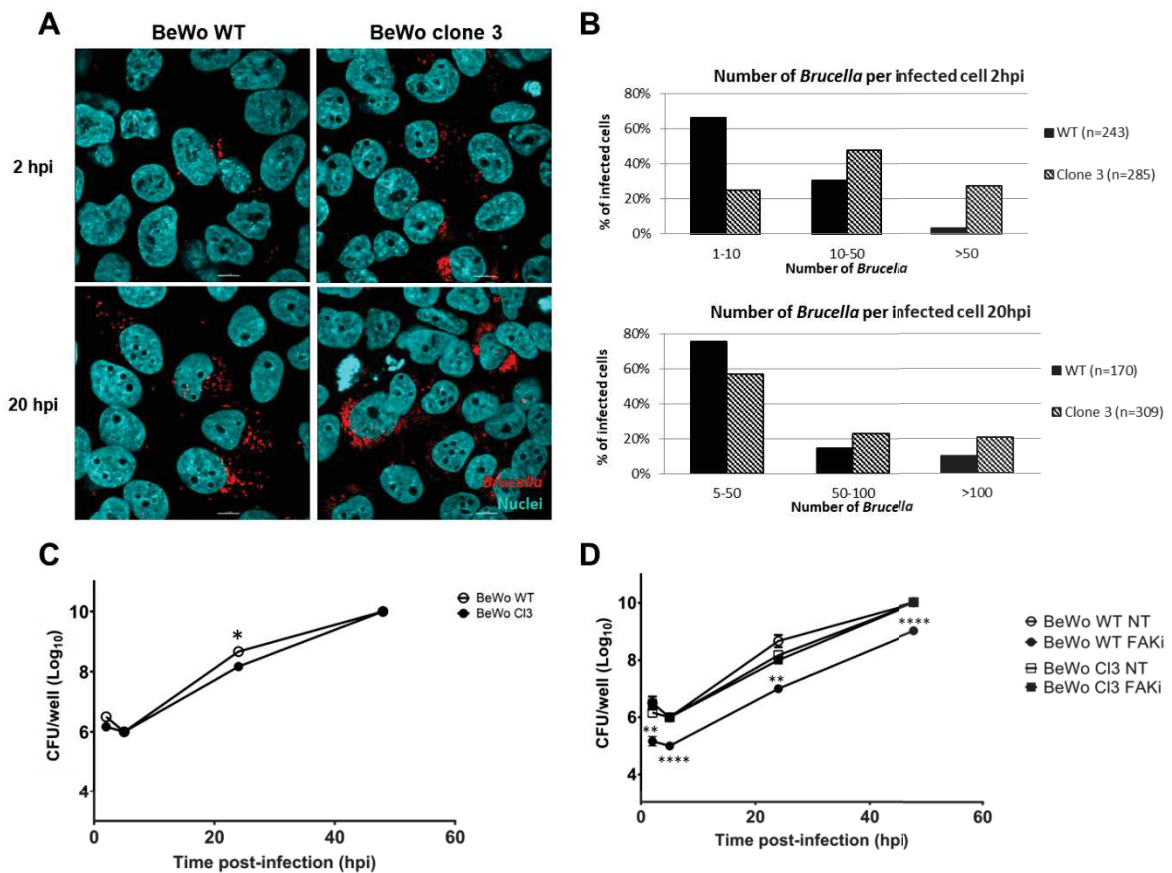


**Figure 4: Expression profile of amino-acid transporters in BeWo cells.** Quantification of different AA transporters expression by qRT-PCR in BeWo WT or clone 3 cells. Data shown are the  $\Delta$ CT regarding expression of housekeeping gene SB34. Amplification of CD98hc transcript was performed using primers B and C in Figure 2.

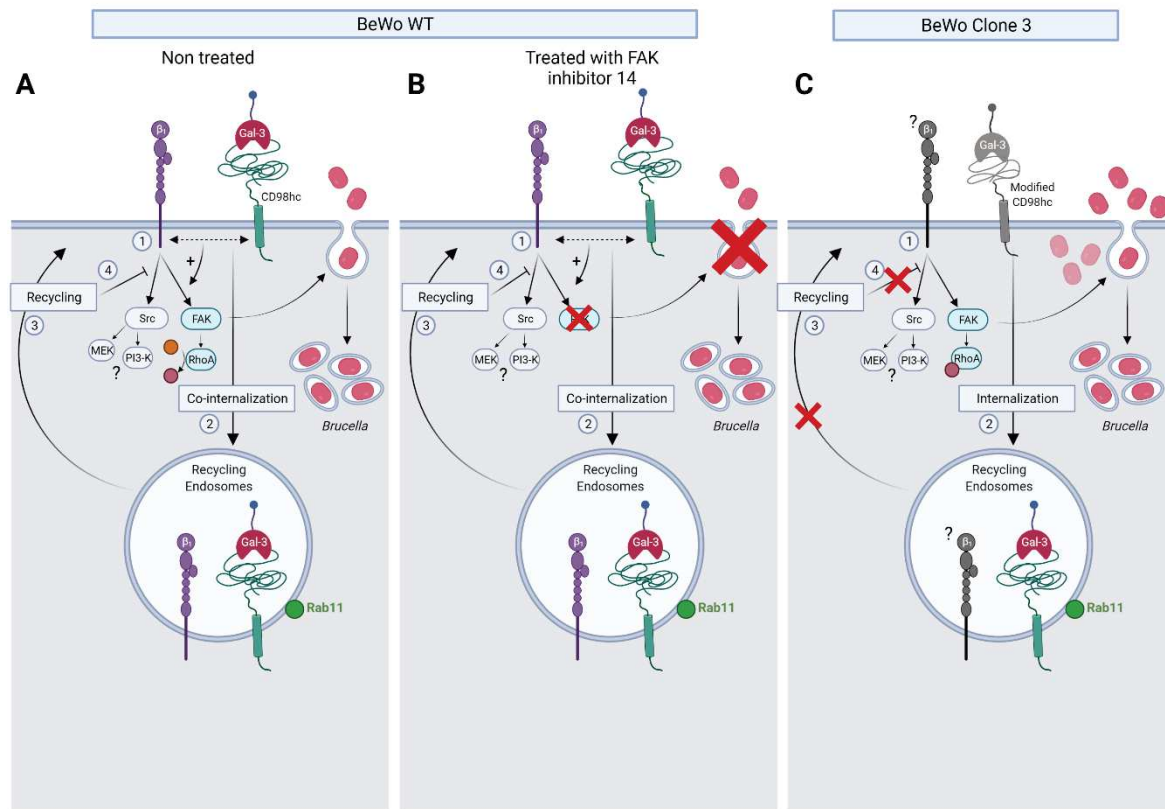


**Figure 5: The FAK/RhoA signaling pathway is latered in clone 3 cells. A)** BeWo cells (either WT or clone 3) were grown on glass coverslips, treated with FAK inhibitor 14 (2  $\mu$ M), fixed with paraformaldehyde 3% and permeabilized with Triton X-100 before staining with phalloidin coupled to Alexa 647 (dark blue) and DAPI (light blue). Cells were observed by confocal microscopy and images were treated with the IMARIS software (version 9.5.1). NT = non-treated **B)** Adhesion-dependent activation of RhoA. BeWo WT and clone 3 were incubated with Fibronectin-coated Dynabeads and stimulated (+) or not (-) with a magnet for 10 min. The RhoA bound to GST-Ras binding domain (GTP-RhoA) was compared with the total RhoA as detected by immunoblotting. +/- indicate whether cells were stimulated or not.

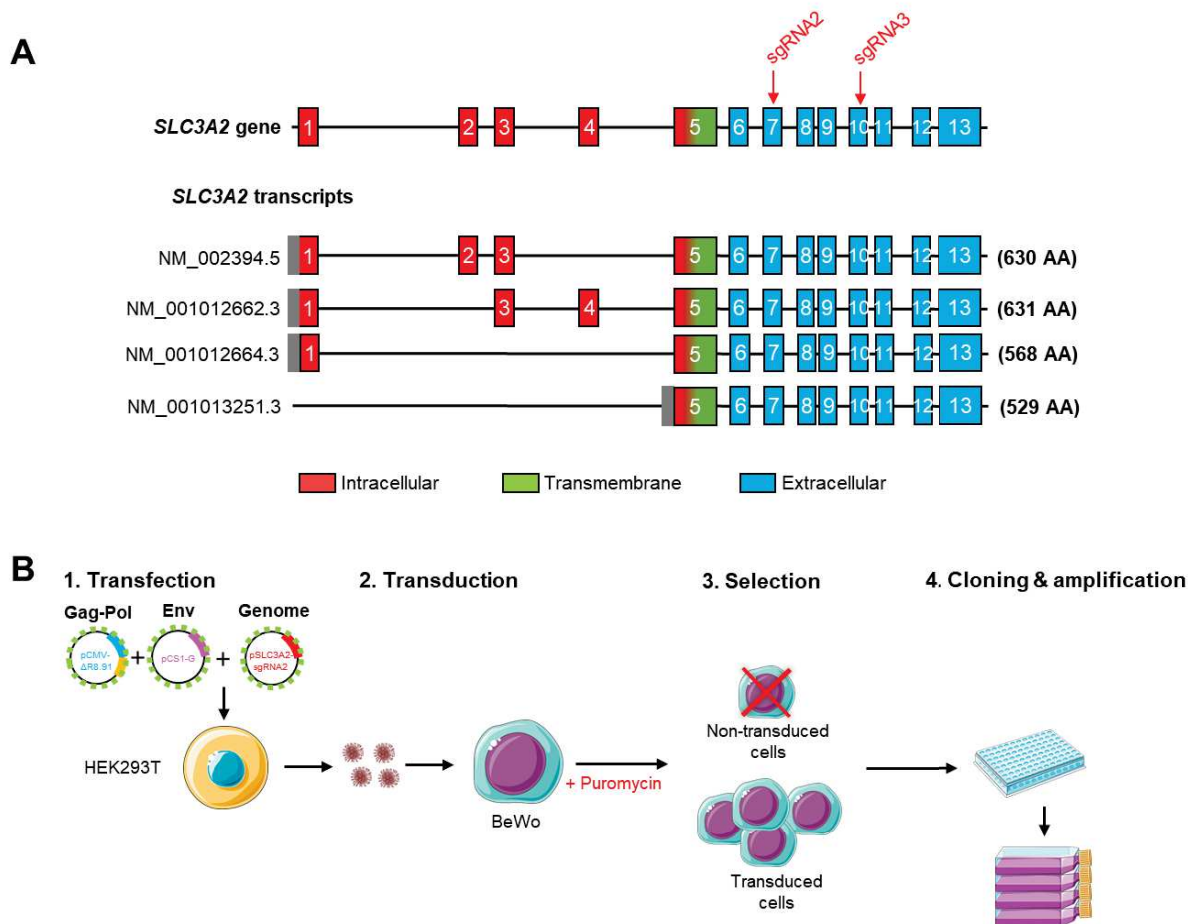




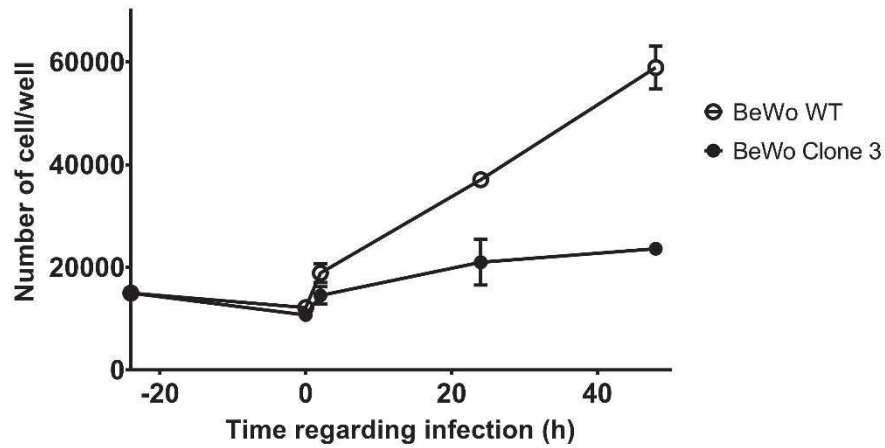
**Figure 6: Intracellular localization of a modified CD98hc is compatible with *Brucella* infection in human trophoblasts. A)** Cells were grown on glass coverslips and infected with *Brucella melitensis* encoding mCherry (red) using a gentamycin protection assay. Infected cells were fixed with paraformaldehyde 3% either at 2 or 20h post-infection. Cells were observed by confocal microscopy. **B)** Quantification of the number of intracellular bacteria performed semi-automatically using the IMARIS software (version 9.5.1). The percentage of infected cells is represented according to the number of bacteria per cell among the different ranges indicated. **C)** BeWo WT or clone 3 cells were infected with *B. melitensis* with an MOI of 500 using gentamycin protection assays. Cells were then lysed at the indicated hours post infection (hpi) for enumeration of intracellular colony forming units (CFU). The data shown are the geometric means of log<sub>10</sub> CFU per well  $\pm$  Standard Errors of Mean (SEM) from at least three independent experiments. **D)** Same as in C except that cell were pre-treated with FAK inhibitor 14 (2  $\mu$ M, overnight) before infection. NT =non-treated.



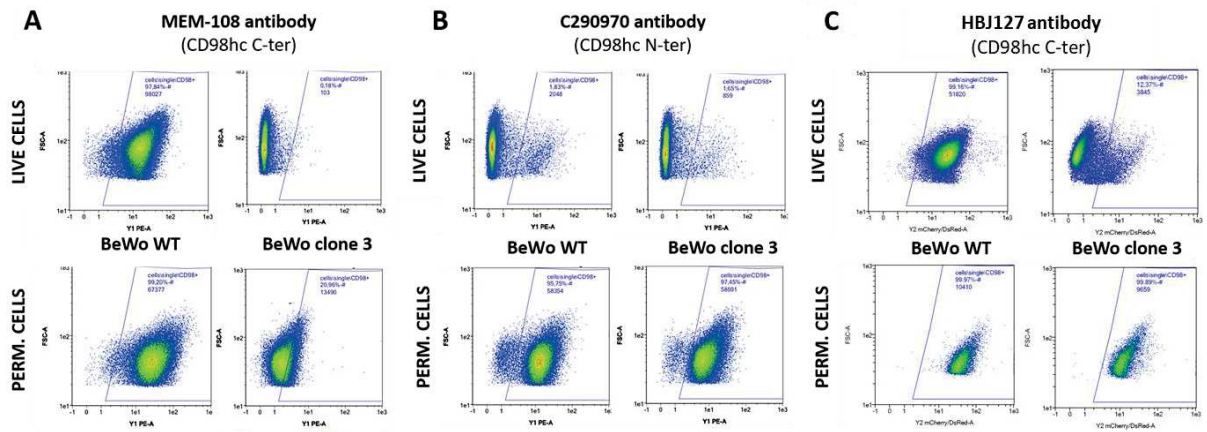
**Figure 7: Schematic representation of the molecular mechanisms that could support *Brucella* infection in wild type (A, B) or clone 3 (C) BeWo cells. A)** In WT cells, CD98hc and  $\beta 1$  integrins are localized at the cell surface. Activation of integrins, which is potentialized by their interaction with CD98hc, triggers downstream signaling pathways (1). The complex is then rapidly internalized in recycling endosomes (2) to allow recycling to the cell surface (3), therefore ending integrin-mediated signaling (4). In these cells, *Brucella* are able to enter and replicate intracellularly. **B)** In WT cells treated with FAK inhibitor 14, the FAK pathway downstream of integrin signaling is inhibited. This results in an inhibition of *Brucella* entry. **C)** In clone 3 cells, the modified CD98hc protein is sequestered in Rab-11 positive recycling endosomes (alongside Galectin-3). If  $\beta 1$  integrins would be co-sequestered with CD98hc, this would result in sustained integrin signaling and RhoA activation. This would result in higher *Brucella* entry by a mechanism that remains to be elucidated.



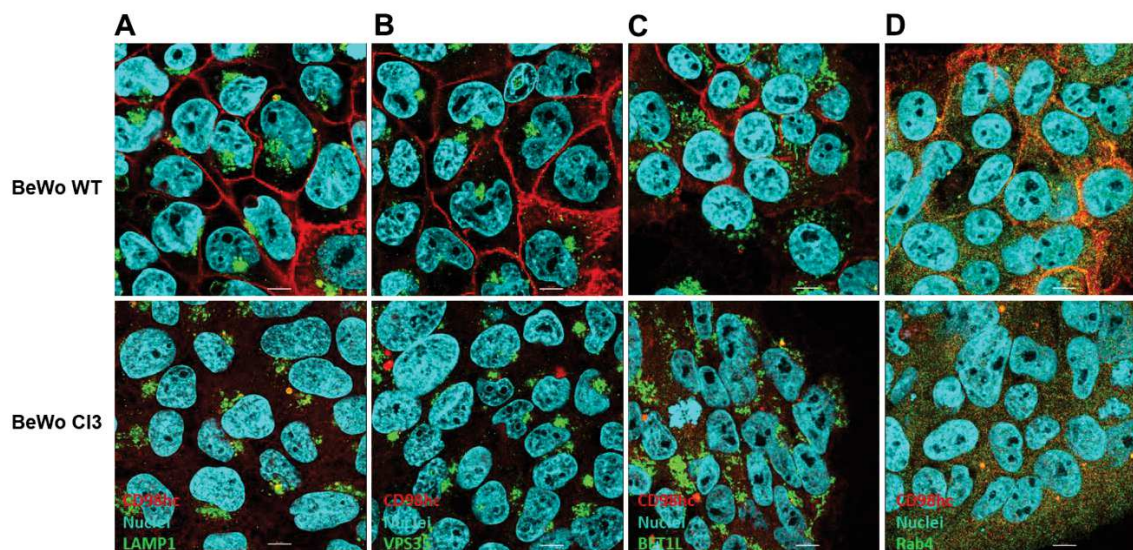
**Figure S1: Strategy to inactivate the *SLC3A2* gene by CRISPR/Cas9 in BeWo cells. A)** Schematic representation of human *SLC3A2* gene and transcripts (according to RCh38.p7 genome assembly). The target site of the sgRNA molecules used in this project are shown in red (adapted from Scalise *et al.*, 2018). Exonic sequences are depicted as boxes, with a color code for sequences encoding the intracellular, transmembrane, or extracellular domains. The different transcripts are shown, and the Genbank accession number is indicated for each variant. UTR sequence is indicated as a grey box. The size (in AA) of the isoforms encoded by each variant is indicated in blue. **B)** Workflow to generate a BeWo cell line with inactivated *SLC3A2*. Viral particles were obtained by co-transfection of HEK293T cells with 3 plasmids encoding the different components of non-replicative lentiviral particles. The viral genome has the coding capacity for the CRISPR/Cas system: the Cas9 enzyme and a small guide RNA (sgRNA) targeting the *SLC3A2* gene. Lentiviruses were recovered from 293T cells supernatant 48 hours post-transfection and applied to BeWo cells. 24 hours after viral transduction, cells were submitted to puromycin selection for 5 weeks. Puromycin-resistant cells were immunolabelled using the anti-CD98hc antibody MEM-108 and CD98hc-negative cells were sorted by flow-cytometry. Individual cellular clones were then amplified and re-tested for CD98hc expression.



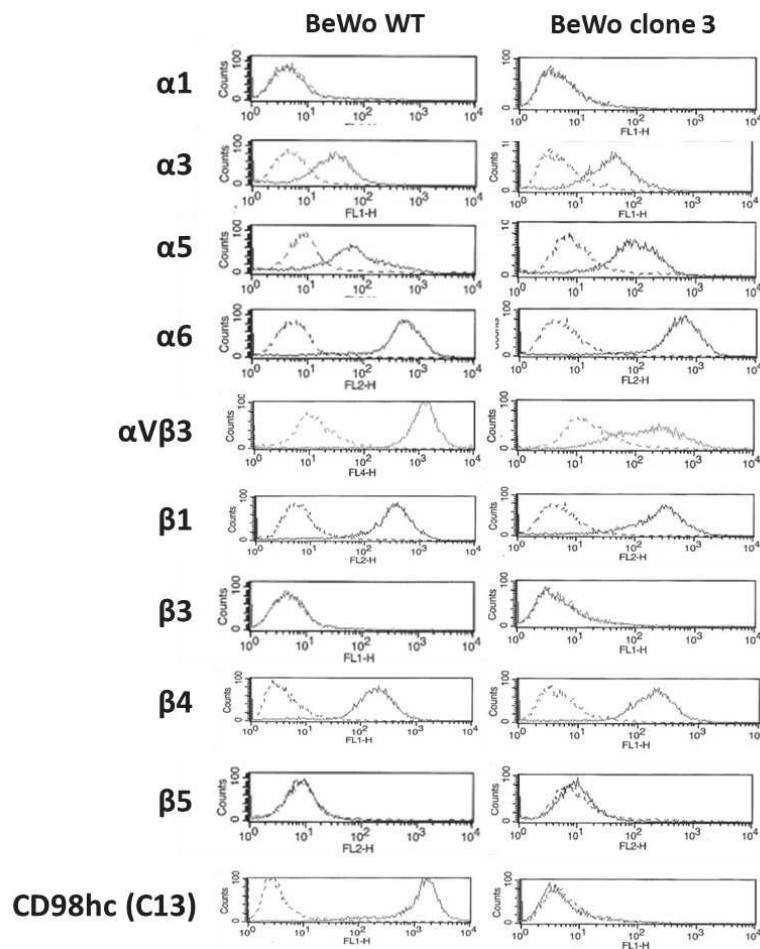
**Figure S2: *In vitro* growth curve of BeWo cells.** Wild-type (WT) or clone 3 BeWo cells were cultured using the experimental conditions for *in vitro* measurement of *Brucella* infection by intracellular CFU counting (gentamycin protection assay). Cells were then collected by trypsinization at different time points post mock-infection and enumerated by flow cytometry. Each time point was done in triplicate. Results are shown as mean numbers of cells per well.



**Figure S3: BeWo clone 3 cells express CD98hc intracellularly.** Expression of CD98hc was monitored in live cells or fixed/permeabilized cells after staining with the indicated anti-CD98hc antibodies and the corresponding secondary antibodies coupled to phycoerythrin or TexasRed. Expression of CD98hc was monitored by flow-cytometry (MACSQuant VYB, Miltenyi).



**Figure S4: CD98hc does not localizes to lysosomes, Golgi or retromer sorting complex in BeWo cells.** BeWo cells (WT or clone 3) were grown on glass coverslips, fixed with paraformaldehyde 3% and permeabilized with Triton X-100 before immunolabelling with the anti-CD98hc antibody HBJ127 in combination with either **(A)** an anti-LAMP1 (clone H4A3, Southern Biotech), **(B)** an anti-VPS35 (sc-374372, Santa Cruz Biotechnology), **(C)** an anti-BET1L (sc-135846, Santa Cruz Biotechnology) **(C)** or an anti-Rab4 antibody (sc-376243, Santa Cruz Biotechnology). CD98hc signal was revealed using a secondary antibody coupled to an Alexa 594 (red) whereas LAMP1, VPS35, BET1L and Rab4 signals were revealed using a secondary antibody coupled to an Alexa 488 (green). Nuclei were stained with DAPI (blue). Cells were observed by confocal microscopy and images were treated with the IMARIS software (version 9.5.1).



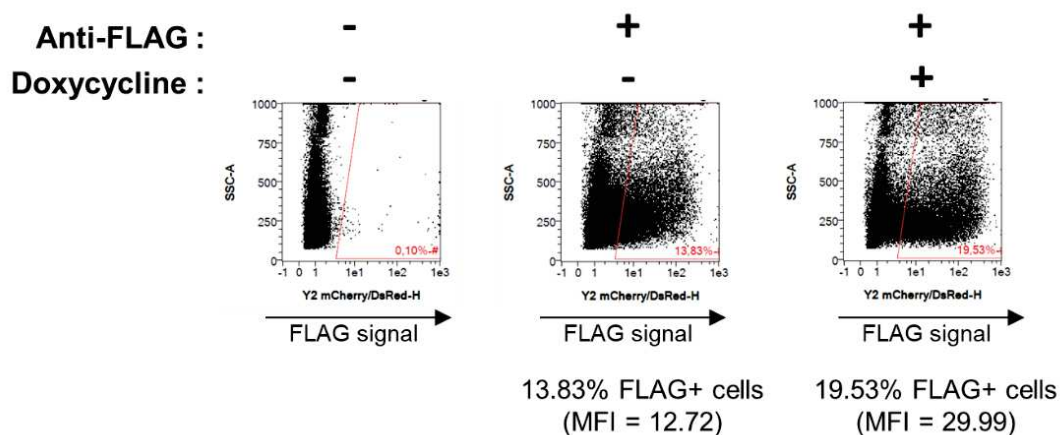
**Figure S5: Expression profile of integrins on BeWo cells.** Flow-cytometry analysis of live BeWo cells (either WT or clone 3) after immunolabelling with specific antibodies to detect surface expression of the indicated integrins subunits. As a control, CD98hc labelling was also performed using another anti-C-ter antibody (clone 4F2C13, ATCC HB-22), whose epitope is unknown.

### 3. Additional results

#### 3.1. Conditional inactivation of *SLC3A2*

Because constitutive inactivation of *SLC3A2* was very challenging, we also developed a conditional inactivation approach. This strategy is based on the conditional expression of Cas9, whose coding sequence is under the control of a doxycycline inducible promoter. This system would allow to control the moment of *SLC3A2* inactivation in cells, which should occur only after addition of doxycycline in the cell culture media, thus ensuring cell survival up to this point.

To do this we used pCW-Cas9-Blast, a plasmidic construct encoding a FLAG-tagged Cas9 that was already validated experimentally (T. Wang et al., 2014). When this plasmid was introduced into HEK293T cells by transient transfection, we detected expression of Cas9 even in the absence of doxycycline, suggesting that this system is leaky (Figure 8). Addition of doxycycline however increased the intensity of the signal, showing higher expression level of Cas9.



**Figure 8: Conditional expression of Cas9 in HEK293T cells.** HEK293T cells were transfected with pCW-Cas9-Blast and treated (or not) with doxycycline (10  $\mu\text{g}/\text{mL}$ , 48h). Cells were then fixed and immunolabelled using an anti-FLAG antibody before analysis by flow cytometry. Cells without the anti-FLAG antibody were used as a negative control. The % of FLAG-positive cells and their MFI (Median Fluorescence Intensity, which reflects the intensity of the signal), are indicated for each condition.

Leakiness of Cas9 expression should not be restraining for our strategy, because the cells in which this would occur would be negatively selected during cell culture (*i.e.* they would die because of *SLC3A2* inactivation). We thus went further and tried stably



inserting this construct in BeWo cells. After lentiviral transduction and blasticidin selection, we obtained a heterogenous population of resistant cells (BeWo-iCas9). However, no FLAG signal was detected even when cells were treated with 200 µg/mL doxycycline, which was the highest concentration defined to have no visual cytotoxicity in these cells (data not shown).

#### 4. Discussion and perspectives

As anticipated, inactivation of CD98hc in human trophoblasts is very challenging.

Using a constitutive inactivation strategy, we could only obtain one cellular clone (BeWo clone 3) that expresses a modified CD98hc protein. This protein has a glycosylation defect and is relocalized in recycling endosomes. These cells also appear to have a deregulated integrin signaling pathway, particularly a defect in FAK signaling. These modifications are however compatible with *Brucella* infection. BeWo clone 3 cells might thus constitute a useful model to help unravelling the molecular mechanisms required for *Brucella* infection.

The conditional inactivation system is a more promising alternative to evaluate the role of CD98hc in trophoblasts infection. We chose a genetic construct that allows inducible expression of Cas9 upon doxycycline treatment. When transiently transfected in HEK293T this construct is functional, although leaky (Figure 8). Unfortunately, the construct did not function when stably inserted in BeWo cells using lentiviral transduction. Inefficacy of the construct in a chromosomal context might be due to its insertion in a heterochromatin-rich regions, which would not allow doxycycline-induced transcription of the Cas9 sequence. This drawback could also be specific to BeWo cells. We will thus try inserting this construct in other cells lines such as HEK293T, which are notoriously easily transduced. Inserting this construct in THP-1 cells (human monocytes) would also be of great use to the scientific community, given the role of macrophages in the pathogenesis of *Brucella* and the importance of CD98hc in myeloid cells.

When functional iCas9 cell lines will be obtained, constructs encoding small guide RNA (sgRNA) molecules targeting *SLC3A2* will be stably inserted in these cells by lentiviral transduction. After puromycin selection, conditional inactivation of *SLC3A2* will be verified both at the transcriptional level (qRT-PCR) and the protein level (using both anti-CD98hc C-ter and N-ter antibodies). We would however use sgRNA molecules different

#### 4. DISCUSSION AND PERSPECTIVES

from the ones used in our constitutive inactivation attempts. These molecules (sgRNA 2 and 3) targeted exons 7 and 10, respectively, which are common to all CD98hc isoforms. However, because they are far away from the 5' of the gene, knocking them out can result in residual expression of CD98hc, as what happened in BeWo clone 3 cells. Therefore, using an *in-silico* approach, we searched for sgRNA sequences targeting with high specificity the exons of *SLC3A2* that are common to several isoforms and predicted to inactivate efficiently the *SLC3A2* gene. We designed two new molecules: sgRNA-A, which targets exon 1 (*i.e.* N-terminus of the longest CD98hc isoforms) and sgRNA-B, which targets exon 5 (*i.e.* the N-terminus of all isoforms). New constructs, encoding these two sgRNAs, were obtained from GenScript.

In case the strategy described above is unsuccessful, several others can be envisaged to inactivate *SLC3A2* in human trophoblasts. One possibility is to first express stably the sgRNA molecules in cells and introduce the Cas9 coding sequence by viral transduction shortly before *Brucella* infection. Alternatively, if BeWo cells cannot handle CD98hc KO, *SLC3A2* inactivation could be concomitant with complementation with genetic constructs encoding recombinant CD98hc proteins (C109 or C330 mutants, or CD98hc/CD69 chimeric proteins) that are compatible with cell survival and dissociate the different functions of CD98hc (Fenczik et al., 2000). Hopefully, we will then be able monitor *Brucella* infection in cells depleted of CD98hc to evaluate the importance of this protein in human trophoblasts infection.



Part II.

Deciphering the function of  
CD98hc required for  
*Brucella* infection

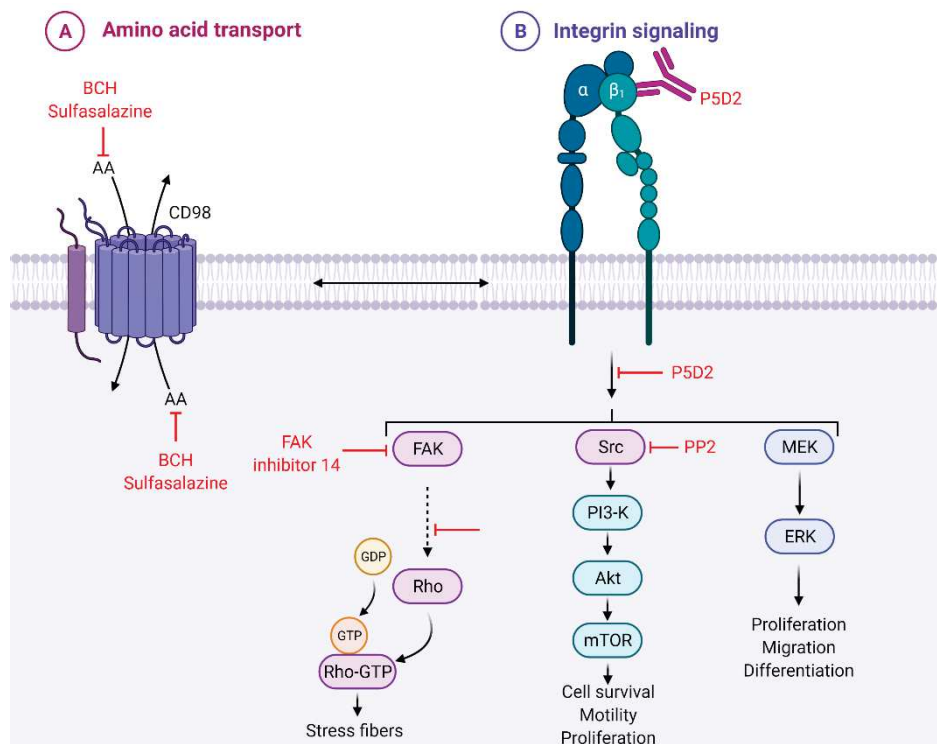
## Part II. Deciphering the function of CD98hc required for *Brucella* infection

### 1. Introduction

In this chapter of my thesis project, we tried elucidating which function of CD98hc is exploited during *Brucella* infection. CD98hc has 2 main functions inside the cell: mediation of integrin signaling and AA transport. The large C-terminal ectodomain of CD98hc carries the function of this protein in AA transport, whereas its short intracellular N-terminus and transmembrane domains are involved in the regulation of integrin-mediated intracellular signaling (Feral et al., 2005). We used both genetic and chemical tools to try deciphering which function is exploited by *Brucella* during its intracellular life.

As a first attempt, we tried overexpression of CD98hc mutants in wild-type cells. We used two point-mutants on conserved cysteine residues that are essential for the formation of disulfide bonds between the heavy and light chains of the heterodimeric AA transporters: C1, whose mutation in murine cells (C109S mutant) inhibits AA transport (Fenczik et al., 2000) or C2, whose mutation in murine cells (C330S mutant) has no effect on CD98hc mediated AA transport.

In parallel, we used chemical molecules that selectively modulate different biological functions mediated by CD98hc (Figure 9).



## 1. INTRODUCTION

**Figure 9: Overview of the different molecules used in this chapter.** Two CD98hc-dependent AA transport systems,  $x_c^-$  or L (LAT1), were inhibited using sulfasalazine or BCH, respectively. Several effectors of the integrin signaling pathways were modulated using either P5D2 (an anti- $\beta$ 1 integrin acting as an agonist in our conditions), or PP2 (a specific inhibitor of the Src kinase involved in early steps of integrin signaling) or FAK inhibitor 14 (which blocks FAK autophosphorylation to prevent its activation and further cytoskeleton rearrangements). Created with BioRender.com.

To assess the importance of CD98hc-dependant AA transport for *Brucella* infection, we used inhibitors targeting two different transport systems:

- Sulfasalazine: this compound inhibits the  $x_c^-$  system (composed of CD98hc and the light chain xCT), which is an exchanger of cystine and L-glutamate. Cells normally import cystine (the oxidized form of cysteine), which is then reduced into cysteine for general synthesis of proteins, or the synthesis of the anti-oxidant glutathione (GSH). By inhibiting cystine uptake, sulfasalazine leads to low levels of intracellular cysteine. This drug was identified in a screening for inhibitory molecules of the  $x_c^-$  system in lymphoma cells. It inhibits the growth of lymphoma cell cultures based on its potent and specific inhibition of the  $x_c^-$  system, leading to intracellular depletion of cysteine (Gout et al., 2001). Notably, sulfasalazine is clinically approved for the treatment of inflammatory bowel disease and rheumatoid arthritis (J. Liu et al., 2020; L. Liu et al., 2021).

- BCH (2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid): this molecule was originally shown to inhibit leucine uptake by the LAT1 transporter in *Xenopus* oocytes (Mastroberardino et al., 1998). Even if BCH was shown to be specific to LAT1 (a transporter of large neutral AA), it can inhibit all members of the LAT family at high concentrations (Q. Wang & Holst, 2015).

To assess the importance of integrin-mediated intracellular signaling during *Brucella* infection, we used several reagents targeting mediators of this pathway:

- P5D2: is an anti- $\beta$ 1 integrin antibody. This function-blocking antibody inhibits cell adhesion in several cell types (Dittel et al., 1993; Yokosaki et al., 1994). However, when applied to already adhered cells, P5D2 acts as an agonist, resulting in phosphorylation of Akt (Tian et al., 2002).

- PP2: is an inhibitor of Src, a kinase shared between several different intracellular signaling pathways. Src is ubiquitously expressed and is part of the human Src kinase family (Roskoski, 2015). The mechanism of inhibition of Src by PP2 is yet to be described. Moreover, even though this inhibitor has been extensively used as a Src “specific”

inhibitor it is in fact able to inhibit the activity of several members of the Src family kinases (Bain et al., 2007).

- FAK inhibitor 14 (1,2,4,5-Benzenetetraamine tetrahydrochloride): is a specific inhibitor of the tyrosine kinase FAK. This kinase is involved in the formation of focal contacts, structures formed at points of contact between integrins expressed on the cell surface and the extracellular matrix (ECM). They bring in contact signaling proteins and cytoskeleton during adhesion, spreading and migration. FAK also mediates activation of Rho-family GTPases within the cell. These molecular switches control actine cytoskeletal processes leading to the formation of stress fibers, filopodia or lamellipodia (Mittra et al., 2005). A key step in FAK activation is its autophosphorylation at the tyrosine 397. This event creates a motif that can be recognized by various proteins including the Src-family kinases (Schlaepfer et al., 2004). FAKi 14 (also known as Y15) prevents autophosphorylation of FAK (Golubovskaya et al., 2008).

## 2. Material and Methods

### *Cell culture*

Human trophoblasts (BeWo cells) were cultured in DMEM/F-12, GlutaMAX™ (Gibco) medium supplemented with 10% heat inactivated fetal bovine serum (FBS).  $\beta$ -mercaptoethanol (0.1mM) was added in the media for clone 3 cells. HeLa cells were grown in RPMI medium (Gibco) supplemented with 10% heat inactivated FBS. Cells were incubated at 37°C and 5% CO<sub>2</sub>.

### *Transfection*

BeWo cells were transfected using either Lipofectamine or nucleofection with pcDNA3.1-based expression vectors (obtained from C. Féral lab). The plasmids used were: pcDNA3.1-FL4F2 (encoding isoform f of human CD98hc), pcDNA3.1-C109S (encoding isoform f of hCD98hc with the C1 mutation), pcDNA3.1-CyLess (encoding isoform f of hCD98hc with both C1 and C2 mutations), empty pcDNA3.1 as a negative control and pmaxGFP (Lonza, Basel, Switzerland) as a positive control for transfection.

For Lipofectamine transfection, BeWo cells ( $4 \times 10^5$  cells per well in 6-well plates) were incubated with a mix of Lipofectamine 2000 (Invitrogen) and DNA for 4 hours at 37°C and 5% CO<sub>2</sub>. Cells were washed and incubated overnight (ON). For nucleofection BeWo cells ( $5 \times 10^6$  cells) were harvested using trypsin and centrifuged at 90g for 10 minutes. Supernatant was removed and the cell pellet resuspended in RT 4D-Nucleofector™

## 2. MATERIAL AND METHODS

Solution SF (Lonza). Cells were distributed in Nucleocuvette™ tubes with the different plasmids. Tubes were placed in the 4D-Nucleofector™ X unit, and the EN-150 program was run. After nucleofection, cells were incubated in 12-well plates containing pre-warmed media and incubated for 24 hours at 37°C and 5% CO<sub>2</sub>. The next day, cells transfected with pmax-GFP were observed using an epifluorescence microscope (DM IRB, Leica) coupled with a cool snap fx black and white camera (Roper Scientific) to determine percentage of transfection.

### *Infection and quantification of intracellular replication*

BeWo cells ( $1 \times 10^5$  cells per well in 24-well plates) were washed with phosphate-buffered saline (PBS) before infection by *Brucella melitensis* expressing mCherry with a MOI of 500 (bacteria per cell) using a gentamycin protection assay. After centrifugation (400g, 10 min, RT), infected cells were incubated at 37°C in a 5% CO<sub>2</sub> atmosphere for 30 min before washes with PBS and addition of 100 µg/mL gentamycin for 1.5h to kill extracellular bacteria. The media was then replaced by fresh media containing 20 µg/mL gentamycin. At 2, 5, 24, or 48h post infection (hpi), intracellular bacterial numbers were determined by lysing the cells with 0.1% Triton X-100. Serial dilutions were plated on TS agar to enumerate colony forming units (CFU). In some experiments, cells were fixed with 3% PFA for 25 minutes and the percentage of infected cells was analyzed by flow cytometry using the MAQS Quant VYB flow cytometer.

HeLa cells ( $2.5 \times 10^5$  cells per well in 24-well plates) were infected with *B. suis* strain 1330 following the same protocol except that MOI was 50 and initial contact with bacteria was 1 hour. After infection, 50 µg/mL of gentamicin was added to kill extracellular bacteria.

### *Inhibitors and antibodies*

BeWo cells were treated with different compounds prior to infection. AA transport inhibitors sulfasalazine (S0883, Sigma-Aldrich) and BCH (A7902, Sigma-Aldrich) were used for 1 hour at 200µM and ON at 2mM respectively. Integrin signaling inhibitors PP2 (P0042, Sigma-Aldrich) and FAK inhibitor 14 (SML0837, Sigma-Aldrich) were used ON at 10µM. The anti-β1 integrin antibody P5D2 (MAB1959, Sigma-Aldrich) was used for 30 minutes at 10 µg/mL. The anti-hCD98hc antibody MEM-108 (11-268-C100, ExBio) and the mouse IgG1 control antibody (11-457-C100, ExBio) were used for 30 minutes at 5µg/mL. All treatment used were visually evaluated to verify the absence of cytotoxicity on cells.



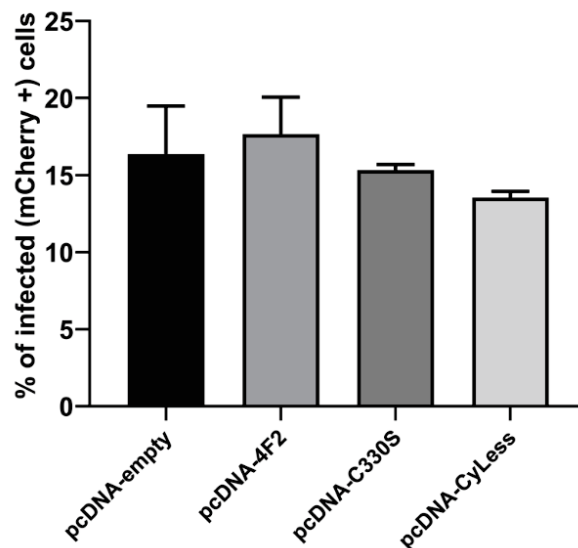
*Statistical analysis*

Statistical data analysis was done using a two-way ANOVA test with a Sidak correction for multiple comparisons, using the GraphPad Prism software version 7 for Windows, GraphPad Software, La Jolla California USA, [www.graphpad.com](http://www.graphpad.com).

### 3. Results

#### 3.1. Overexpression of CD98hc mutants

We assessed the impact of overexpressing different CD98hc mutants on *Brucella* infection in wild-type trophoblasts. Expression vectors encoding these mutants were transfected in BeWo cells, which were then infected by fluorescent *B. melitensis*. Unfortunately, we did not detect any changes in the % of infected cells (Figure 10). This could be due to a low transfection rate in BeWo cells. Indeed, using a GFP-encoding plasmid for optimization, a maximum of  $\pm 30\%$  of cells could be transfected, using either Lipofectamine or nucleofection (data not shown). Also, the transfection was very transient as only  $\pm 7\%$  of cells remained GFP-positive 96h post-transfection. This low transduction efficiency may thus not be sufficient to observe eventual effects of the expression of CD98hc mutants on *Brucella* infection.

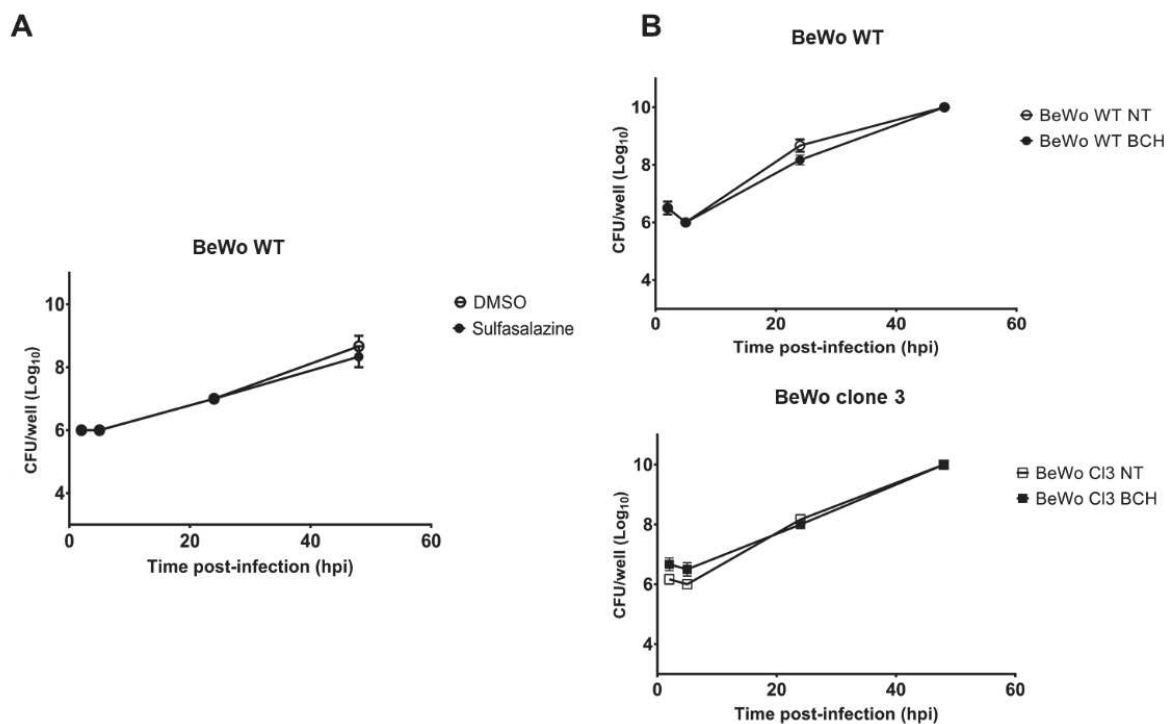


**Figure 10: Effect of overexpressing CD98hc mutants on *Brucella* infection.** BeWo cells were nucleofected with different expression vectors encoding wild-type or mutant CD98hc. Twenty-four after transfection, cells were infected with fluorescent *B. melitensis* with a MOI of 500 using gentamycin protection assay. Cells were then harvested 48hpi and the percentage of infected cells was quantified by flow cytometry. The data shown are the geometric means of 2 experiments  $\pm$  Standard Error of the Mean (SEM).

### 3. RESULTS

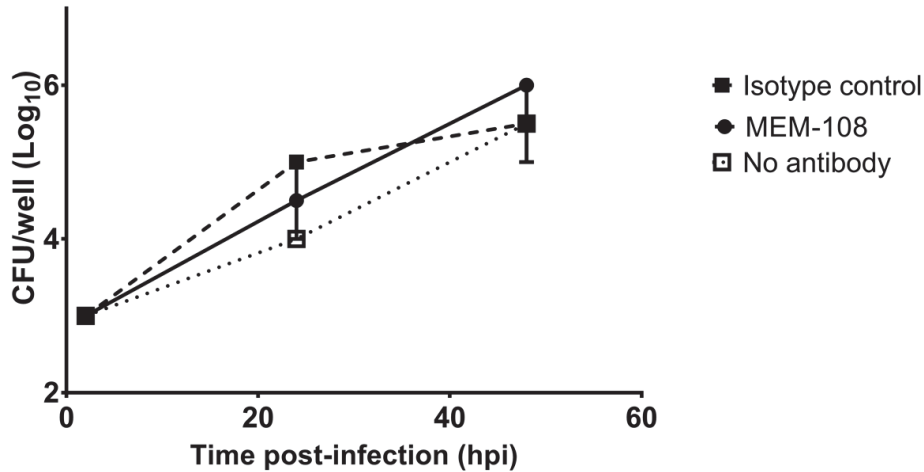
#### 3.2. Effect of AA transport inhibitors on infection

We first evaluated the effect of two selective inhibitors of CD98hc-dependant AA transporters: sulfasalazine (an inhibitor of  $xc^-$ , a cystine/glutamine exchanger) and BCH (an inhibitor of LAT1, a transporter of large neutral AA) (Table 3). In WT BeWo cells, none of these compounds influenced *Brucella* infection (Figure 11). The effect of BCH was also evaluated in BeWo clone 3, as these cells present a growth defect and seem to have altered AA transport (see Part II). As in WT BeWo cells, BCH has no effect on *Brucella* infection in clone 3 cells (Figure 11).



**Figure 11: Effect of AA transport inhibitors on *Brucella* infection in BeWo cells.** BeWo cells were infected with *B. melitensis* with a MOI of 500 using gentamycin protection assay. Cells were then lysed at the indicated hours post infection (hpi) for enumeration of intracellular colony forming units (CFU). **A.** For Sulfasalazine (200  $\mu$ M) or **B.** BCH (2 mM) treatments, a 1 hour or overnight pre-incubation was performed before infection, respectively. The data shown are the geometric means of Log<sub>10</sub> CFU per well  $\pm$  Standard Error of the Mean (SEM).

Our observations that *Brucella* infection is affected by neither AA transport inhibitors nor expression of a CD98hc protein with modified extracellular C-terminus (in BeWo-clone 3 cells) suggest that their intracellular life rather depend on the N-terminus CD98hc, which is responsible for the role of this protein as a modulator of integrin-mediated signaling. Moreover, pre-incubating HeLa cells with the MEM-108 antibody, which targets the C-terminus of CD98hc, did not impair *Brucella* infection (Figure 12).

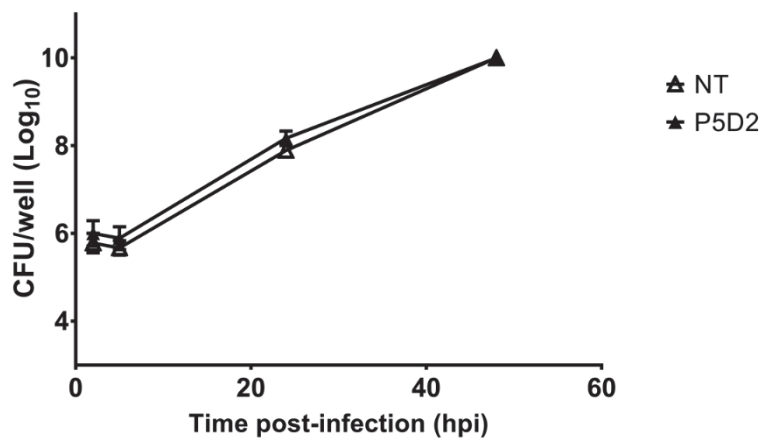


**Figure 12: Effect of an anti-CD98hc C-ter antibody (MEM-108) on *Brucella* infection.** HeLa cells were pre-incubated with MEM-108 (5  $\mu\text{g}/\text{mL}$  in cell culture media) or, as a control, a mouse IgG1 isotype for 30 min at 37°C in a 5% CO<sub>2</sub> atmosphere. Cells were then infected by *B. suis* with a MOI of 50 using a gentamycin protection assay (NB: the antibodies were still present @ 2,5  $\mu\text{g}/\text{mL}$  in the inoculum, i.e. until addition of gentamycin). Cells were then lysed at the indicated hours post-infection (hpi) for enumeration of intracellular colony forming unit (CFU). The data shown are the geometric means of Log<sub>10</sub> CFU per well  $\pm$  Standard Error of the Mean (SEM).

We thus next focused on a possible link between the role of CD98hc as a mediator of integrin-signaling and *Brucella* infection in human trophoblasts.

### 3.3. Effect of integrin-mediated signaling modulators on infection

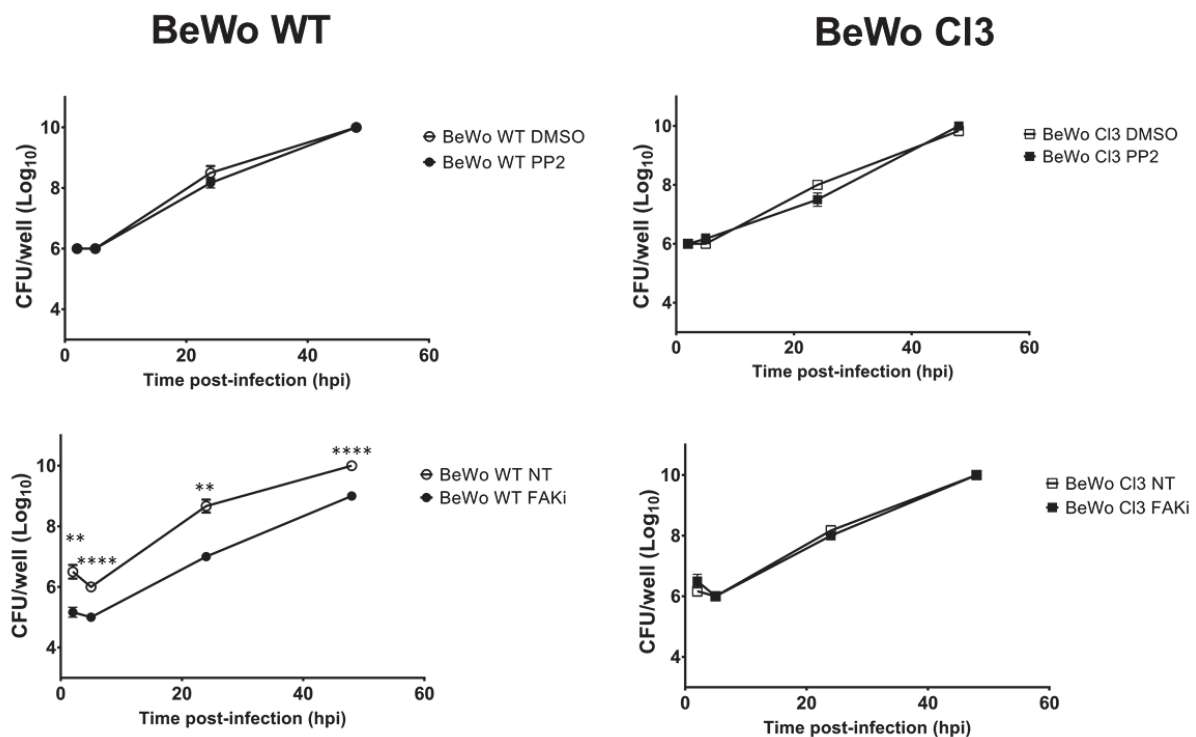
We quantified *Brucella* infection in BeWo cells after modulation of several effectors of the integrin signaling pathway. First, pre-incubating cells with P5D2 (an anti- $\beta$ 1 integrin antibody), had no effect on infection (Figure 13). Also, no effect was observed after an overnight pre-treatment of cells with PP2, a specific inhibitor of the kinase Src (Figure 14).



#### 4. DISCUSSION AND PERSPECTIVES

**Figure 14: Effect of an anti- $\beta$ 1 integrin antibody on *Brucella* infection.** BeWo cells were pre-treated with P5D2 (10  $\mu$ g/mL, 30 min pre-incubation) and then infected with *B. melitensis* with a MOI of 500 using gentamycin protection assay. Cells were then lysed at the indicated hours post infection (hpi) for enumeration of intracellular colony forming units (CFU). The data shown are the geometric means of  $\text{Log}_{10}$  CFU per well  $\pm$  Standard Error of the Mean (SEM) from three independent experiments. NT = non-treated.

Interestingly, pre-treatment with FAK inhibitor-14 (FAKi-14), a specific inhibitor of the kinase FAK, decreased *Brucella* entry by 100-fold in BeWo WT (Figure 14). In this condition, internalized bacteria seemed however to replicate with the same rate as in untreated cells as the curves are parallel between 5 and 48hpi. Moreover, inhibition of *Brucella* entry by FAKi-14 was not observed in BeWo-clone 3, suggesting that the activity of this kinase or the signaling pathways downstream are somehow affected in these cells.



**Figure 13: Effect of Src or FAK inhibitors on *Brucella* infection.** BeWo cells (either WT or clone 3) were pre-treated overnight with PP2 (10  $\mu$ M) or FAK inhibitor 14 (10  $\mu$ M) and then infected with *B. melitensis* with a MOI of 500 using gentamycin protection assay. Cells were then lysed at the indicated hours post infection (hpi) for enumeration of intracellular colony forming units (CFU). The data shown are the geometric means of  $\text{log}_{10}$  CFU per well  $\pm$  Standard Error of the Mean (SEM) from three independent experiments. DMSO = control condition; NT = non-treated.

#### 4. Discussion and perspectives

To gain insights as to which function of CD98hc is important for *Brucella* infection we first used a genetic approach to overexpress different CD98hc mutants in BeWo cells. Unfortunately, we could not detect any changes in the percentage of infected cells (Figure 10). This can be due to the low number of transfected cells using either Lipofectamine

2000 or nucleofection and the very transient expression of the mutant proteins. Also, as endogenous CD98hc is highly expressed in wild-type BeWo cells, overexpression of these mutant proteins might not be sufficient to see any changes in *Brucella* infection. An alternative would be to express CD98hc mutant proteins in CD98hc-KO cells. The murine derm fibroblasts (mDF) constitute an appropriate cellular model for such studies. Indeed, while the wild-type cells are efficiently infected by *Brucella*, CD98hc-KO mDF cells, which could be obtained from transgenic mice after conditional KO in adult animals, are resistant to infection (Keriel et al., 2015). In such cells, that do not express any CD98hc protein and have a clear phenotype in infection, expression of mutant CD98hc proteins may thus allow discriminating between the different functions of CD98hc to understand which one is/are required during *Brucella* infection. In the same line of thoughts, the use of CD98hc/CD69 chimeric constructs, which encompass different domains of CD98hc and thus dissociate its 2 main functions (Fenczik et al., 2000), could help.

As overexpression of mutant CD98hc proteins in wild-type trophoblasts did not allow us to assess which function of CD98hc is important for *Brucella* infection, we tried a biochemical approach. Inhibiting the CD98hc-dependant transport of large neutral AA (through LAT1) or of cystine (through xc<sup>-</sup>) did not affect *Brucella* infection of human trophoblasts, suggesting that the intracellular lifestyle of these bacteria does not rely on this function of the host protein. The situation seems different for *Salmonella*: during infection, LAT1 is essential for targeting of mTOR to the *Salmonella*-containing vacuole, ensuring bacterial survival. Treatment of *in vitro* infected cells with BCH blocks recruitment of mTOR (Schuster et al., 2015) and induces intracellular killing of *Salmonella* (Tattoli et al., 2012).

In this study we could only assess the implication of the LAT1 and xc<sup>-</sup> systems as no specific inhibitors exist for other CD98hc-dependent AA transporters. Therefore, these experiments cannot exclude the implication of other AA transporters in *Brucella* infection. However, the observation that BeWo cells 3 cells are permissive to *Brucella* infection (Figure 14) strongly suggest that the role of CD98hc as a mediator of AA import from the extracellular space is not essential for infection. Indeed, the modified CD98hc protein expressed in these cells is strictly intracellular and can thus not ensure its role as a chaperone for the light chains and guide them to the plasma membrane (Verrey et al., 1999). In this hypothesis, which is currently being verified experimentally, none of the

#### 4. DISCUSSION AND PERSPECTIVES

CD98hc-dependent AA transporters would function in clone 3 cells. Another line of evidence is that clone 3 cells require the presence of  $\beta$ -mercaptoethanol in the cell culture media to survive (data not shown) suggesting that these cells have an altered AA transport. This compensation mechanism, which is thus CD98hc-independent and remains to be characterized, is compatible with infection of clone 3 cells by *Brucella*.

Our data suggest that *Brucella* rather exploits the ability of CD98hc to mediate integrin signaling for infecting cells. More specifically, the FAK pathway appears to be essential as inhibition of FAK results in a significant decrease in bacterial entry in WT BeWo cells (Figure 14). During integrin signaling, it seems that activation of FAK via its autophosphorylation is required for the binding of SH2-domain containing proteins (including Src) to the newly created domain (Mitra et al., 2005). However, depending on the signaling pathway it is unclear whether Src is downstream of FAK or the other way around. In our case, the absence of effect of Src inhibition by PP2 during *Brucella* infection could suggest that Src is not upstream of FAK in the pathway. Of course, the lack of effect can also be due to the experimental setup. As PP2 is cytotoxic for cells, it was not possible to submit them to a treatment longer than 1 hour with this compound.

Treatment of the cells with P5D2 did not alter *Brucella* infection in our experimental conditions. This antibody blocks integrin-mediated adhesion in various cell types (Dittel et al., 1993; Yokosaki et al., 1994). The lack of effect in our case might be due to the treatment of cells already attached on a surface. Indeed, it was shown that the blocking activity of the antibody is observed, in fibroblasts, when preincubated with cells prior to adherence (Tian et al., 2002). When fibroblasts were treated after adhesion, the antibody acted as an agonist of  $\beta$ 1-integrin. Therefore, pre-treating cells in suspension prior to *Brucella* infection might be a better way to assess the effect of this antibody. Also, engagement of integrins varies depending on the material used to grow cells (Streuli, 2016). Thus, infection of cells grown on a matrix such as fibronectin or even on Matrigel of different stiffness would provide a better experimental setup to assess the role of integrin signaling in *Brucella* infection.

Another interesting approach for deciphering which CD98hc function is important for *Brucella* infection is the ability of human CD98hc to bind to a soluble form of CASK (Calcium/Calmodulin-dependent serine protein kinase). This protein belongs to the MAGUK family (Membrane-Associated Guanylate Kinases), which assembles specific

multiprotein complexes at cell junctions and regulate adhesion and plasticity. CASK is membrane-associated kinase mediating many protein-protein interactions in several cell types (*i.e.*, podocytes, neurons). It possesses several protein-protein interaction domains: a N-ter domain (with homology to the protein kinase calcium/calmodulin-dependent) and 3 characteristic MAGUK domains (PDZ Class II, SH3 domain and GUK). PDZ are peptide-binding domains of 80–90 residues comprising six beta-strands and two alpha-helices compactly arranged into a globular structure. A secreted form of human CASK (sCASK) exists; it is detected in the serum of patients affected by FSGS (Focal and Segmental Glomerulo Sclerosis), a severe disease affecting the renal glomeruli (Yan et al., 2007). *In vitro*, recombinant sCASK induces morphological rearrangement in cultured podocytes (*i.e.*, alterations of the cytoskeleton, increased motility, and monolayer permeability) and intravenous injection of sCASK causes proteinuria in mice (Beaudreuil et al., 2019). Human CD98hc can bind to this soluble, extracellular sCASK (Yan et al., 2007) and the morphological rearrangements observed in cultures podocytes treated with sCASK are CD98hc-dependent (Beaudreuil et al., 2019). This interaction occurs between the PDZ domain of CASK (residues 481-572) and the C-ter of human CD98hc (PDZ-binding domain, GLLLRFPYAA, residues 520–529) (Giansanti et al., 2015; Yan et al., 2007). Based on these observations, it would be interesting to evaluate *Brucella* infection in cells pretreated with recombinant sCASK. The full-length protein being toxic (structural and functional alterations in podocytes *in vitro*; proteinuria in mice and humans), we would use the free PDZ domain of CASK alone, which is sufficient for hCD98hc binding and is not toxic for cultured cells up to  $\mu\text{M}$  concentrations (Giansanti et al., 2015).

All together, these results show the role of FAK during *Brucella* infection of human trophoblasts. The study of the molecular mechanisms involved will be performed by Western Blot analysis to understand the signaling pathways at play during *Brucella* infection particularly using BeWo-clone 3 as a cellular tool.

Part III.

Fighting *Brucella*  
infections: a drug  
repositioning strategy



## Part III. Fighting *Brucella* infections: a drug repositioning strategy

### 1. Introduction

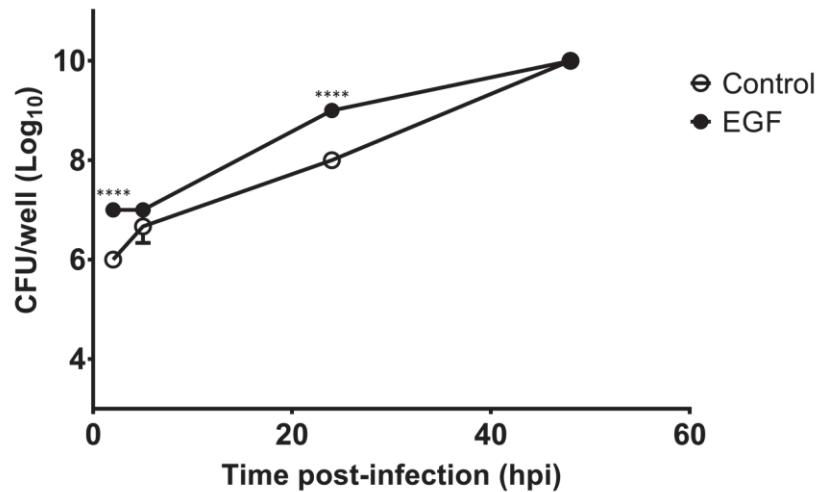
There is currently no vaccine to combat human brucellosis. Moreover, the recommended treatment (*i.e.* more than 6 weeks of double antibiotherapy) has many side effects and can lead to disease relapse because of the intracellular localization of bacteria. Alternative treatments to combat *Brucella* infections are thus needed.

In this chapter of my thesis project, we decided to investigate the efficiency of already existing drugs against *Brucella* infections. Such a drug repositioning/repurposing strategy is often adopted for neglected diseases due to the reduced number of required clinical trial steps (reduction of time and costs for the medicine to reach the market) or the existing pharmaceutical supply chains (facilitation of the formulation and distribution steps). This strategy also allows scientific research projects to convert rapidly into disease-oriented research. We focused on drugs that are linked to my thesis project, meaning CD98hc and intracellular signaling (CD98hc-dependant or not), and chose to evaluate the effect of statins and Gefitinib for the reasons explained below.

1. Statins: These molecules are a class of lipid-lowering medications used by patients with high risk of cardiovascular disease. They are the most common cholesterol-lowering drugs. They inhibit the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, a rate-limiting enzyme in the cholesterol biosynthesis pathway (Rikitake & Liao, 2005). Interestingly, HMG-CoA reductase is also involved in Rho GTPases signaling, a pathway downstream of integrins. Statins were indeed shown to block synthesis of geranylgeranylpyrophosphate (GGPP) and farnesylpyrophosphate (FPP), two important intermediates for post-translational modifications of proteins including G-proteins and GTP-binding proteins (Rho, Ras, Rap, Rab). These proteins being the end mediators of the integrin pathway, we wanted to assess the effect of statins on *Brucella* infection.

2. Gefitinib: We and others observed a link between activation of the epidermal growth factor receptor (EGFR, aka ErbB-1 or HER1) and *Brucella* infection. Indeed, pre-treating HeLa cells with EGF significantly increased bacterial internalization in these cells (J. Celli, personal communication and A. Keriél, unpublished data). This effect was confirmed in BeWo cells (Figure 15).

## 1. INTRODUCTION



**Figure 15: Effect of EGF, an EGFR ligand, on *Brucella* infection in human trophoblasts.** BeWo cells were pre-treated with EGF and infected with *B. melitensis* using a MOI of 500 in a gentamycin protection assay. Cells were then lysed at the indicated hours post infection (hpi) for enumeration of intracellular colony forming units (CFU). The data shown are the geometric means of Log<sub>10</sub> CFU per well  $\pm$  Standard Error of the Mean (SEM) from one experiment.

EGF is a protein that stimulates cell proliferation, differentiation, and survival. Upon EGF binding with high affinity to EGFR on the cell surface, the receptor undergoes a transition from an inactive monomeric form to active dimers, thus stimulating its intrinsic intracellular protein-tyrosine kinase activity (Oda et al., 2005). The tyrosine kinase activity, in turn, triggers several signal transduction cascades, mainly the MAPK/ERK (also known as the Ras-Raf-MEK-ERK pathway), Akt and JNK pathways. As deregulation of EGFR signaling is associated with several human diseases (mainly cancers, in case of EGFR over-expression or -activity), several drugs targeting this receptor have been developed. Some of them are specific inhibitors of the EGFR tyrosine kinase activity, thus halting the downstream signaling cascades. One example is Gefitinib, a drug prescribed for the treatment of NSCLC (non-small cell lung cancer) (Paez et al., 2004). This molecule specifically binds to the tyrosine kinase domain of EGFR, thus preventing ATP from binding and blocking subsequent receptor autophosphorylation. This results in the inhibition of EGFR signal transduction pathways.

Interestingly, physical interactions between EGFR and CD98hc as well as crosstalks between the respective intracellular signaling pathways of EGFR and integrins have recently been described (Chen et al., 2018). Moreover, activated EGFR, CD98hc and integrins all cluster in surface structures known as “lipid rafts” or cholesterol-enriched

domains (Del Pozo, 2004; Hofman et al., 2008; Schroeder et al., 2012). These observations prompted us to assess the effect of Gefitinib on *Brucella* infection.

## 2. Material and Methods

### *Cell culture*

Human trophoblasts (BeWo cells) were cultured in DMEM/F-12, GlutaMAX™ (Gibco) medium supplemented with 10% heat inactivated fetal bovine serum (FBS). Murine macrophages (J774.1 cells) were grown in DMEM GlutaMAX™ medium (Gibco) supplemented with 10% heat inactivated FBS. Cells were incubated at 37°C and 5% CO<sub>2</sub>.

### *Infection and quantification of intracellular replication*

BeWo cells ( $1 \times 10^5$  cells per well in 24-well plates) were washed with phosphate-buffered saline (PBS) before infection by *Brucella* strains expressing mCherry with a MOI of 500 (bacteria per cell) using a gentamycin protection assay. After centrifugation (400g, 10 min, RT), infected cells were incubated at 37°C in a 5% CO<sub>2</sub> atmosphere for 30 min before washes with PBS and addition of 100 µg/mL gentamycin for 1.5h to kill extracellular bacteria. The media was then replaced by fresh media containing 20 µg/mL gentamycin. At 2, 5, 24, or 48h post infection (hpi), intracellular bacterial numbers were determined by lysing the cells with 0.1% Triton X-100. Serial dilutions were plated on TS agar to enumerate colony forming units (CFU). In some experiments, cells were fixed with 3% PFA for 25 minutes and percentage of infected cells or total number of cells were analyzed by flow cytometry using the MAQS Quant VYB flow cytometer (Miltenyi Biotec).

J774 cells ( $5 \times 10^5$  cells per well in 24-well plates) were infected with *Brucella* following the same protocol except that MOI was 50. After infection, 50 µg/mL of gentamicin was added to kill extracellular bacteria.

### *Immunolabelling*

BeWo ( $1 \times 10^5$  cells per well) and J774 cells ( $5 \times 10^5$  cells per well) were grown ON in 12-well plates containing sterile coverslips before infection as described above. At 48hpi, cells were fixed with 3% PFA, permeabilized with 0.5% Triton-X100 and stained with phalloidin coupled to Alexa 647 (A22287, ThermoFischer Scientific). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, 1 µg/ml) and the coverslips mounted using ProLongGold (Life Technologies). Slides were observed with the Olympus FV10C-O confocal microscope and images were treated with the IMARIS software version 9.5.1.

## 2. MATERIAL AND METHODS

### *Drugs*

BeWo and J774 cells were treated with the different compounds prior to or during infection. Simvastatin (S6196, Sigma Aldrich) was used on BeWo cells for 1 hour at 1 $\mu$ M. The EGFR inhibitor Gefitinib (SML1657, Sigma Aldrich) was used at 5  $\mu$ M at different timing. To assess effect of a pretreatment, BeWo cells were treated for 1h prior to infection. To assess the effect of a continuous treatment, BeWo and J774 cells were treated for 1h before and during infection. To assess a potential curative effect of Gefitinib, BeWo and J774 cells were treated with the drug starting at 5 or 24hpi and treatment was continuous during infection for up to 12 days in the “long term” experiments. In all experiments, media containing Gefitinib (or DMSO as a control) was changed every 24h. For all the different treatment conditions used, cytotoxicity in cells were visually evaluated previously to infection experiments.

### *In vivo experiments*

Adult BALB/c mice were infected by intraperitoneal injection of *B. melitensis* and then treated with Gefitinib for 7 consecutive days (cf details of the different experiments in the figures legend). At different timepoints, some animals were euthanized by CO<sub>2</sub> asphyxiation and spleen and livers were collected. Organs were weighted and homogenized in PBS to determine bacterial load by CFU counting.

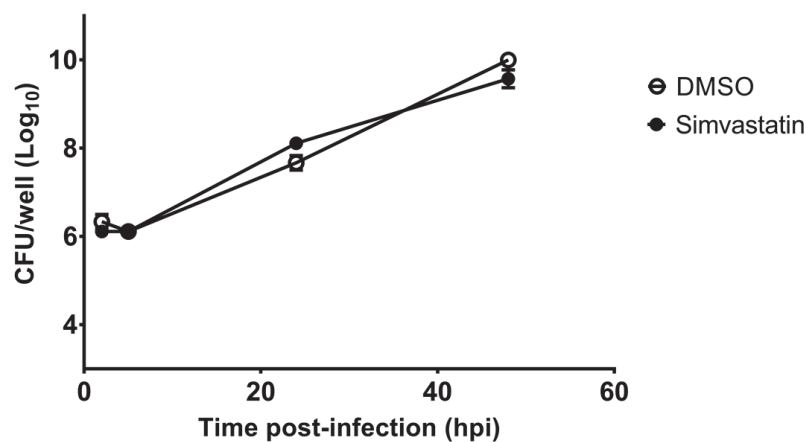
### *Statistical analysis*

Statistical data analysis was done using a two-way ANOVA test with a Sidak correction for multiple comparisons, using the GraphPad Prism software version 7 for Windows, GraphPad Software, La Jolla California USA, [www.graphpad.com](http://www.graphpad.com).

### 3. Results

#### 3.1. Effect of statins on *Brucella* infection

We first treated BeWo cells with simvastatin, a molecule part of the statin family. Unfortunately, we did not observe any effect of statin (simvastatin, 1  $\mu$ M) on *Brucella* infection in BeWo cells (Figure 16), even when using a continuous treatment (data not shown). To conclude, even though statins block the synthesis of important intermediates for post-translational modifications of downstream effectors in the integrin pathway, this had no impact on *Brucella* infection of human trophoblasts.



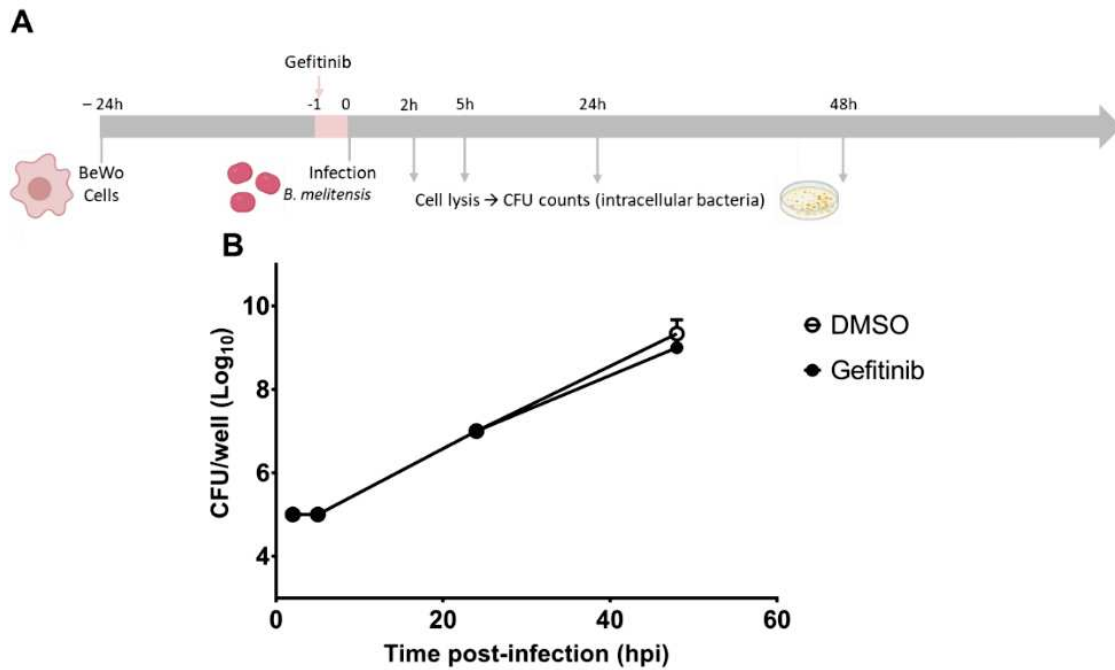
**Figure 16: Effect of simvastatin on *Brucella* infection.** BeWo WT cells were pre-treated for 1 hour with simvastatin (1  $\mu$ M) and then infected with *B. melitensis* with a MOI of 500 using a gentamycin protection assay. Cells were then lysed at the indicated hours post infection (hpi) for enumeration of intracellular colony forming units (CFU). The data shown are the geometric means of Log<sub>10</sub> CFU per well  $\pm$  Standard Error of the Mean (SEM) from three independent experiments. DMSO = control condition.

#### 3.2. Effect of Gefitinib on *Brucella* infection

##### 3.2.1. Preventive effect of Gefitinib against *Brucella* infection in human trophoblasts and murine macrophages

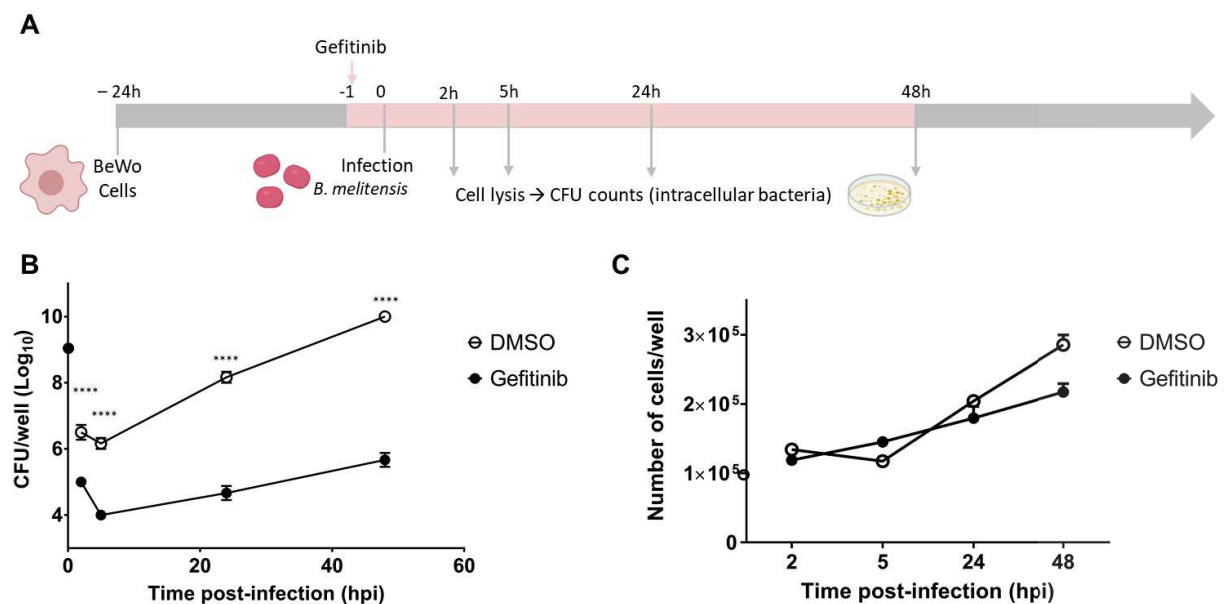
We first assessed the effect of Gefitinib by pre-treating BeWo cells for 1 hour before infection with *B. melitensis*. Using this experimental setup, Gefitinib had no significant effect on *Brucella* infection (Figure 17).

### 3. RESULTS



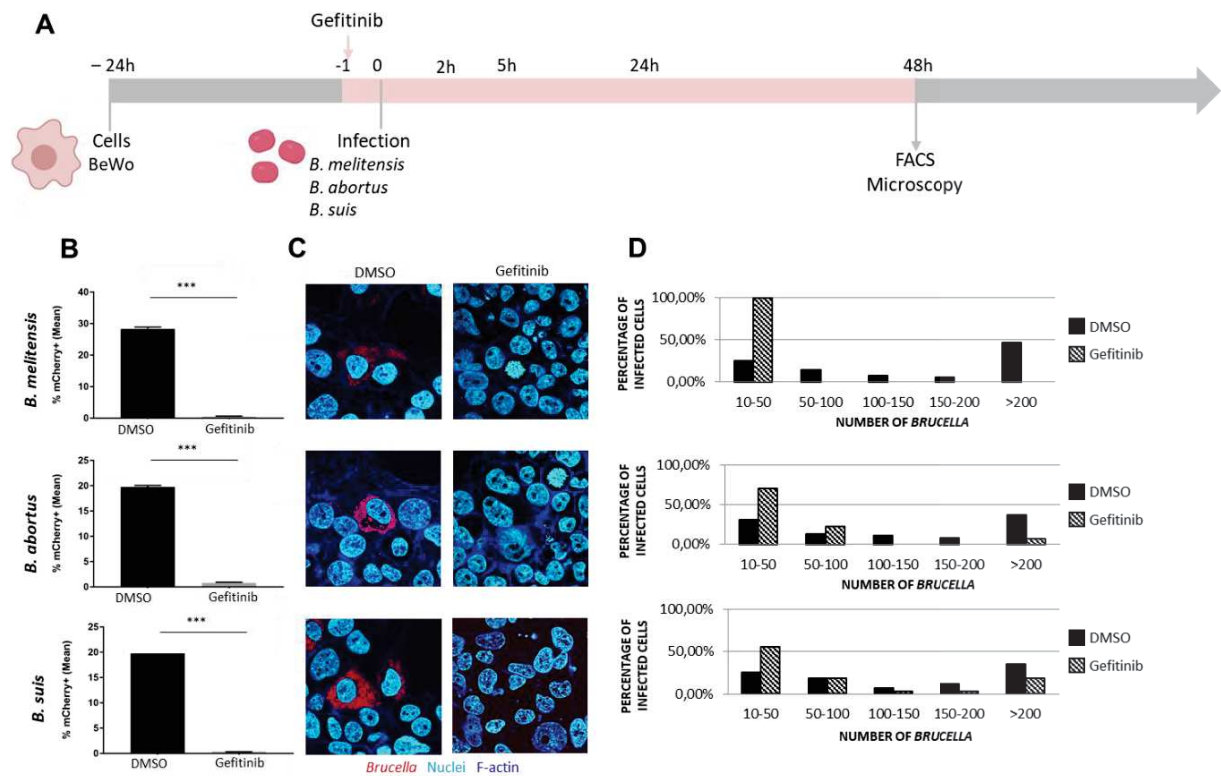
**Figure 17: Effect of Gefitinib, an EGFR inhibitor, on *Brucella* infection in human trophoblasts.** **A.** Workflow of the experiment. BeWo cells were pre-treated with Gefitinib (5  $\mu$ M) for 1 hour before infection with *B. melitensis* using a MOI of 500 in a gentamycin protection assay. Cells were then lysed at the indicated hours post infection (hpi) for enumeration of intracellular colony forming units (CFU). **B.** The data shown are the geometric means of Log<sub>10</sub> CFU per well  $\pm$  Standard Error of the Mean (SEM) from one experiment.

As no effect was observed when cells were treated 1 hour before infection, we prolonged the treatment by maintaining Gefitinib in the cell culture media throughout the infection. In parallel, cells were enumerated at each time point to evaluate a potential effect on cells numbers. In BeWo cells, such a treatment alters the entry of *B. melitensis* (1 Log<sub>10</sub> difference at 2 hpi) and inhibits very strongly its intracellular replication (5 Log<sub>10</sub> at 48 hpi) (Figure 18).



**Figure 18: Protective effect of Gefitinib on *Brucella* infection in human trophoblasts.** **A.** Workflow of the experiment. BeWo cells were pre-treated with Gefitinib and infected with *B. melitensis* using a MOI of 500 in a gentamycin protection assay. Cells were then lysed at the indicated hours post infection (hpi) for enumeration of intracellular colony forming units (CFU). Treatment with Gefitinib (5  $\mu$ M) was continuous during the infection assay. **B.** The data shown are the geometric means of Log<sub>10</sub> CFU per well  $\pm$  Standard Error of the Mean (SEM) from three independent experiments. **C.** In parallel, cells were counted at each timepoint to evaluate a possible effect of Gefitinib on cell numbers.

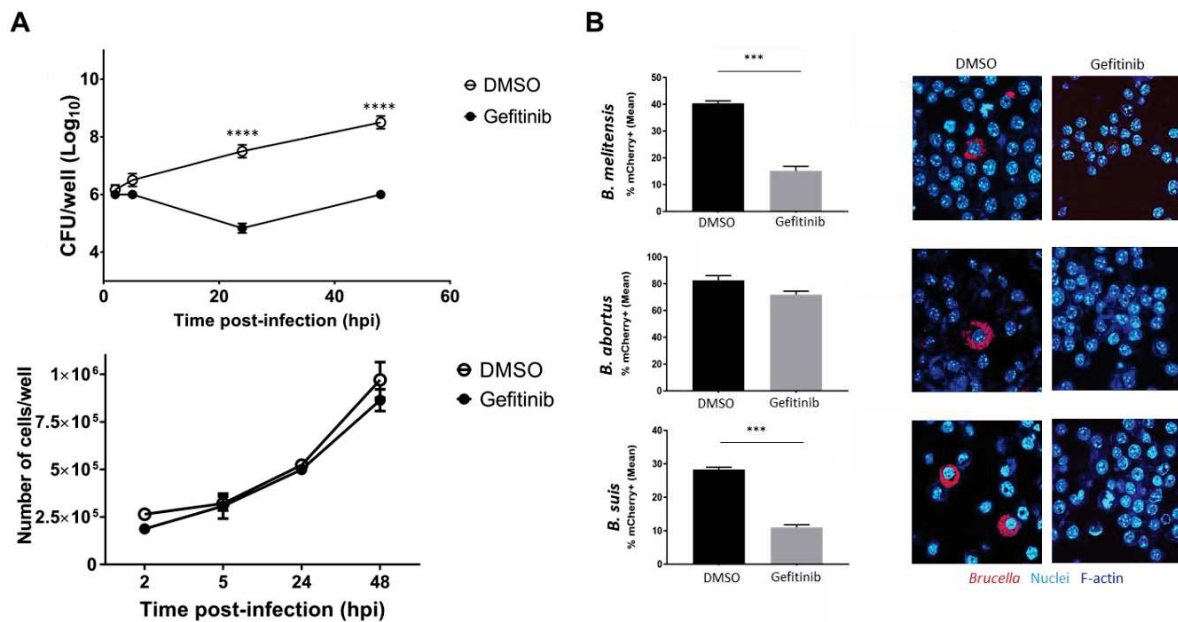
To confirm these observations, the effect of Gefitinib on infection was measured using as a readout the percentage of infected cells (using flow cytometry) and the number of intracellular bacteria (using confocal microscopy). We found that BeWo cells treated with Gefitinib rarely contain fluorescent bacteria and, when they do, their numbers are dramatically reduced compared to non-treated cells (Figure 19). Importantly, we found that Gefitinib is also efficient against the two other main zoonotic species of *Brucella* (i.e., *B. abortus* and *B. suis*).



**Figure 19: Gefitinib protects human trophoblasts against *Brucella* infections.** **A.** Workflow of the experiment. BeWo cells were pre-treated with Gefitinib and infected with *B. melitensis*, *B. abortus* or *B. suis* using a MOI of 500 in a gentamycin protection assay. Cells were then fixed at the indicated hours post infection (hpi) for analysis by flow cytometry (% of infected cells, **B**) or confocal microscopy (**C**). The number of intracellular bacteria was quantified semi-automatically using the IMARIS software (version 9.5.1). **D.** The percentage of infected cells is represented according to the number of bacteria per cell among the different ranges indicated. Treatment with Gefitinib (5  $\mu$ M) was continuous during the infection assay.

### 3. RESULTS

To exclude that the effect of Gefitinib on *Brucella* was cell-type dependent we repeated these experiments in murine macrophages, another cell type favored by *Brucella* during natural infection. The protective effect of a continuous treatment of Gefitinib against *Brucella* infection was also confirmed in J774 cells, a murine macrophagic cell line commonly used in *Brucella* research (Figure 20). For *B. abortus*, the decrease in % of infected cells was not significant but paired with a decrease in mean fluorescence intensity (correlated with bacterial replication) in treated cells.



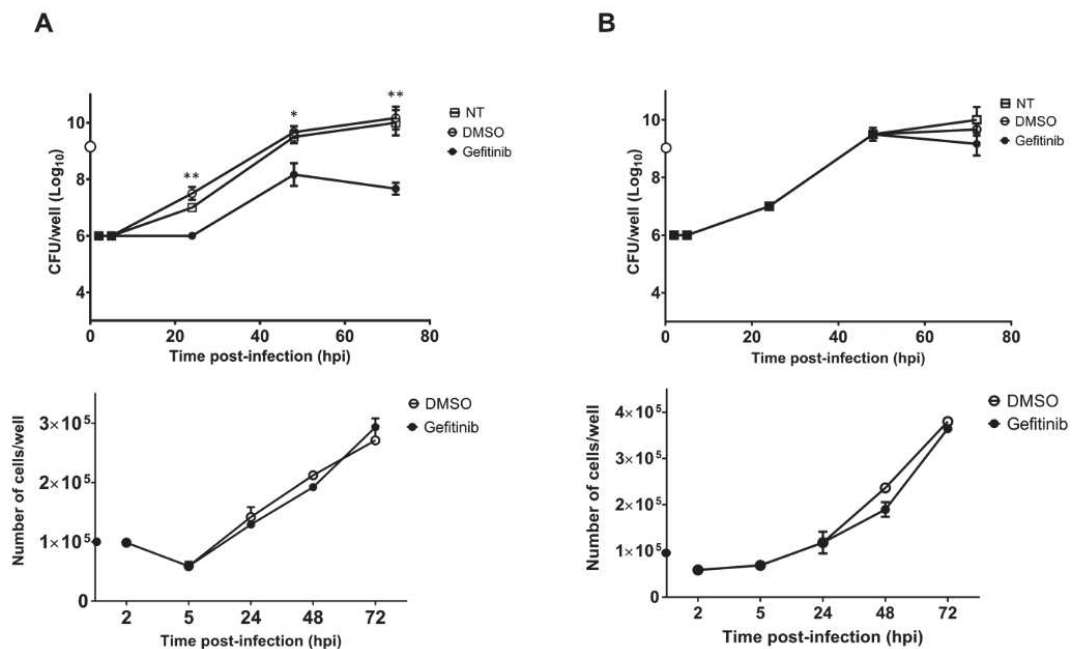
**Figure 20: Protective effect of Gefitinib on *Brucella* infection in murine macrophages.** **A.** J774 cells were pre-treated with Gefitinib and infected with *B. melitensis* using a MOI of 50 in a gentamycin protection assay. Cells were then lysed at the indicated hours post infection (hpi) for enumeration of intracellular colony forming units (CFU). Treatment with Gefitinib (5  $\mu$ M) was continuous during the infection assay. The data shown are the geometric means of Log<sub>10</sub> CFU per well  $\pm$  Standard Error of the Mean (SEM) from three independent experiments. In parallel, cells were counted at each timepoint to evaluate a possible effect of Gefitinib on cell numbers. **B.** Alternatively, cells were pre-treated with Gefitinib and infected with *B. melitensis*, *B. abortus* or *B. suis* using a MOI of 50 in a gentamycin protection assay. Cells were then fixed at the indicated hours post infection (hpi) for analysis by flow cytometry (% of infected cells, left) or confocal microscopy (right) and the number of intracellular bacteria was quantified semi-automatically using the IMARIS software (version 9.5.1). Treatment with Gefitinib (5  $\mu$ M) was continuous during these infection assays.

Taken together these results demonstrate the protective role of a continuous treatment of Gefitinib on both human trophoblasts and murine macrophages against infection by the 3 main zoonotic species of *Brucella* (*B. melitensis*, *B. abortus* and *B. suis*).



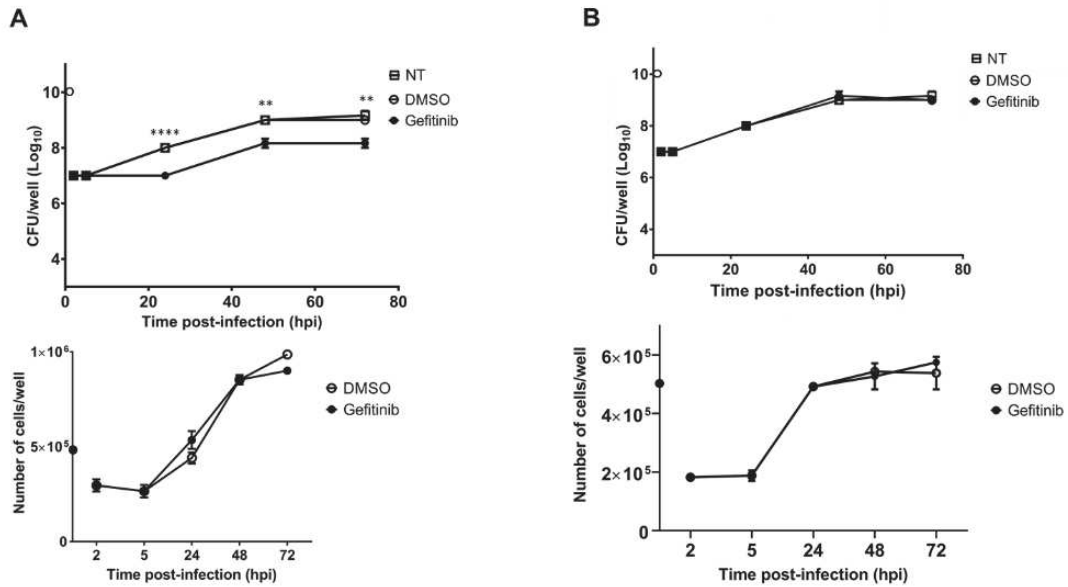
### 3.2.2. Curative effect of Gefitinib against *Brucella* infection in human trophoblasts and murine macrophages

Given the remarkable efficacy of a continuous Gefitinib treatment throughout infection, we wondered if the drug would have a curative effect when added after infection. We chose to start treatment either 5hpi (when bacteria are initiating replication) or 24hpi (when cells are full of bacteria). In BeWo cells, when the drug was added 5h post infection, bacterial burden was significantly lower from 24 hpi, reaching 3 Log<sub>10</sub> at 48 hpi (Figure 21). When treatment was started at 24 hpi no significant differences were observed. The same tendency was observed in J774 cells (Figure 22).



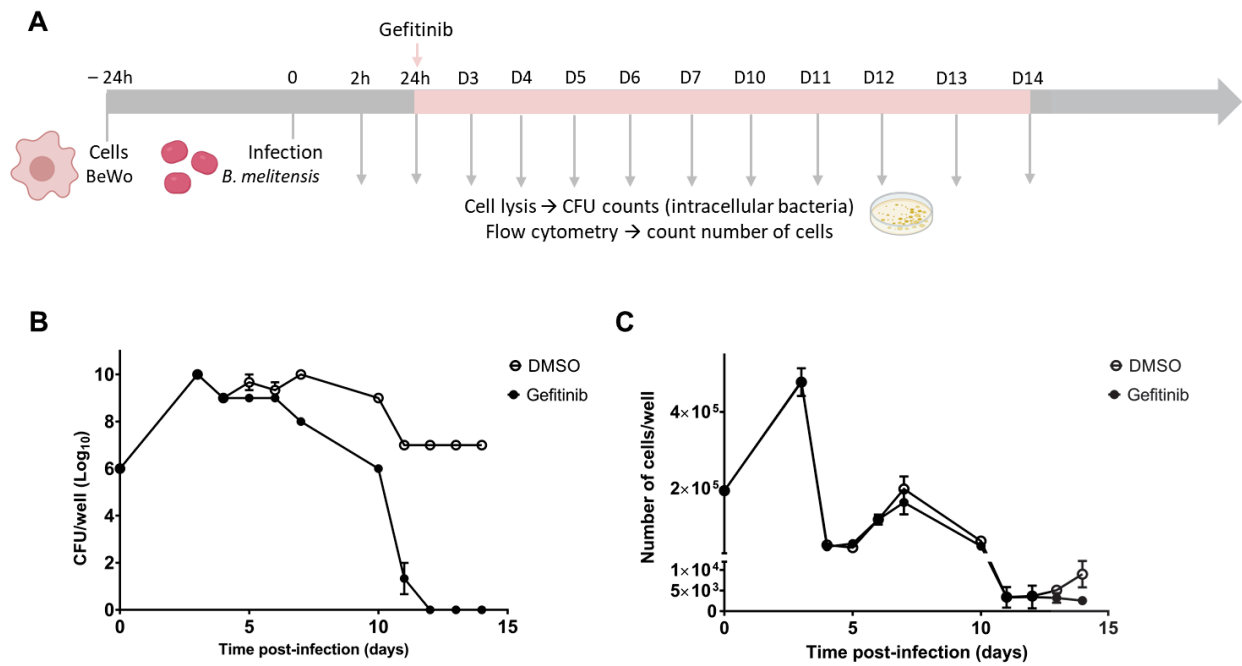
**Figure 21: Curative effect of Gefitinib on *Brucella* infection in human trophoblasts.** BeWo cells were infected with *B. melitensis* using a MOI of 500 in a gentamycin protection assay. Infected cells were then treated with 5  $\mu$ M Gefitinib after 5 (A) or 24h (B). They were then lysed at the indicated hours post infection (hpi) for enumeration of intracellular colony forming units (CFU). The data shown are the geometric means of Log<sub>10</sub> CFU per well  $\pm$  Standard Error of the Mean (SEM) from three independent experiments. In parallel, cells were counted at each timepoint to evaluate a possible effect of Gefitinib on cell numbers.

### 3. RESULTS



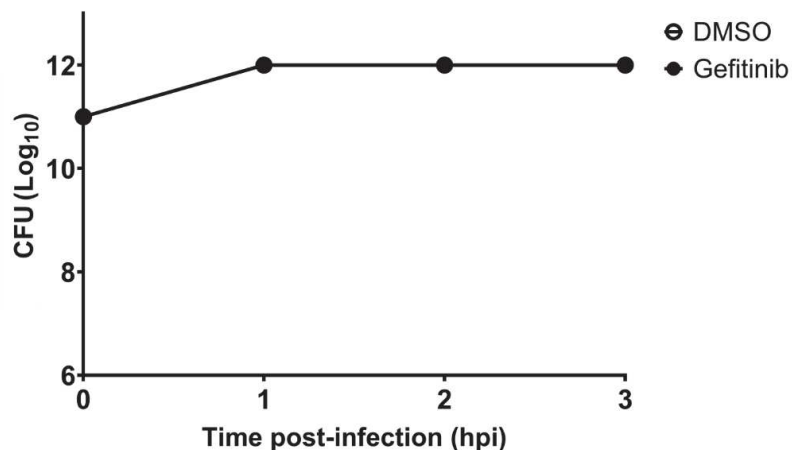
**Figure 22: Curative effect of Gefitinib on *Brucella* infection in murine macrophages.** J774 cells were infected with *B. melitensis* using a MOI of 500 in a gentamycin protection assay. Infected cells were then treated with 5  $\mu$ M Gefitinib after 5 (A) or 24h (B). They were then lysed at the indicated hours post infection (hpi) for enumeration of intracellular colony forming units (CFU). The data shown are the geometric means of Log<sub>10</sub> CFU per well  $\pm$  Standard Error of the Mean (SEM) from three independent experiments. In parallel, cells were counted at each timepoint to evaluate a possible effect of Gefitinib on cell numbers.

When BeWo cells were treated with Gefitinib starting 24hpi, a slight decrease in CFU was observed 48hpi (1 Log<sub>10</sub> difference) (Figure 21). To investigate whether bacterial number would continue to decrease after 48hpi, we followed bacterial burden for a longer period. To do so, infected cells were treated with Gefitinib at 24hpi and then replated every 2 days up to day 14. Gefitinib decreased progressively the CFU counts until 12 days of treatment, from when no live intracellular bacteria could be detected (Figure 23). This indicates that Gefitinib is able to clear *Brucella* infection in human trophoblasts.



**Figure 24: Curative effect of Gefitinib on *Brucella* infection in human trophoblasts in long term experiments.** **A.** Workflow of the experiment. BeWo cells were infected with *B. melitensis* using a MOI of 500 in a gentamycin protection assay. Infected cells were then treated with 5  $\mu$ M Gefitinib starting 24 hpi and for up to 14 days. Cells were re-plated every 2-3 days and lysed at the indicated hours post infection (hpi) for enumeration of intracellular colony forming units (CFU). **B.** The data shown are the geometric means of Log<sub>10</sub> CFU per well  $\pm$  Standard Error of the Mean (SEM) from one experiment. **C.** In parallel, cells were counted at each timepoint to evaluate a possible effect of Gefitinib on cell numbers.

To ensure that bacterial clearance was because of an effect of Gefitinib on infected cells and not directly on bacteria, we incubated *B. melitensis* with DMSO or Gefitinib for 3 consecutive days. Every day bacterial suspension was diluted and incubated in fresh TS containing Gefitinib or DMSO. Gefitinib had no effect on *Brucella* viability or growth (Figure 24).



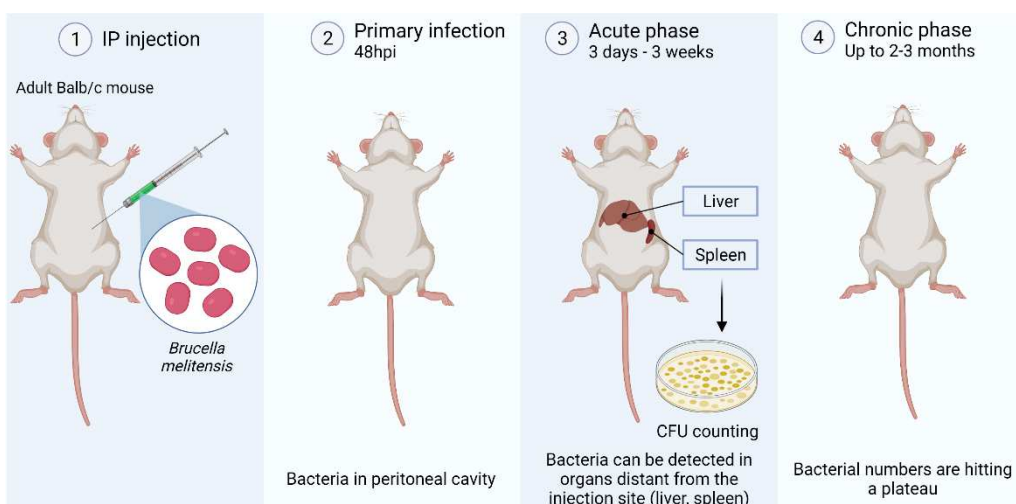
**Figure 23: Effect of Gefitinib on *B. melitensis*.** Bacterial suspension of *B. melitensis* was treated either with DMSO or Gefitinib 5  $\mu$ M for 3 days. Every day, bacterial suspension was plated for enumeration of colony forming units (CFU). Media was changed and bacterial suspension was diluted to OD 0.1 before further incubation. The data shown are the geometric means of Log<sub>10</sub> CFU  $\pm$  Standard Error of the Mean (SEM) from one experiment.

### 3. RESULTS

Besides its inhibitory action on *Brucella* infection when added at the time of infection, Gefitinib also acts as a curative treatment by restricting the growth of *B. melitensis* and clearing infection when the cells are treated 24hpi. Moreover, this effect is clearly due to the action of Gefitinib on the infected cells and not the bacterium itself. These *in vitro* findings suggested that Gefitinib could be a candidate as an alternative treatment for brucellosis.

#### 3.2.3. Effect of Gefitinib in a murine brucellosis model

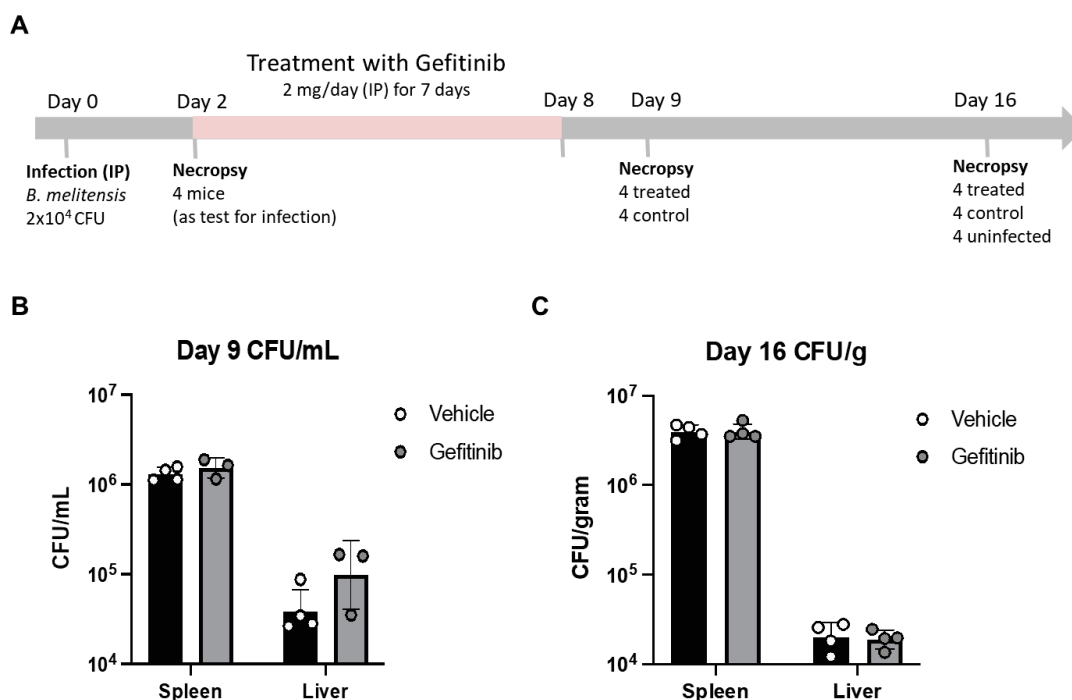
Given the remarkable effect of Gefitinib on *Brucella* infection *in vitro*, we initiated a collaboration with the group of Pr Renee Tsois (UC Davis, CA, USA) to evaluate Gefitinib *in vivo*, in a murine model of brucellosis. In this model adult mice are infected by intraperitoneal (IP) injection of *Brucella* (Figure 25). During the first 48hpi, bacteria reside around the injection site, in the peritoneal cavity, and the inflammatory response is quite low (Grilló et al., 2012). After this initial stage, infection progresses to the acute phase from day 3 until approximately 3 weeks after infection. During this time, bacteria reach organs that are distant from the injection site (mainly liver and spleen) where they multiply actively. The inflammatory response peaks at this stage with production of INF- $\gamma$ , IL-12, IL-6 and RANTES. The next stage of murine brucellosis is the chronic phase. During this phase that last up to 3 months, bacterial numbers reach a plateau. Lastly, the chronic phase is followed by a declining phase which corresponds to a slow elimination of the bacteria. This can last beyond 7 months.



**Figure 25: Mouse model of brucellosis.** Animals are infected with a peritoneal injection of *Brucella*. In mice, brucellosis is divided in 3 different phases. The first phase is the primary infection. After 48hpi, bacteria are found within the peritoneal cavity, around the injection site. Starting 3 days and lasting until 3 weeks pi, mice enter the acute phase of infection during which bacteria reach organs distant from the injection site (liver and spleen) and multiply rapidly. This phase is also characterized by a peak in inflammatory response. Lastly, in the chronic phase, lasting up to 3 months, bacterial numbers reach a plateau. Created with Biorender.com.

### PART III. FIGHTING BRUCELLA INFECTIONS: A DRUG REPOSITIONING STRATEGY

In our experimental setup, adult BALB/c mice were infected by IP injection of *B. melitensis*. In our first trial, mice were then treated with Gefitinib by IP injection for 7 consecutive days starting 2 days after infection. Mice were sacrificed a day 9 or day 16 to assess *Brucella* CFUs in spleen and liver (Figure 26). With this experimental setup, no differences were observed between treated and control mice.



**Figure 26: Evaluation of IP injection of Gefitinib in a murine model of brucellosis.** **A.** Workflow of the experiment. Adult BALB/c mice were infected IP with *B. melitensis* ( $2 \cdot 10^4$  CFU in PBS). 2 days after infection, mice were sacrificed to assess successful infection. Other mice were treated IP with Gefitinib (2mg/day) or the suspension buffer as a control for 7 consecutive days. **B.** At day 9, treated and control mice were sacrificed. Liver and spleen were collected for enumeration of colony forming units per mL of resuspended tissue (CFU/mL). **C.** At day 16, treated, control and non-infected mice were sacrificed. Liver and spleen were collected and weighted for enumeration of intracellular colony forming units per gram of tissue (CFU/g). Increase in spleen weight is also an indicator of splenomegaly, a known phenomenon in the murine model of brucellosis. The data shown are the geometric means of CFU per mL or g  $\pm$  Standard deviation from one experiment.

There was however a technical issue with this preliminary assay. Gefitinib is indeed notoriously very difficult to get into solution (Reddy & Vahini, 2020). This compound is soluble in DMSO, but animal experiment ethical committees limit injection of DMSO to a maximum of 10%. To obtain an injectable Gefitinib solution, the powder thus had to be dissolved sequentially in DMSO (qsp 10% of the final volume), then in PEG300 (40% final), then in Tween-80 (5% final) and finally in PBS (45% final). It is important to mention that several attempts were necessary to successfully solubilize Gefitinib with this procedure. It can thus not be excluded that IP injection of such solution did not allow Gefitinib to diffuse to the different tissues.

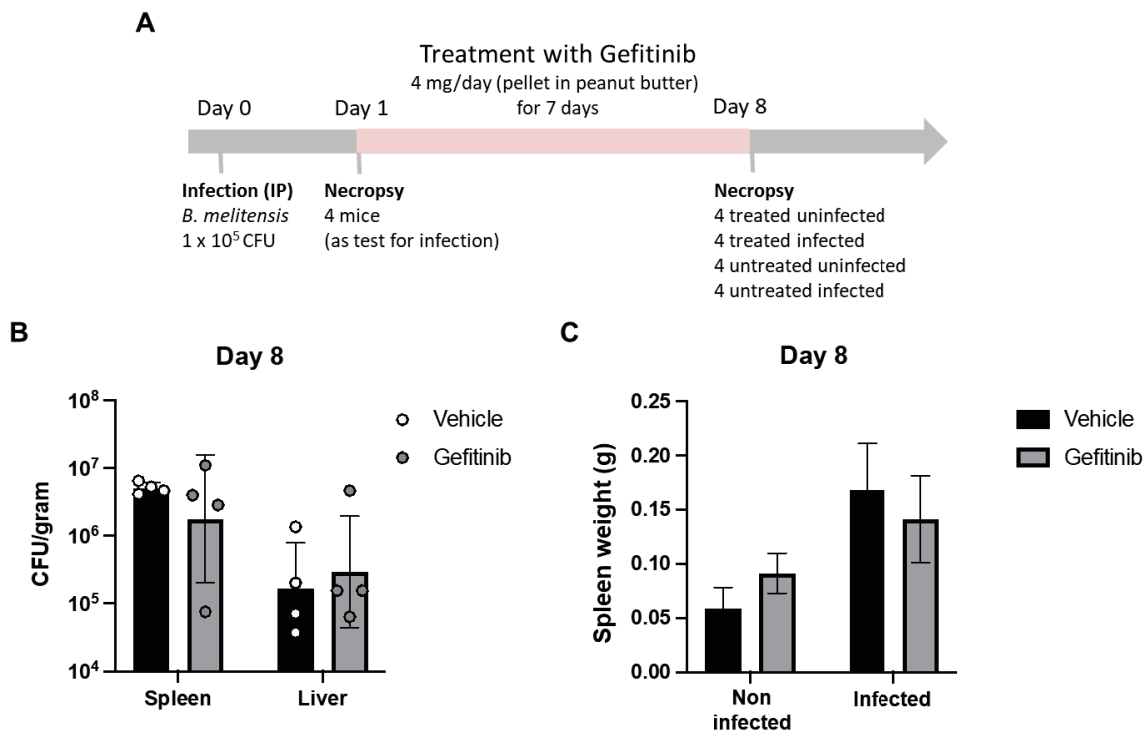
### 3. RESULTS

Given the technical issue described above we cannot exclude that the absence of effect of the treatment is due to an altered bioavailability of Gefitinib and cannot conclude on these data. We therefore developed another protocol with a different route of administration for the drug.

In many *in vivo* studies using Gefitinib the drug is given orally and passage through the stomach is believed to be necessary for its bioavailability and diffusion in the organism. Moreover, using oral administration allows to increase the dose of drug as up to 150 mg of Gefitinib/kg of body weight/day (*i.e.*  $\pm$  4mg/day for 25g mice) has little side effects in mice. We thus designed a protocol in which Gefitinib is given by oral administration via formulation in appetent food. Self-administration of compounds is a non-invasive method that minimizes injury potential and results in attenuated stress response compared to intragastric gavage (Gonzales et al., 2014). Additionally, Gefitinib was found to have efficient anti-oncogenic activity in mice when administered orally in peanut butter (Lorch et al., 2007).

In the second trial, we thus mixed Gefitinib powder into peanut butter and shaped the mixture into frozen pellets, allowing convenient distribution to mice and accurate dosing of the drug. Infected mice were given pellets once a day for 7 consecutive days starting 1-day post infection. Compared to the first assay, Gefitinib treatment was thus started sooner and with a higher dose. Mice were sacrificed a day 8 pi to assess *Brucella* CFUs and organ weight in spleen and liver. Again, no significant differences were observed between treated and control mice (Figure 27). It is however to note that one mouse in the treated group contained low number of CFU in the spleen. Also, spleen weights seemed to be lower in mice treated with Gefitinib, although non-significantly (Figure 27C). It is thus possible that the absence of significant effect could be due to the low number of mice per group. Also, some animals developed feeding avoidance of peanut butter pellets which may have somehow affected consumption of Gefitinib in some of them.

At this point we are still optimizing the technical details of the protocol such as route of administration and drug dose. We are confident that Gefitinib is a promising drug candidate for the treatment of brucellosis and experiments are ongoing to continue studying its effect in an *in vivo* model of murine brucellosis.



**Figure 27: Evaluation of oral administration of Gefitinib in a murine model of brucellosis.** **A.** Workflow of the experiment. Adult BALB/c mice were infected IP with *B. melitensis* ( $1.10^5$  CFU in PBS). 1 day after infection, mice were sacrificed to assess successful infection. The other mice were treated orally with Gefitinib (4mg/day) in peanut butter or peanut butter alone for 7 consecutive days. Mice were isolated at the time of feeding to ensure accurate drug dosing. A habituation period to peanut buffer was also performed for 6 days before infection. **B.** At day 8, treated and control mice were sacrificed. Liver and spleen were collected and weighted for enumeration of intracellular colony forming units per mL of resuspended tissue (CFU/g). The data shown are the geometric means of CFU per mL or g  $\pm$  Standard deviation from one experiment. **C.** Are shown the spleen weights at day 8.

## 4. Discussion and perspectives

We assessed the efficacy of simvastatin and Gefitinib against *Brucella* infection in a drug repositioning strategy. Treatment of BeWo cells with simvastatin did not impact *Brucella* infection (Figure 16). As statins inhibit the HMG-CoA reductase, which is involved in Rho GTPases signaling, it would be interesting to test the effect of simvastatin in BeWo clone 3 cells as RhoA seems constitutively activated in these cells. On the contrary, Gefitinib remarkably inhibits *Brucella* replication in both human trophoblasts and murine macrophages, an effect observed with the 3 major zoonotic species (Figures 18-20). Gefitinib treatment also inhibits *B. melitensis* infection when applied after infection in both cell types, and completely cured infection when long term treatment was applied on human trophoblasts (Figures 21-23). These observations were however obtained with established cell lines. The BeWo cells derive from a human choriocarcinoma (Pattillo & Gey, 1968) and the macrophage-like cells J774.1 from a murine sarcoma (Forman &

#### 4. DISCUSSION AND PERSPECTIVES

Moller, 1973). Even if there is no indication that the transformation of these two cell lines involved de-regulation of EGFR phosphorylation, as it is the case in the cancer cells specifically targeted by Gefitinib, it cannot be excluded that this drug can protect from *Brucella* infection only in tumor cells. It would thus be useful to reproduce these experiments in primary cells. Murine bone marrow derived macrophages (BMDM) are commonly used cellular models to study *Brucella* infections. Precursor cells are recovered from the femur and tibia bone marrow of adult mice and differentiated into adherent macrophages using granulocyte-macrophage colony-stimulating factor (GM-CSF). The primary macrophages obtained can be used for infection after 7 days in culture.

Given the protective effect of Gefitinib against *Brucella* infection *in vitro*, we started assessing the effect of Gefitinib in mice in collaboration with the group of Pr Renee Tsois (UC Davis, USA). After the first trial, when Gefitinib was given IP, we saw no effect on CFU numbers (Figure 26). However, we cannot yet conclude on these data. Because the drug is very hard to get into solution, we cannot exclude that Gefitinib did not diffuse to the different organs. Molecular analyses (qRT-PCR, Western blot) will be performed to assess whether the drug had any effect on the cells of target organs during this experiment. The oral route of administration was more promising, but no significant effect was observed on bacterial burden (Figure 27). We are currently repeating this experiment with a higher dose of Gefitinib (8 mg/day) and a larger number of animals. Longer treatment with Gefitinib could also be envisaged.

Gefitinib alone might not be sufficient to treat brucellosis, and the clinical application for this drug is rather to be used alongside antibiotics, with the idea to reduce the duration and side effects of the treatment currently used (*i.e.* combination of rifampicin and doxycycline for 6 weeks). We thus propose next to evaluate the time required to eradicate *Brucella* in mice using antibiotics alone or in combination with Gefitinib. After doing a literature review of the studies that used both antibiotics in the murine model of brucellosis, we propose a protocol in which antibiotics will be given in the drinking water at a dose of 2mg/day for doxycycline and 0.5g/day for rifampicin. We will first determine the kinetic of bacterial killing by this treatment. Mice will be infected IP and treatment will be started 7 dpi. Mice will be sacrificed at day 0, 4, 7, 10 or 14 post-treatment to determine bacterial numbers in spleen and liver. Based on these results, we will decide on the best timing for the co-treatment with Gefitinib by oral administration.



If the effect of Gefitinib on *Brucella* pathogenesis is confirmed *in vivo*, a long-term perspective would be to evaluate in clinical trials the drug alone or in combination to the recommended antibiotherapy to decrease its duration and prevent disease relapse.

# General discussion & concluding remarks

## General discussion & concluding remarks

My research project concentrated on the infection of human trophoblasts by the bacterial pathogens *Brucella*. Understanding the mechanisms by which *Brucella* enter and multiply in these placental cells, and how can this affect their biology, are important issues to address. Although underestimated for long time, acquisition of brucellosis during pregnancy is now recognized as a risk for pregnancy outcome. Studies performed in the Middle East, South America and Africa showed that the rate of spontaneous abortions in infected women range from 18.6 to 73.3%. *Brucella* infection also increases the risk of pre-term delivery (ranging from 6.9 to 72%) which is the leading cause of death in children under 5 years old. On top of these complications that are life threatening to the fetus, infection can be passed down from the mother to the fetus during pregnancy or during delivery, and infants that develop congenital or neonatal brucellosis face long-term feeding and growth issues.

Most brucellosis cases occur during the 1<sup>st</sup> or 2<sup>nd</sup> trimester. These early stages of pregnancy are critical for proper development of the placenta since maternal tissues undergo extensive remodeling to allow embryo implantation and development. It is now known that *Brucella* can infect the different types of human trophoblasts (cytotrophoblasts, syncytiotrophoblasts and extravillous trophoblasts) and that infection can be transmitted between these cells (García-Méndez et al., 2019c; Salcedo et al., 2013). Even though the exact mechanisms of transmission of infection from the mother to the fetus are not known, we could imagine that *Brucella* could reach the placenta via the maternal decidua. Inside the placenta, the bacteria would infect and replicate inside trophoblasts, thus de-regulating their fusion and migration abilities and disrupting normal placental development. Infection could then spread from cell-to-cell inside the chorionic villi and finally reach the fetus. Thus, *Brucella* infection opposes serious risks for both the mother and the baby.

Fortunately, these consequences can be avoided as prompt diagnosis and treatment significantly reduce the risk of adverse pregnancy outcomes. However, brucellosis is a disease of poverty. Countries that are endemic for brucellosis do not have the means to correctly diagnose and treat infection leading to a poor medical supervision of patients, among which pregnant women and their babies are at serious risk. Furthermore, countries dealing with brucellosis also face other infectious diseases that benefit from a

better public exposure such as malaria. This context makes it difficult to raise awareness towards brucellosis in endemic countries.

In our studies we focused on CD98hc, a multifunctional host protein important for *Brucella* infection in epithelial cells and fibroblasts. We tried evaluating its role in infection of human trophoblasts by inactivating its gene (*SLC3A2*). Very few studies describe successful inactivation of *SLC3A2* in cells. We based our strategy on a study published by Wang and colleagues (S. Wang et al., 2017) in which they inactivated *SLC3A2* in human gastric cells by CRISPR/Cas9. They found that CD98hc KO decreased the invasive capacity of these cells but had no effect on their viability or proliferation. In our hands, using the same sgRNA sequences, inactivation of *SLC3A2* in BeWo cells was unsuccessful. The single cellular clone that survived our attempts had different pattern for CD98hc expression depending on the antibody used for labelling, and deep analyses showed that these cells express a CD98hc protein with modified C-ter and altered sub-cellular localization. In the study by Wang and colleagues, inactivation of *SLC3A2* was only verified by Western Blot. It can thus not be excluded that these cells expressed a CD98hc protein that could not be recognized by the antibody they used; sequencing of *SLC3A2* would have been useful to confirm the KO of this gene.

We anticipated that knocking out *SLC3A2* may be very difficult in cells. This gene is indeed considered as essential because its disruption is lethal in mouse embryos (Tsumura et al., 2005). In this study, disruption of CD98hc did not allow transmission of the mutated allele to the progeny. However, one aberrant clone that resulted in an extra 1.0-kb transcript in the genomic DNA of heterozygous mouse was transmitted to progeny. The predicted protein would have an intracellular, a transmembrane and part of the extracellular domain. No mice homozygous for this aberrant targeting were observed suggesting that expression of this aberrant protein was lethal between day 3.5 and 9.5 of embryonic development. These results showed that intact CD98hc protein is necessary for embryonic development. The putative protein described here also resembles what we found in BeWo-clone 3 cells, suggesting that these cells are an important tool to understand CD98hc functions.

In a pioneering work, inactivation of *SLC3A2* was performed in mouse embryonic stem (ES) cells by replacing exon 1 (*i.e.* exon 5 with the nomenclature used in this thesis), which encodes the transmembrane domain of CD98hc, by a neomycin selection cassette (Feral

et al., 2005). The CD98hc KO ES cells obtained had serious morphology changes, impaired cell proliferation and migration. These cells also require addition of  $\beta$ -ME in their culture media to survive, as for the BeWo-clone 3 cells described in our work. However, further study, carried out years later, revealed that these cells express intracellularly a CD98hc protein with truncated N-ter and transmembrane domains (C. Feral & A. Keriél, unpublished observations). Recent characterization of these cells also showed that their reduced proliferation rate is due to an imbalanced AA intracellular content and that these cells had a compensation mechanism involving increased expression of several CD98hc-independent AA transporters (Ballina et al., 2016). The same group next used the Cre/lox system to generate CD98hc-null murine embryonic fibroblasts (MEF) (Féral et al., 2007). The authors first generated transgenic mice in which exons 1 and 2 (*i.e.* exons 5 and 6 according to the nomenclature used in this thesis) were flanked by loxP sites. MEF were then derived from homozygote embryos and CD98hc was deleted *in vitro*, by infecting these cells with an adenoviral vector encoding the Cre recombinase. Excision of exons 1 and 2 was verified by PCR, and cells were further sorted to select fibroblasts lacking detectable CD98hc expression. CD98hc-null MEF had reduced proliferation rate and died in absence of  $\beta$ -ME in their culture media. More recently, the KO of *SLC3A2* was successfully performed in murine derm fibroblasts (mDF) (Tissot et al., 2018). These cells were obtained from transgenic mice with loxP-flanked *SLC3A2* and constitutive expression of Cre controlled by Fsp1 (fibroblast specific promoter), ensuring tissue-specific inactivation of *SLC3A2* in the dermis. mDF were obtained from the derm of adult mice and transfected with SV40 LargeT/pBR vector for immortalization. Proper deletion of exons 1 and 2 was assessed by PCR. The genome of these cells was also sequenced (C. Feral, personal communication). These are the cells that had a very strong impairment in *Brucella* infection (Keriél et al., 2015).

In another study, Digomann and colleagues used CRISPR/Cas9 to inactivate *SLC3A2* in the squamous cell carcinoma cells Cal33 RR (Digomann et al., 2019). They used sgRNA molecules targeting sequences localized upstream and downstream of the gene. Verification of deletion was performed by both PCR and sequencing of the regions adjacent to the sgRNA targets. Homozygous KO was impossible, so they used a monoallelic KO. These cells appeared had de-regulated AA metabolism and increased oxidative stress; a phenotype similar to CD98hc murine null cells described above. Also, these cells were cultured in an enriched DMEM media that did not contain  $\beta$ -ME.

Finally, another group used zinc-finger nucleases (ZFN) to disrupt CD98hc expression in the colon adenocarcinoma LS174T cell line (Cormerais et al., 2016). The zinc-finger (ZF) motif consists of 1  $\alpha$ -helix and 2  $\beta$  sheets stabilized by 2 cysteine and 2 histidine residues and that bind a zinc ion (Hauschild-Quintern et al., 2013). This motif can bind nucleotide triplets in the major groove of DNA using residues in the  $\alpha$ -helix. ZF can be designed to bind to any 3 bp combination. Also, several ZF motifs can be combined to form a larger site-specific ZF DNA recognition domain. A ZFN is composed of a site-specific ZF DNA binding domain fused to the non-specific cleavage domain of the FokI endonuclease. As this nuclease must dimerize to cleave DNA, 2 ZFN molecules are used together. After binding to the target sequence, a double strand DNA break occurs. Cellular repair is based on the same mechanisms following a CRISPR/Cas9 mediated DNA break. After transfection with the ZFN targeting *SLC3A2*, CD98hc-negative or low expressing cells were sorted and cloned. Then, each clone was analyzed for CD98hc expression by immunoblot using the antibody H300 (recognizing sequences in CD98hc C-ter). Negative clones were re-cloned and analyzed by DNA sequencing to ensure *SLC3A2* KO. Surprisingly, the CD98hc KO cells did not exhibit any deficiencies in cell proliferation or survival. We performed an *in-silico* analysis using the sequences provided in this manuscript and found that one of the CD98hc KO cellular clone retained the capacity to encode for a truncated protein expressing only the first 119 residues of the N-ter domain. This putative protein would thus not contain the conserved cysteine C1 and would not be able to associate with CD98hc-dependent light chains. Using the same strategy, they also obtained LAT1 KO cells which presented several proliferation and AA metabolism impairments. LAT1 KO but not CD98hc KO cells required a “super rich” culture medium to have normal proliferation rate and AA homeostasis. However,  $\beta$ -ME was not needed for their survival. The authors also describe a CD98hc/LAT1 double mutant that appears to have a very similar phenotype as LAT1 KO cells. Given the importance of both proteins for the cell’s biology, it is very surprising that they could survive. Contrary to what is described in other papers, no compensatory mechanisms were found in CD98hc KO cells. Perhaps a broader analysis of all CD98hc-dependent and -independent AA transporters would highlight the mechanisms by which these cells survive. All the studies mentioned above highlight the difficulty of inactivating essential genes in cells. Some discrepancies between the studies raise questions about the veracity of the gene inactivation, although it cannot be excluded that this may depend on the importance of CD98hc for the biological

functions of different cell types. Cellular alternatives would be to develop conditional inactivation system based on a Tet-ON or Tet-OFF approach to allow on-demand inactivation of target genes *in vitro*. This way, the cells could be maintained normally, and inactivation could be triggered at the desired time.

In my research project, we also tried deciphering which function of CD98hc could be hijacked by *Brucella* for their intracellular replication in trophoblasts. We used inhibitors to selectively block either integrin signaling or AA transport. This work has provided clues as to which of these functions is important for *Brucella* infection, but the precise molecular mechanisms remain to be elucidated. However, we did not assess the role of CD98hc as a regulator of membrane fusion. This function was revealed using anti-CD98hc antibodies that target the surface protein on cells. Incubation of cells with such antibodies resulted in either induction or inhibition of cell-to-cell fusion, depending on the cell type and infection context (reviewed in part I of this thesis). CD98hc is usually considered as a surface glycoprotein exerting its different functions at the plasma membrane, but *in situ* immunolabelling also show cytoplasmic localization. It is thus conceivable that this protein would also be present in intracellular membranes (“endomembranes”) and play a role in their fusion. This is consistent with the observation that, during HSV-1 infection, CD98hc is relocated to the nuclear envelope where it mediates membrane fusion to allow virus de-envelopment (Hirohata et al., 2015). This hypothesis is also coherent with our observation that CD98hc is associated with intracellular *Brucella* in BeWo cells early during infection (2hpi) until later stages (20hpi) (data not shown). Endomembrane fusions are well described in the endocytic pathway, where different organelles communicate via vesicular carriers that are generated on one organelle and fuse with another one (Naslavsky & Caplan, 2018). During its intracellular life, *Brucella* reside in a vacuole (BCV) that matures by sequential interactions with several membranous compartments such as endosomes or the endoplasmic reticulum. The role of CD98hc in *Brucella* infection may thus reside in its ability to regulate endomembranes fusion, thus controlling the interaction of the BCV with intracellular organelles. Alternatively, modulation of membrane stiffness could also be involved, as this can impact endomembrane fusion (Saric & Freeman, 2021). Boulter and colleagues showed that the absence of CD98hc in cells modifies the lipid composition of their membranes and, as a consequence, their stiffness (Boulter et al., 2018). The ability of the BCV to fuse with

intracellular organelles could thus also depend on the rigidity of endomembranes. Both hypotheses are currently under investigations.

Finally, in another part of my research project, we transposed our observations on the molecular mechanisms of host-pathogen interactions to evaluate potential inhibitors of *Brucella* infection in a drug repositioning strategy. The recommended treatment for human brucellosis consists of a combination of two antibiotics for 6 weeks, which has many side effects. Moreover, disease relapse is often observed because of the intracellular localization of bacteria, highlighting the limits of the current antimicrobials. Alternative treatments to combat brucellosis are thus urgently needed. Rather than targeting the pathogen itself, efforts of the scientific community in the last years tend to focus on modulation of host factors to control infections. This approach, called host-directed therapy (HDT), is defined as any product that stimulates host defense mechanisms, modulate excessive inflammation or both, leading to an improved clinical outcome (Zumla et al., 2016). HDT can improve host cellular response against pathogens, stimulate immune response or target disease-causing factors. This can be done using monoclonal antibodies, vitamins, cytokines, cellular therapy, recombinant proteins, or repurposed drugs. Repurposing commonly used and affordable drugs is an advantageous strategy used for alternative treatments of neglected infectious diseases such as brucellosis as this approach cuts down cost and time between research and marketing. In this work we identified Gefitinib, an EGFR inhibitor used for the treatment of non-small cell lung cancer, as a very effective drug against *Brucella* infection *in vitro*. Its *in vivo* efficacy is currently under investigation and next steps would be to decipher the molecular mechanisms by which Gefitinib controls *Brucella* infection. As long-term perspectives, Gefitinib could constitute a new therapeutic arsenal to be used in conjunction with antibiotics to fight brucellosis.





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# Appendix



# Appendix

## Résumé en français

### Introduction

La brucellose est une zoonose causée par les bactéries du genre *Brucella*. Ces pathogènes peuvent infecter une large gamme de mammifères (principalement les bovins, caprins et suidés). Chez l'animal, les symptômes majeurs de la maladie sont les avortements et la mort des petits, causant d'importantes pertes économiques chez les éleveurs. Cette infection peut également être transmise à l'Homme par la consommation de produits laitiers non pasteurisés, le contact direct avec des tissus infectés et/ou des sécrétions issues d'animaux infectés ainsi que par aérosolisation. La brucellose humaine se traduit par un syndrome grippal accompagné d'une fièvre ondulante caractéristique et peut conduire à de sévères complications telles que la neurobrucellose et la spondylarthrite en l'absence de traitement. Bien que suspectées depuis le 20<sup>ème</sup> siècle, les preuves de conséquences obstétricales chez la femme enceinte dues à l'infection par *Brucella* ont seulement émergé durant les 10 dernières années. De nombreuses études épidémiologiques démontrent maintenant que l'infection par *Brucella* augmente le risque de complications obstétricales (accouchement prématuré, avortement spontané ou mort foetale) chez la femme enceinte (A. Al-Tawfiq & A Memish, 2013; Alnemri et al., 2017; Elshamy & Ahmed, 2008; Kurdoglu et al., 2010; Vilchez et al., 2015). De plus, l'infection peut être transmise au fœtus *in utero* probablement via le placenta, causant une forme congénitale de brucellose (Samartino & Enright, 1993). Récemment, il a été démontré que *Brucella* peut infecter des cellules placentaires humaines spécialisées : les trophoblastes (Salcedo et al., 2013).

Au sein du placenta, les trophoblastes jouent des rôles essentiels dans l'implantation de l'embryon et les interactions avec l'utérus maternel. Trois types de trophoblastes sont décrits : les cytotrophoblastes indifférenciés (CTB), les syncytiotrophoblastes multinucléés (STB) et les trophoblastes extravilleux (EVT). Les CTBs peuvent se différencier en SYN (après fusion cellulaire) ou en EVT (en envahissant la paroi utérine). Les SYN constituent la couche la plus externe du placenta et médient les échanges d'oxygène et de nutriments entre le sang maternel et le fœtus alors que les EVT ont des capacités phagocytiques impliquées dans le remodelage des tissus maternels requis pour



l'ancrage du placenta et l'approvisionnement en sang du fœtus. Ces deux types de trophoblastes produisent des hormones qui contrôlent le développement placentaire.

Les fonctions trophoblastiques sont étroitement régulées étant donné que des défauts peuvent être délétères et causés des complications lors de la grossesse. La protéine humaine CD98hc (ou 4F2) semble essentielle pour la fusion cellulaire des CTB et la migration des EVT (Kabir-Salmani et al., 2008; Kudo & Boyd, 2002). CD98hc est la chaîne lourde commune d'hétérodimères présents à la surface cellulaire et médiant le transport d'acides aminés (AA). CD98hc interagit aussi avec plusieurs intégrines et module la transduction du signal intracellulaire de cette voie (Fenczik et al., 2000). De façon intéressante, CD98hc a été démontré comme étant essentielle pour l'infection par *Brucella* des fibroblastes dermiques murins (mDF) (Keriel et al., 2015). Des expériences préliminaires d'ARNi dans des lignées trophoblastiques humaines (BeWo et JEG-3) n'ont pu que partiellement réprimer l'expression de CD98hc. Néanmoins, une faible mais significative inhibition de l'infection par *Brucella* a été détectée dans ces expériences (K. B. Garcia Mendez, résultats non publiés), suggérant que CD98hc pourrait jouer un rôle dans l'infection des trophoblastes humains. De plus, nous avons observé que les infections à *Brucella* affectent plusieurs fonctions trophoblastiques (production d'hormones, capacité de fusion de la CTB et capacité d'invasion de l'EVT (García-Méndez et al., 2019d).

Mon projet de thèse porte donc sur le rôle de CD98hc dans l'infection des trophoblastes humains. Nous évaluerons d'abord l'importance de CD98hc pour l'infection à *Brucella* dans ce type de cellules et tenterons ensuite d'identifier quelle(s) fonction(s) de la protéine pourrait être détournée par des bactéries intracellulaires.

## Résultats

### Part I. Importance de CD98hc dans l'infection par *Brucella* des trophoblastes humains

La stratégie la plus simple pour évaluer le rôle d'une protéine dans un processus consiste à inactiver son gène. Cependant, étant donné que la suppression du gène *SLC3A2* (codant pour CD98hc) entraîne une mort embryonnaire précoce chez la souris (Tsumura et al., 2005), suggérant que CD98hc est essentiel à la viabilité cellulaire, l'inactivation de ce gène dans les cellules peut être très difficile. Nous avons donc d'abord essayé de surexprimer des formes mutantes de CD98hc dans des trophoblastes humains. Des

vecteurs d'expression codant pour ces mutants ont été transfectés dans des CTB humains (lignée cellulaire BeWo), qui ont ensuite été infectés par *Brucella melitensis*. Malheureusement, nous n'avons pu détecter aucun changement dans le pourcentage de cellules infectées.

Nous avons ensuite essayé d'inactiver *SLC3A2* à l'aide de la technologie CRISPR/Cas9. Le système CRISPR/Cas ciblant *SLC3A2* (c'est-à-dire les séquences codant pour l'enzyme Cas9 et un sgRNA spécifique) a été introduit dans les cellules BeWo par transduction lentivirale. Nous avons utilisé deux ARN guides différents (sgRNA2 et sgRNA3) ciblant respectivement l'exon 7 ou 10, et déjà validées expérimentalement (Tsumura et al., 2005). Un seul clone cellulaire a pu être amplifié (BeWo-clone 3, transduit par sgRNA2 uniquement). En utilisant un anticorps monoclonal ciblant l'extrémité C-terminale de la protéine CD98hc humaine (anticorps MEM-108), les cellules du clone 3 étaient négatives pour CD98hc en utilisant la cytométrie en flux et la microscopie confocale. Cependant, l'utilisation d'anticorps ciblant l'extrémité N-terminale des isoformes plus longues (anticorps C290970) ou un autre épitope dans le domaine C-terminal (anticorps HBJ126) a révélé que les cellules BeWo-clone 3 expriment CD98hc, suggérant que l'épitope de l'anticorps MEM-108 a été altéré dans la protéine CD98hc modifiée.

Nous avons donc analysé le génome des cellules BeWo-clone 3 pour comprendre les modifications générées par le système CRISPR/Cas. L'analyse a révélé que les deux allèles avaient bien été modifiés au niveau de l'exon 7 et que cela a entraîné la délétion de l'exon entier dans un allèle et une modification de quelques nucléotides dans l'autre. Ces changements entraîneraient théoriquement l'expression d'une protéine CD98hc manquant une hélice alpha dans son C-ter, ou une substitution de 3 résidus. L'expression d'une protéine CD98hc modifiée a ensuite été confirmée par des analyses moléculaires. Une analyse par Western Blot a confirmé que la protéine exprimée dans les cellules BeWo-clone 3 est plus petite que la protéine WT. De plus, la protéine exprimée dans les cellules BeWo-clone 3 présentent un défaut de glycosylation.

Fait intéressant, la localisation subcellulaire de CD98hc est altérée dans les cellules BeWo-clone 3 : la protéine est localisée à la surface cellulaire dans les cellules WT alors qu'elle est intracellulaire dans les cellules clone 3. En utilisant des expériences de co-immunomarquage, nous avons découvert que CD98hc est associé à Rab11 avec son partenaire Galectin-3 dans les BeWo-clone 3, indiquant que la protéine modifiée

s'accumule dans les endosomes de recyclage de ces cellules. Enfin, nous avons évalué l'effet des modifications de CD98hc sur la capacité des *Brucella* à infecter et à se répliquer dans les cellules BeWo. Les cellules ont été infectées avec *B. melitensis* et l'infection a été mesurée par cytométrie en flux 48 heures après l'infection (hpi). Nous n'avons trouvé aucune différence dans le pourcentage de cellules infectées entre les cellules WT et clone 3. Dans l'ensemble, nos données montrent que les cellules BeWo-clone 3 peuvent être efficacement infectées par *Brucella*.

Comme l'inactivation de CD98hc était prévue pour être difficile nous avons en parallèle développé un système d'inactivation conditionnel basé sur une expression de l'endonucléase Cas9 après ajout de doxycycline. Le système fonctionne lorsqu'il est intégré de façon transitoire mais aucune expression de Cas n'est détectée après ajout de doxycycline dans les cellules ou le système est intégré stablement.

## **Part II. Etude des fonctions de CD98hc requises pendant l'infection par *Brucella***

Dans ce chapitre de mon projet de thèse, nous avons tenté d'élucider quelles fonctions de CD98hc sont exploitées lors de l'infection par *Brucella* en inhibant sélectivement le transport des AA dépendant de CD98hc ou la signalisation intracellulaire médiée par les intégrines. Nous avons d'abord évalué l'effet de deux inhibiteurs sélectifs des transporteurs d'AA CD98hc-dépendants : la sulfasalazine (un inhibiteur de xc<sup>-</sup>, un échangeur arginine/glutamine) et BCH (un inhibiteur de LAT1, un transporteur d'AA neutres). Aucun de ceux-ci n'a modifié l'infection par *Brucella* dans les cellules BeWo WT. L'effet du BCH a également été évalué dans le clone 3 de BeWo, car ces cellules semblent avoir un transport d'AA altéré et présentent un défaut de croissance. Une incubation sur la nuit avec du BCH n'a pas affecté l'infection par *Brucella*.

Nous nous sommes ensuite concentrés sur un lien possible entre le rôle de CD98hc en tant que médiateur de la signalisation des intégrines et l'infection à *Brucella* dans les trophoblastes humains. Nous avons quantifié l'infection à *Brucella* dans les cellules BeWo après modulation de plusieurs effecteurs de la voie de signalisation des intégrines. Premièrement, la pré-incubation des cellules avec P5D2 (un anticorps anti-intégrine  $\beta$ 1 agissant comme un agoniste) n'a eu aucun effet sur l'infection. Aussi, aucun effet n'a été observé après un pré-traitement d'une nuit des cellules avec PP2, un inhibiteur spécifique de la kinase Src. Fait intéressant, le prétraitement avec FAK1-14, un inhibiteur spécifique

de la kinase FAK, a diminué l'entrée de *Brucella* de 100 fois dans les cellules BeWo WT. Dans cette condition, les bactéries internalisées semblaient cependant se répliquer avec le même taux que dans les cellules non traitées. De plus, l'inhibition de l'entrée de *Brucella* par FAKi-14 n'a pas été observée dans les cellules BeWo-clone 3, suggérant que l'activité de cette kinase ou les voies de signalisation en aval sont dérégulées dans ces cellules.

### **Part III. Combattre les infections à *Brucella* : stratégie de repositionnement thérapeutique**

Il n'existe actuellement aucun vaccin pour lutter contre la brucellose humaine. De plus, le traitement recommandé (plus de 6 semaines de double antibiothérapie) a de nombreux effets secondaires et peut conduire à une rechute de la maladie en raison de la localisation intracellulaire de la bactérie. Des traitements alternatifs pour lutter contre les infections à *Brucella* sont donc nécessaires. Dans ce chapitre de mon projet de thèse, nous avons décidé d'étudier l'efficacité de médicaments déjà existants. Une telle stratégie de repositionnement des médicaments est souvent adoptée pour les maladies négligées en raison du nombre réduit d'étapes d'essais cliniques requises (réduction du temps et des coûts pour que le médicament atteigne le marché) ou des chaînes d'approvisionnement pharmaceutiques existantes (facilitation de la formulation et de la distribution pas). C'est également une stratégie permettant aux projets de recherche scientifique d'évoluer rapidement vers une recherche axée sur les maladies. Nous nous sommes concentrés sur les médicaments liés à mon projet de thèse, à savoir CD98hc et la signalisation intracellulaire (CD98hc-dépendante ou non). Nous avons tout d'abord évalué l'effet de molécules traditionnellement utilisées pour le traitement du cholestérol mais agissant également sur des molécules de la voie de signalisation des intégrines : les statines. Malheureusement, nous n'avons observé aucun effet de la simvastatine sur l'infection à *Brucella* dans les cellules BeWo, même en utilisant un traitement continu.

Nous nous sommes ensuite concentrés sur un médicament ciblant l'EGFR. En effet, la dérégulation de la signalisation de l'EGFR étant associée à plusieurs maladies humaines (principalement des cancers, en cas de surexpression ou d'activité de l'EGFR), plusieurs médicaments ciblant ce récepteur ont été développés. Certains d'entre eux sont des inhibiteurs spécifiques de l'activité tyrosine kinase de l'EGFR, stoppant ainsi les cascades de signalisation en aval. Un exemple est le Gefitinib, un médicament prescrit pour le traitement de certains cancers du poumon (Paez et al., 2004). Il est intéressant de noter

que les interactions physiques entre l'EGFR et le CD98hc et les interactions entre les voies de signalisation intracellulaires respectives de l'EGFR et des intégrines ont récemment été décrites. Ces observations nous ont incités à évaluer l'effet du Gefitinib sur l'infection à *Brucella*.

Dans les cellules BeWo, le traitement continu par Gefitinib modifie l'entrée de *B. melitensis* (différence 1 Log<sub>10</sub> à 2 hpi) et inhibe très fortement sa répllication intracellulaire (5 Log<sub>10</sub> à 48 hpi). En mesurant l'effet du traitement par le Gefitinib par cytométrie en flux ou microscopie confocale, nous n'avons trouvé pratiquement aucune cellule BeWo infectée et très peu de bactéries dans les rares cellules infectées. De plus, le Gefitinib était également efficace contre les deux autres principales espèces zoonotiques de *Brucella* (*B. abortus* et *B. suis*). L'effet du Gefitinib a ensuite été évalué en tant que traitement curatif. Dans les cellules BeWo, lorsque le médicament a été ajouté 5 h après l'infection, la charge bactérienne était significativement plus faible à partir de 24 hpi, atteignant 3 Log<sub>10</sub> à 48 hpi. Lorsque le traitement a été ajouté 24 hpi, aucune différence significative n'a été observée. La même tendance a été observée dans les cellules J774. Nous avons ensuite suivi la charge bactérienne pendant une période plus longue en passant les cellules infectées tous les 2 jours. Le traitement au Gefitinib a été initié 24 hpi, à un moment où les cellules BeWo sont déjà pleines de bactéries. Après 12 jours de traitement, nous n'avons pu détecter aucune bactérie intracellulaire vivante, ce qui indique que le traitement curatif au Gefitinib a pu éliminer l'infection à *Brucella* dans les trophoblastes humains.

Compte tenu de l'effet important du Gefitinib sur l'infection à *Brucella* *in vitro*, nous avons initié une collaboration avec le groupe du Pr Renee Tsolis (UC Davis, CA, USA) pour évaluer le Gefitinib *in vivo*, dans un modèle murin de brucellose. Dans ce modèle, des souris BALB/c adultes ont été infectées par injection intrapéritonéale (IP) de *B. melitensis* (2,10<sup>4</sup> CFU). Dans la première expérience, des souris ont reçu une injection IP de Gefitinib (2 mg) ou la solution de contrôle pendant 7 jours consécutifs à partir de 48 hpi. Les souris ont été sacrifiées au jour 9 ou au jour 16 pour évaluer les UFC de *Brucella* dans la rate et le foie (Figure 23). Malheureusement, aucune différence dans l'infection à *Brucella* n'a été observée entre les souris témoins ou traitées. Parce que le médicament est très difficile à mettre en solution, nous avons conçu un autre protocole dans lequel le Gefitinib est administré par voie orale. Le Gefitinib a été mélangé à du beurre de cacahuète et façonné en pastilles qui ont été administrées chaque jour pendant 7 jours à partir d'un jour après

## APPENDIX

l'infection. Malheureusement, aucune différence dans l'infection à *Brucella* n'a été observée entre les souris témoins ou traitées.







## Abstract

Brucellosis, an infectious disease caused by bacteria of the *Brucella* genus, is one of the major zoonosis around the globe. Initially an animal disease, the infection can be passed onto humans and increases the risk of obstetrical complications in pregnant infected women. During pregnancy, specialized placental cells called trophoblasts ensure the main functions of the placenta. Several of these functions involve the host protein CD98hc. This surface glycoprotein was shown by our group to be important during *Brucella* infection in different cell types. In this work, we studied the importance of CD98hc in *Brucella* infection of human trophoblasts and tried to decipher which specific function(s) of the protein was (were) hijacked by the bacteria. Knocking out the *SLC3A2* gene (coding for CD98hc) turned out to be impossible, showing that this gene is essential in human trophoblasts. We could however obtain a cellular clone expressing a CD98hc protein with conformational and glycosylation changes in its C-terminal domain and that seems to be trapped in recycling endosomes. Nonetheless, such modifications were compatible with *Brucella* infection, giving clues about how bacteria could use CD98hc during infection. In another chapter of this work, we identified Gefitinib, an EGFR inhibitor used in the treatment of non-infectious diseases, as a promising drug to fight against brucellosis. This drug was shown to be effective *in vitro* against the 3 main zoonotic species of *Brucella* in human trophoblasts as well as murine macrophages. These findings provide new insights into the role of CD98hc in *Brucella* infection and new leads toward the development of improved brucellosis treatments.

## Résumé

La brucellose, une maladie infectieuse causée par les bactéries du genre *Brucella*, est une des plus importantes zoonoses mondiales. Maladie animale, l'infection peut être transmise à l'Homme et augmente le risque de complications obstétricales chez la femme enceinte. Durant la grossesse, les cellules placentaires spécialisées appelées trophoblastes occupent les fonctions centrales au sein du placenta. Plusieurs de ces fonctions impliquent la protéine de l'hôte CD98hc. Cette glycoprotéine de surface a été démontrée par notre équipe comme étant importante pour l'infection de différents types cellulaires par *Brucella*. Dans cette étude, nous souhaitons évaluer l'importance de CD98hc dans l'infection par *Brucella* dans les trophoblastes humains et avons essayé de déterminer quelle(s) fonction(s) spécifique(s) de CD98hc étai(en)t détournées par la bactérie. Inactiver *SLC3A2* (codant pour CD98hc) s'est révélé impossible, montrant le caractère essentiel de ce gène dans les trophoblastes humains. Nous avons cependant obtenu un clone cellulaire qui exprime une protéine avec des changements de conformation et de glycosylation dans son domaine C-ter et qui semble séquestrée dans les endosomes de recyclage. Toutes ces modifications sont cependant compatibles avec l'infection de ces cellules par *Brucella*, donnant ainsi des pistes sur la façon dont ces bactéries utilisent CD98hc au cours de l'infection. Dans un autre chapitre de ce travail, nous avons identifié le Géfitinib, un inhibiteur de l'EGFR utilisé dans le traitement de maladies non infectieuses, comme médicament prometteur pour lutter contre la brucellose. Le Géfitinib est en effet efficace *in vitro* contre les 3 principales espèces zoonotiques de *Brucella* dans les trophoblastes humains et les macrophages murins. Ces résultats fournissent de nouvelles perspectives quant au rôle de CD98hc dans l'infection par *Brucella* et de nouvelles pistes pour améliorer le traitement de la brucellose.