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THESE DE DOCTORAT

En vue d'obtenir le titre de docteur

Les modifications de l'hématopoïèse lors de la brucellose

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Résumé

La brucellose est une zoonose dont l'agent étiologique est une bactérie facultative intracellulaire du genre Brucella. Elle se transmet facilement à l'homme principalement par la consommation de lait cru ou l'inhalation d'aérosols contaminés. Chez l'homme, *Brucella* établit une infection chronique qui persiste pendant des mois ou des années avec différentes manifestations cliniques. *Brucella* persiste dans la rate, le foie, les ganglions lymphatiques et la moelle osseuse. La moelle osseuse est un organe du système immunitaire privilégié et aussi la niche principale de l'hématopoïèse. Cependant, il n'a pas été fait état, à nos jours, de travaux de recherches fondamentales investiguant l'effet de la bactérie dans cet organe.

En utilisant le modèle murin, nous avons démontré, pour la première fois, que *Brucella* persiste dans la moelle osseuse. Les cellules infectées par *Brucella* dans la moelle osseuse sont les monocytes, les neutrophiles et les progéniteurs communs granulo-monocytaires (GMPs). Ces résultats sont particulièrement intéressants parce que les GMPs possèdent des pompes à efflux comme le système ABCG2 qui expulse les médicaments. Cela suggère que les cellules infectées dans la MO peuvent être la source des rechutes de *Brucella* observées chez les patients traités aux antibiotiques. Comme la bactérie est présente dans la moelle, nous nous sommes intéressées à l'effet de l'infection par cette bactérie sur la biologie des cellules souches hématopoïétiques (CSHs).

En effet les CSHs, ne sont pas juste les cellules génératrices des cellules immunitaires mais elles peuvent aussi répondre directement à une infection. Les CSHs expriment à leur surface des récepteurs de reconnaissance de motifs moléculaires et sont capables de reconnaitre des pathogènes. De plus, les CSHs comme d'autres cellules hématopoïétiques expriment à leur surface le récepteur membranaire SLAMF1 (CD150). SLAMF1 est le récepteur du virus de la rougeole et a été identifié comme un senseur bactérien chez les macrophages.

De plus, nous avons découvert que le domaine extracellulaire de SLAMF1 se lie avec la protéine de la membrane externe de Brucella Omp25. L'axe Omp25-SLAMF1 empêche l'activation des cellules dendritiques dérivées de la moelle osseuse (BMDCs) sans affecter la réplication de *Brucella in vitro*. Le rôle de SLAMF1 dans les CSHs est inconnu. Nous avons montré que les CSHs peuvent également reconnaître la protéine membranaire Omp25 de *Brucella* via SLAMF1.

En effet, la reconnaissance directe de Omp25 par SLAMF1 induit la différentiation des CSHs envers la lignée myéloïde, conduisant à une production accrue de progéniteurs et de cellules myéloïdes matures pendant la phase aiguë de l'infection. Ces résultats soulignent pour la

première fois que les CSHs sont capables de détecter directement les motif moléculaire associé aux pathogènes (PAMPs). Sachant que les cellules myéloïdes constituent la principale niche de *Brucella*, l'augmentation de la production de cellules myéloïdes issues des CSHs semble être l'une des stratégies utilisées par la bactérie pour échapper au système immunitaire.

Parallèlement, nous avons découvert que lors de la phase aigue de la brucellose l'axe Omp25-SLAMF1 atténue la réponse immunitaire adaptative, comme l'illustre l'augmentation du nombre de lymphocytes T $CD4^+$ et $CD8^+$ activés chez les animaux infectés par la souche mutante Δomp25 ou chez les animaux n'exprimant pas SLAMF1.

Il est intéressant de noter que l'axe Omp25-SLAMF1 n'a aucun effet sur la réplication bactérienne chez les souris infectées lors de la phase aigue de l'inféction. Par contre, lors de la phase chronique, on observe une augmentation des bactéries chez les souris sauvages qui est dépendante de l'axe Omp25-SLAMF1. Nous suggérant que cette augmentation de charge bactérienne en phase chronique est due à la production accrue de cellules myéloïdes par les CSHs et à l'inhibition de l'inflammation lors de la phase aiguë.

Dans l'ensemble, ces résultats mettent en lumière des mécanismes encore méconnus par lesquels *Brucella* subvertit le système immunitaire. Ils suggèrent que l'interaction Omp25-SLAMF1 est essentielle pour que *Brucella* induise directement l'engagement myéloïde des CSHs et établisse un profil inflammatoire bas pendant lors de la phase aiguë de l'infection. Cela favorise l'installation des bactéries dans leur niche réplicative et leur permet d'induire une infection chronique.

Mots clés : moelle osseuse , cellules souches hématopoiètiques, cellules dendritiques, SLAMF1/CD150, inféction, *Brucella abortus*, Omp proteins

Abstract

Brucellosis, a worldwide re-emerging zoonotic disease, is caused by a facultative intracellular pathogenic bacterium of genus *Brucella*. *Brucella* can be transmitted to humans via contaminated food or infected aerosol particles. In humans, the disease is long lasting, displaying a variety of clinical and pathological manifestation that persist for months and years. *Brucella* persists in spleen, liver, lymph nodes, and bone marrow. Bone marrow is an immune privileged organ and the principal niche for hematopoiesis. However, the impact of *Brucella* infection in this organ has not been studied, as yet.

Using the mice model of brucellosis, we showed that *Brucella* infects and persists in the bone marrow of infected mice. Monocytes, neutrophils and granulo-myeloid progenitors (GMPs) were identified as the cells harboring *Brucella* in the BM. These findings are particularly interesting because GMPs possess drug efflux pumps like ABCG2 system, suggesting that BM infected cells can be the source of *Brucella* relapses observed in antibiotic treated patients.

Since the bacterium is present in the bone marrow, we got interested on studying the effect of *Brucella* infection on hematopoietic stem cells (HSCs) biology. HSCs are not just the suppliers of immune system, but can also directly respond to infection. Indeed, HSCs express pattern recognition receptors (PRR) and are able to sense pathogens. Furthermore, HSCs express as mature hematopoietic cells at their cell surface the receptor SLAMF1 (CD150). SLAMF1 is the entry receptor for Measles-virus and has been identified as a direct bacterial sensor on macrophages. Furthermore, we discovered that SLAMF1 binds to *Brucella* Omp25. SLAMF1/Omp25 axis limits bone marrow-derived dendritic cells (BMDCs) activation without affecting *Brucella* replication *in vitro*. The role of SLAMF1 in HSCs is unknown. We showed that HSCs can also recognize *Brucella* Omp25 via SLAMF1 and studied the functional consequences on hematopoiesis.

We discovered that the direct recognition of *Brucella* Omp25 by HSCs via SLAMF1 induces a functional commitment of HSC toward the myeloid lineage, leading to an increased production of downstream myeloid progenitor and mature myeloid cells during acute infection. These results highlight for the first time that HSCs are able to directly sense pathogen-associated molecular patterns (PAMPs) such as Omp25 through SLAMF1. In addition, knowing that myeloid cells are the principal niche for *Brucella*, the increased myeloid cell production of HSCs seems to be one of the strategies used by *Brucella* to evade host immune detection.

In parallel, we discovered that Omp25-SLAMF1 axis dampens the adoptive immune response during the acute phase of brucellosis as illustrated by higher activated $CD4^+$ and $CD8^+$ T cell numbers in animals infected with the Δomp25 mutant strain or in the animals lacking *Slamf1*. Interestingly, Omp25-SLAMF1 axis has no effect on bacterial replication in acutely infected mice. But bacterial load significantly increases in a Omp25-SLAMF1-dependent fashion at the chronic phase of infection, as a consequence of the increased HSCs myeloid production and of the hitherto modulated inflammation during the acute phase of infection.

Altogether, these findings shed light on yet unrecognized mechanisms by which *Brucella* subvert the immune system. They suggest that the Omp25-SLAMF1 interaction is essential for *Brucella* to directly induce myeloid commitment of HSCs and establish a low inflammatory profile during the acute phase of infection in order to support bacterium settling in its furtive replicative niche and progression to chronicity.

Key words : bone marrow, hematopoietic stem cells, dendritic cells, infection, SLAMF1/CD150, *Brucella abortus*, Omp proteins

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Abbreviations

BCV: *Brucella*-Containing Vacuole
Btp: *Brucella* TIR-Containing Protein
BM: Bone Marrow
BMP: Bone Morphogenic Protein

CβG: Cyclic β 1,2-Glucan
CLN: Cervical Lymph Node

ERES: Endoplasmic Reticulum Exit Site
EEA: Early Endosomal Antigen

GTP: Guanine Triphosphate

HSC: Hematopoietic Stem Cell
HSPC: Hematopoietic Stem and Progenitor Cell
Hsp: Heat Shock Protein 60

iNOS : Inducible Nitric Oxide Synthase 2
Ig : Immunoglobulin
IL : Interleukin
IFN: Interferon
IRAK: Interleukin-1 receptor-associated kinase
IRF: Interferon regulatory factor
IRF-1: Interferon regulatory factor 1
IRF-8: Interferon regulatory factor 8

ITSM: Immunoreceptor Tyrosine-based Switch Motifs

LAMP-1: Lysosomal-Associated Membrane Protein 1
LBP: LPS Binding Protein
LPS: Lipopolysaccharide

MPP: Multipotent Progenitors

NADPH: Nicotinamide Dinucleotide Phosphate Hydrogen
NF-κB: Nuclear factor-kappa B
NK: Natural Killer
NLR: Nod-Like Receptors

OM: Outer Membrane
OMV: Outer Membrane Vesicle
OMP: Outer Membrane Protein

PI3: Phospho-Inositide 3 kinase
PrPC: Cellular Prion Protein
PRR: Pattern recognition receptor

ER: Endoplasmic Reticulum
RANK: Receptor Activator of Nuclear NF-Kb
RANKL: Receptor Activator of

Nuclear NF-κb Ligand

ROS: Reactive Oxygen Species

SR-A: A Scavenger Receptor

SLAM: Signaling Lymphocytic Activation Molecule

TGFβ: Transforming Growth Factorβ

TIR: Toll/IL-1R Homology

TNF-α: Tumor Necrosis Factor α

TCR : T Cell Receptor

TLR: Toll like receptor

T_{reg}: Regulatory T cell

T4SS : Type IV secretion system

UPR: Unfolded protein response

WHO: World Health Organization

WIE: World Organization for Animal Health

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Introduction

Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less.

Marie Curie

Chapter I: Brucellosis

A brief history of *Brucella*

Brucella is a Gram-negative, non-motile, non-sporulant and non-encapsulated coccobacilli. *Brucella* organisms are *α-Proteobacteria* phylogenetically related to plant pathogens and symbionts such as *Rhizobium* or *Ochrobactrum sp.*, to intracellular pathogens such as *Bartonella* or *Rickettsia* and to opportunistic and free living bacteria like *Ochrobactrum* and *Caulobacter*. Compared to enterobacteria, *Brucella* is very resistant to cationic antibiotics due to its peculiar outer membrane composition. It can live outside the host in soil up to 32 days (Moreno, Cloeckaert et al. 2002) .

Brucella is considered to be a facultative intracellular pathogen responsible of a highly contagious zoonotic disease called brucellosis (Malta Fever). The first cases of brucellosis were identified in Malta island, in 1884 by a doctor of the british army, named David Bruce (Bruce 2011). British solders based in Malta at that time were affected by an illness causing an undulant fever. In 1887, Bruce isolated a microorganism that he named *micrococcus* from necropsy of kidneys, livers and spleens of soldiers who suffered from Malta fever. To test if *micrococcus* was the agent causing the Malta fever, Bruce infected seven monkeys. Only three of them survived from infection. The surviving monkeys developed an undulating fever mimicking human symptoms of Malta fever. Thus, in 1887, Dr. Bruce identified for the first time the agent of Malta fever, that he named *Miccrococus melitensis*.

Later on, in 1897, in Denmark, Dr. Bang isolated *Bacillus abortus* from cattle. He noticed that the pathogen can also infect goats, sheep and horses. The disease became known as the Bang disease (Bang 1897).

In 1918, Alice Evans isolated a microorganism from human fetus that was closely related to those of Bruce and Bang. She did several bacterial tests and she concluded that both bacteria were similar and they were neither coccus nor bacillus but coccobacilli. Evans also claimed that the coccobacillus genus caused a zoonotic disease and was the agent responsible for undulant fever, animal abortion and Malta fever. At the beginning, her findings were dismissed by the scientific community. Scientists claimed that if the two pathogens were the same, surely another (male) scientist would have discovered that before Alice. Evans discoveries were accepted only when Paul de Kruif included her in his widely-read book “Men against Death” (Evans and Chinn 1947, Colwell 1999, Colwell, Dantas-Torres et al. 2011). The new genus of coccobacillus responsible for Malta fever and cattle abortion was hence known as *Brucellae species.*

Nowadays, at least 12 *Brucella* species are known (Table 1). These bacteria can infect a wide range of animals including humans.

Brucella species	Colony type	Natural host	Zoonoses
B. melitensis	Smooth	Goat and sheet	+++
B. abortus	Smooth	Cattle	++
B. suis 1-3	Smooth	Pig	++
B. suis 2	Smooth	Wild boar, Hare	+
B. suis 4	Smooth	Reindeer, Caribou	++
B. suis 5	Smooth	Rodent	-
B. ovis	Rough	Sheep	-
B. neotamea	Smooth	Desert Rat	+
B. canis	Rough	Dog	+
B. ceti	Smooth	Dolphins	+
B. pinnipedialis	Smooth	Selas	+
B. microti	Smooth	Wild voles	?
B. inopinata	Smooth	Human	++
B. papionis	?	Baboon	?
B. vulpis	?	Red Fox	?

Table.1 List of different *Brucella* species and their natural host
Adapted from (Atluri, Xavier et al. 2011)

Brucellosis epidemics

Brucellosis is considered by the World Health Organization (WHO) as one of the most dangerous world spread zoonotic disease (Fig.1) and ranks among top 7 “neglected” zoonosis that threaten human health and causes poverty (Bundle and McGiven 2017).

Every year, 500 000 new cases of brucellosis are declared. Hence, brucellosis stays an endemic infectious disease in regions such as India, China, Mongolia, Mid Orient and Sub Saharan Africa. However, brucellosis cases are still detected in developed countries due the wildlife stocks brucellosis and the absence of efficient vaccines.

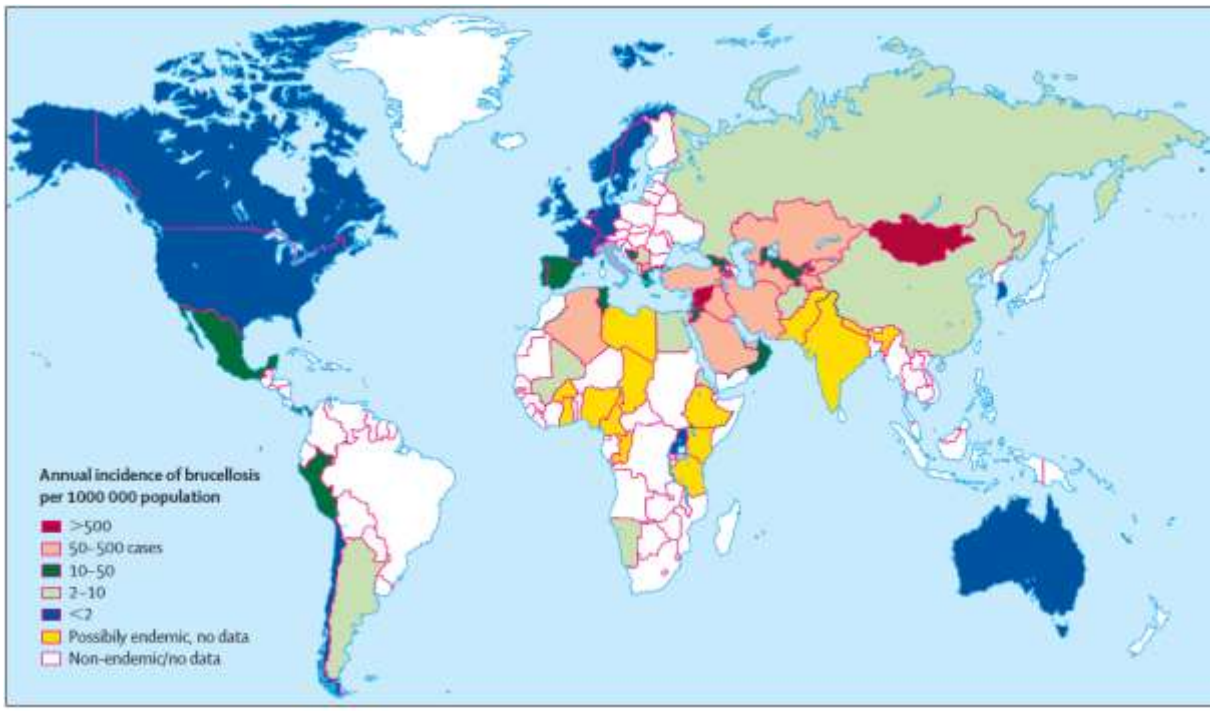

Figure 1 : Worldwide incidence of human brucellosis
Adapted from (Pappas, Akritidis et al. 2005)

Animal brucellosis

Brucella spp. infects domestic animals, mainly livestock, but also wild animals, both terrestrial and aquatic. Each species of *Brucella* tends to be associated with a specific animal host, but other species can be infected, especially when they are kept in close contact (Moreno, Cloeckaert et al. 2002).

In animals, *Brucella* affects the reproductive system (placenta, mammary glands and epididymis) leading to a decrease of fertility in males and abortion and low milk production in females as shown in Table 2 (Byndloss and Tsolis 2016). Transmission within animals occurs via aerosols, ingestion of contaminated placenta or milk and during breeding making *Brucella* highly contagious.

Due to the losses in livestock, brucellosis can cause huge economical losses and there is no efficient vaccines. The existing vaccines are virulent for humans, induce abortion in animals and do not confer protection for all *Brucella* species (Goodwin and Pascual 2016, Bundle and McGiven 2017). The only way to prevent *Brucella* epidemics following the World Organization for Animal Health (OIE) requirements is to organize frequent veterinary controls, kill the infected animals in developed countries and mass vaccination in endemic regions.

Human brucellosis

Not all *Brucella* species are responsible of zoonosis. Human is infected mostly by *B. melitensis*, *B. abortus*, *B. suis* and *B. canis*. Seventy percent of human infections are due to *Brucella melitensis*.

Brucellosis typically manifests as a range of non-specific clinical signs including malaise, undulant fever and fatigue. When the infection is untreated, brucellosis enters into a chronic phase. Most of chronically infected patients suffer from bone lesions like arthritis, osteomyelitis. Other symptoms can be observed to *Brucella* infected patients as endocarditis, splenomegaly, orchitis. Human-to-human transmission can occur trans-placentally, by bone marrow transplantation and blood transfusions. Transmission can also occur through direct contact with infected animals, their tissues (e.g. placenta or aborted tissues), and/or infected animal products (e.g. dairy) (Franc, Krecek et al. 2018). Currently, no vaccine is available to prevent human brucellosis. Indeed, brucellosis patients are treated with a combination antibiotic therapy for least 2 months. But, treatment failures and relapses have been reported. Therefore,

a better understanding of the pathologies in animals and humans remains an important task for the future development of efficient tools applied to brucellosis treatment.

***Brucella* species**	**Clinical Signs**
B. melitensis	**Female**: abortion, weak offspring, reduced milk yield **Male**: infertility, orchitis, epididymitis
B. abortus	**Female**: abortion, weak offspring, reduced milk yield **Male**: infertility, orchitis, epididymitis
B. suis	**Female**: abortion, weak offspring, reduced milk yield **Male**: infertility, orchitis, epididymitis, osteoarticular disorders
B. ovis	**Female**: abortion, weak offspring,bacterimia **Male**: infertility, orchitis, epididymitis, bacterimia
B. canis	**Female**: abortion, weak offspring **Male**: infertility, orchitis

Table.2: Clinical signs of *Brucella* species in domestic animals
Adapted from (Atluri, Xavier et al. 2011)

Brucella- adhesion, survival and persistence in the host

As shown in the previous section *Brucella* is able to induce a persistent chronic infection. To persist and survive in the host, the bacterium has developed a whole arsenal of strategies.

Brucella entry in the host

Brucella abortus and *Brucella melitensis* enter the host primarily via the digestive system or the respiratory tract (Atluri, Xavier et al. 2011). The presence of a relatively long latency phase (usually 2 to 4 weeks) suggests that there is no inflammation following *Brucella* entry into the body. Indeed, *Brucella* surface protein SP41 binds to epithelial receptors carrying sialic acid residues allowing the bacteria to cross the epithelial barrier of the respiratory epithelium. Furthermore, *Brucella* adherence to these receptors leads to cytoskeletal arrangements promoting bacterial invasion (Castaneda-Roldan, Avelino-Flores et al. 2004, Castaneda-Roldan, Ouahrani-Bettache et al. 2006). Once the epithelial barrier is crossed, *Brucella* is engulfed by phagocytic cells, in particular alveolar macrophages and migratory dendritic cells (DCs) and thereafter drained into lymph nodes and secondary lymphoid organs (Archambaud, Salcedo et al. 2010).

In the case of intra-gastric infection, it has been shown in mice that *Brucella* urease is decisive for surviving the acidic environment of the stomach. Indeed, mutant strains for the *ure*1 gene are unable to colonize mice through the gastric route (Sangari, Seoane et al. 2007).

Moreover, infection of mice by intestinal loop model revealed the presence of bacteria in the lysoDCs of follicle associated epithelia (FAE), interfollicular region and in the lamina propria underlying the Peyer's Patches. These results suggested that *B. abortus* uses the tolerogenic properties of DCs to subvert the immune response (Salcedo, Marchesini et al. 2008) .

However, patients infected by oral route suffer mostly from lymphadenitis suggesting that *Brucella* is not a proper enteropathogen. Von Bargen *et al*, showed that bacteria selectively and quickly colonize the cervical lymph nodes (CLN), where they persist for up to 50 days. Intriguingly, *Brucella* induces a local immune response in CLNs without triggering systemic immune response during oral infection. Hence these observations argue that CLNs are a reservoir for *Brucella* promoting bacterial dissemination and chronicity (Gorvel 2014, von Bargen, Gagnaire et al. 2014)

Brucella intracellular life

Most studies of intracellular life of *Brucella* are focused on macrophages. The internalization of the bacterium in these cells involves two different strategies when bacteria are or are not opsonized.

The internalization of non-opsonized bacteria is dependent on the presence of lipid rafts (Naroeni and Porte 2002, Watarai, Makino et al. 2002), Phosphoinositide 3-kinase (PI3-kinase) and Toll Like Receptor-4 (TLR4) (Guzmán-Verri, Chaves-Olarte et al. 2001, Pei, Turse et al. 2008). Two receptors on the surface of macrophages have been identified as having a role in this process, the class A scavenger receptor (SR-A) that interacts with lipopolysaccharide (LPS) (Kim, Watarai et al. 2004) and the PrPC (cellular prion protein) that interacts with the chaperone protein Hsp60 (Watarai 2004). However, the path involving PrPC is controversial since conflicting results have been published (Fontes, Alvarez-Martinez et al. 2005). Internalization is facilitated by the activity of GTPases Cdc42, Rho, Rac, which are recruited at the entry site and interact with the microtubule network and actin cytoskeleton (Guzmán-Verri, Chaves-Olarte et al. 2001, Celli 2019). In the case of opsonized bacteria, internalization is independent of lipid rafts and involves IgG Fc receptors (Naroeni and Porte 2002).

Once inside the cell, *Brucella* resides in a vacuole called BCV for "*Brucella*-Containing Vacuole". Immediately after internalization, BCV transiently interacts with early endosomes and acquires EEA-1 (early endosomal antigen) and Rab 5 markers (Pizarro-Cerdá, Méresse et al. 1998, Celli, de Chastellier et al. 2003, Pizarro-Cerda and Cossart 2006). After this stage BCVs acquires markers of late endosomes, LAMP1, LAMP2, CD63 and the small GTPase Rab7 (Comerci, Martínez‐Lorenzo et al. 2001, Boschiroli, Ouahrani-Bettache et al. 2002).

The interaction with the lysosome is transient and causes the decrease of the pH in the BCV, called in this step eBCV for endosomal BCV. The lipopolysaccharide (LPS) and the cyclic β1,2-glucan (CβG) seem to play an important role in avoiding prolonged contact with the lysosome and protect *Brucella* from antimicrobial effectors (Marchesini, Herrmann et al. 2011). BCV acidification is suggested to be required for the induction of virulence factors from *Brucella* type IV secretion system VirB T4SS. Blocking acidification with drugs such as bafilomycin or monezin inhibits intracellular replication of *Brucella* without affecting the bacterium entry in the cell (Porte, Liautard et al. 1999, Boschiroli, Ouahrani-Bettache et al. 2002, Starr, Ng et al. 2008).

eBCVs target then ERES (Endoplasmic Reticulum Exit Site), an ER sub-compartment where secretory transport is initiated through the formation of COPII-coated cargo vesicles. Indeed, *Brucella* infection upregulates expression of Sar1 and the COPII components Sec23 and Sec24D by an unknown mechanism (Taguchi, Imaoka et al. 2015). Upregulation of these molecules increase the production of secretory vesicles that are suggested to fuse with the eBCV allowing the direct entry of the eBCV to the ER without passing from the classical endosome-Golgi-ER retrograde trafficking processes. The newly formed vacuoles acquire ER membrane-associated markers such as Calreticulin, Calnexin and Sec61β (Pizarro-Cerdá, Méresse et al. 1998, Celli, de Chastellier et al. 2003) that present also structural and functional features of the ER (Sedzicki, Tschon et al. 2018). Modifications of eBCV and its localization in ER correspond to the onset of bacterial replication. For this reason the BCV closely connected to ER is called rBCV for replicative BCV (Celli, Salcedo et al. 2005, Miller, Smith et al. 2017).

After extensive bacterial proliferation in the ER, an additional stage of *Brucella* intracellular cycle consists in the generation of aBCVs (autophagic BCVs) that allow bacteria to finish the intracellular cycle and exit the cell (Starr, Child et al. 2012). Indeed, between 48 and 72 h post-infection rBCVs capture crescent-like membrane structures, leading to the formation of multi-membrane vacuoles structurally reminiscent of auto-phagosomes. aBCVs are released by the cell promoting thus infection of the surrounding cells.

Alternative replicative niches have also been described particularly in human trophoblasts where *B. abortus*, *B. papionis* and *B. suis* replicate in large lysosomal inclusions enriched in LAMP1 (Salcedo, Chevrier et al. 2013, Garcia-Mendez, Hielpos et al. 2019) .

Despite the ability to multiply in high numbers and acquire large amounts of membrane in infected cells, infection with *B. abortus* does not lead to cell death in professional phagocytes. Infected cells undergo normal cell division, showing that basic cellular processes are not affected by *Brucella* infection (Atluri, Xavier et al. 2011).

To conclude *Brucella* intracellular trafficking to the ER is one of the first line of defense of this bacterium against the host.

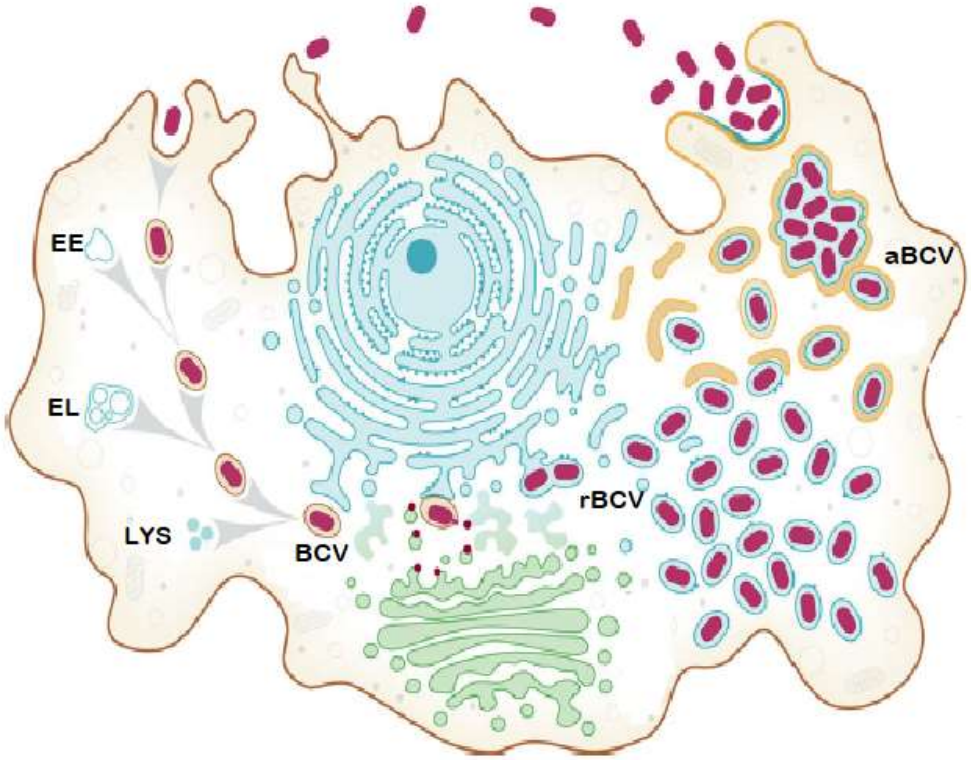

Figure 2: *Brucella* enters the cell through lipid rafts in a vacuole called BCV (*Brucella*-containing vacuole). Once internalized, BCV will interact with early endosome (EE), late endosome (EL) and lysosomes (LY) endosomes. Thereafter, *Brucella* targets ERES (Endoplasm Reticulum Exit Site) and then merges with endoplasmic reticulum vesicles to form the replicative BCV (rBCV). After cell host apoptosis, the replicative BCV is converted into an autophagosome (aBCV) vacuole and allows the bacteria to escape from the cell.

Adapted from (Celli 2019)

Brucella strategies to evade the immune surveillance

Brucella ability to establish persistent and chronic infections consists in the capacity of the bacterium to colonize the host and to escape host immune response (Monack, Mueller et al. 2004).

We have already seen that *Brucella* controls BCV trafficking by avoiding lysosomal degradation. Immune cells, such as macrophages and neutrophils are able to produce reagents such as ROS (Reactive Oxygen Species) or RNS (Reactive Nitrogen Species) during oxidative burst or antimicrobial peptides for the early elimination of pathogens. *Brucella* LPS plays a key role in the resistance to microbial response by inhibiting the action of complement and antimicrobial peptides. Resistance to complement is directly related to its LPS O-chain. Indeed, mutants with rough LPS are more sensitive to complement (Fernandez-Prada, Nikolich et al. 2001). *Brucella* LPS also contributes to resistance to bactericidal cationic peptides as lactoferrin or defensin NP-2 (De Tejada, Pizarro-Cerda et al. 1995, Freer, Moreno et al. 1996).

In addition, neutrophils play a key role in innate immunity. They are often the first cells recruited on inflammatory sites. Recruited at the site of inflammation they release ROS (Reactive Oxygen Species) and lytic enzymes in order to kill the microbes. However, *Brucella* is engulfed by neutrophils but resists to the microbicide activity of these cells.

In contrast to mouse neutrophils, *B. abortus* induces the early death of human neutrophils through the action of its LPS and in particular its lipid A by a mechanism dependent on CD14 and NADPH activation (Barquero-Calvo, Mora-Cartin et al. 2015).

The chronicity during *Brucella* infection is due to the capacity of the bacterium to replicate inside a wide range of cells (endothelial cells, trophoblasts, B cells, T cells, osteoblasts, osteoclasts, etc) and therefore modulate their properties in order to dampen the adaptive immune system.

Brucella abortus infection is associated with a Th-1 response (TNFα, IL-12, IFNγ) during acute infection. A Th-17 response combined to Th-1 response has also been observed during acute infection (Mamber, Lins et al. 2016). However, the role of Th-17 response has to be clarified. In the late phase of infection, *Brucella* inhibits IL-12 production by DCs and induces a Th-2 response that correlates with IL-10 and TGFβ production. IFNγ is extremely important for the host to control the bacterial replication. *Ifnar*$^{-/-}$ mice infected with *Brucella* died due to

inflammation and the increased number of bacterial replication. TNFα and IL-12 are also important for

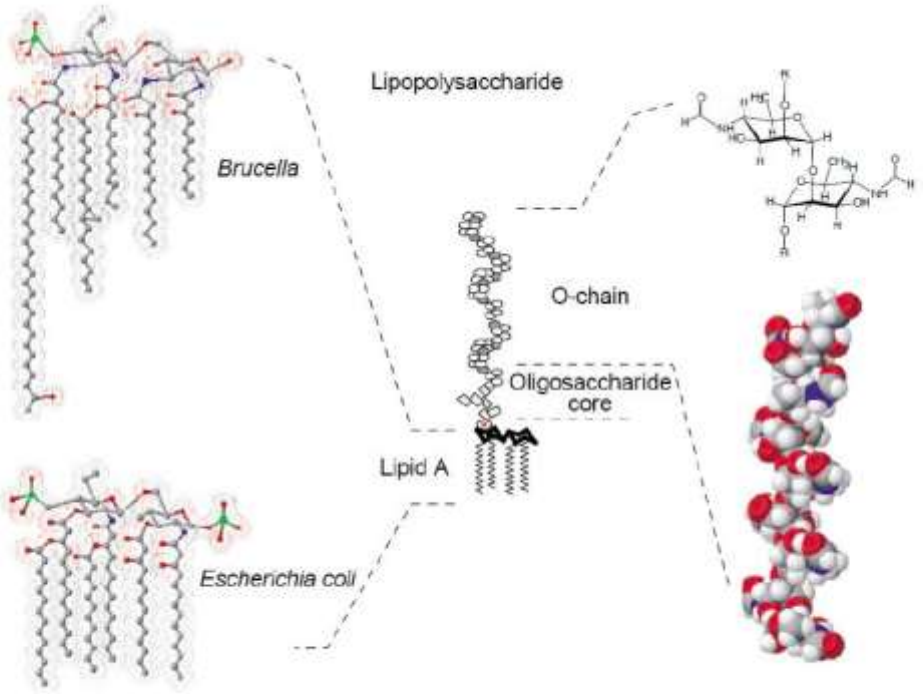

Figure 3: *Brucella* LPS structure. *B. abortus* lipid A possesses a diaminoglucose backbone linked by amide bonds to long acyl groups (C18–C19, C28), which differs from the classical LPS of *E. coli.* The O-chain is an N-formyl-perosamine homopolymer (top-right). The oligosaccharide core is composed of 3-deoxy-D-manno-2 octulosonic acid (Kdo), mannose and glucosamine (bottom-right).

Adapted from (Lapaque, Moriyon et al. 2005)

Brucella infection, but the lack of these cytokines in mice induces a less severe phenotype (Zhan and Cheers 1995, Grillo, Blasco et al. 2012).

Adaptive immunity has an important role in *Brucella* persistence. Transfer of $CD4^+$ and $CD8^+$ T cells from infected mice provides protection in recipient mice (Baldwin and Goenka 2006). CD3 and TCR-β deficiency, but not TCR-δ or B cell deficiency, completely abrogates protection in the spleen, thus demonstrating that α/β T cells constitute the core of the adaptive immune system involved in protective immunity against *Brucella* (Mamber, Lins et al. 2016).

B cells have also an important role in *Brucella* persistence. At the end of the acute phase of infection plasma B cells produce a predominance of IgG3a and IgG2a with low amounts of IgG1 promoting opsonisation of bacteria and macrophage uptake. However, this process does not lead to bacterial clearance but to macrophage infection and *Brucella* persistence (Elzer, Kovach et al. 1995).

It is also important to mention that Natural Killer cells (NK) do not seem to play any role in *Brucella* infection in the mouse model. Nevertheless, in humans NK cells are suggested to interfere with bacterial infection. It has been shown that human NK cells stimulated *in vitro* with *B. suis* produce IFNɣ and kill *Brucella* infected macrophages (Ko, Gendron-Fitzpatrick et al. 2002, Dornand, Lafont et al. 2004). Despite these discrepancies, major similarities in defense mechanisms are observed, which makes the mouse model a very good model to study and understand *Brucella* infection.

In the next section, I am going to be concentrate on the role of *Brucella* outer membrane in pathogenicity and also in the modulation of DCs properties to dampen the adaptive immune system and allow the bacteria to persist in the host.

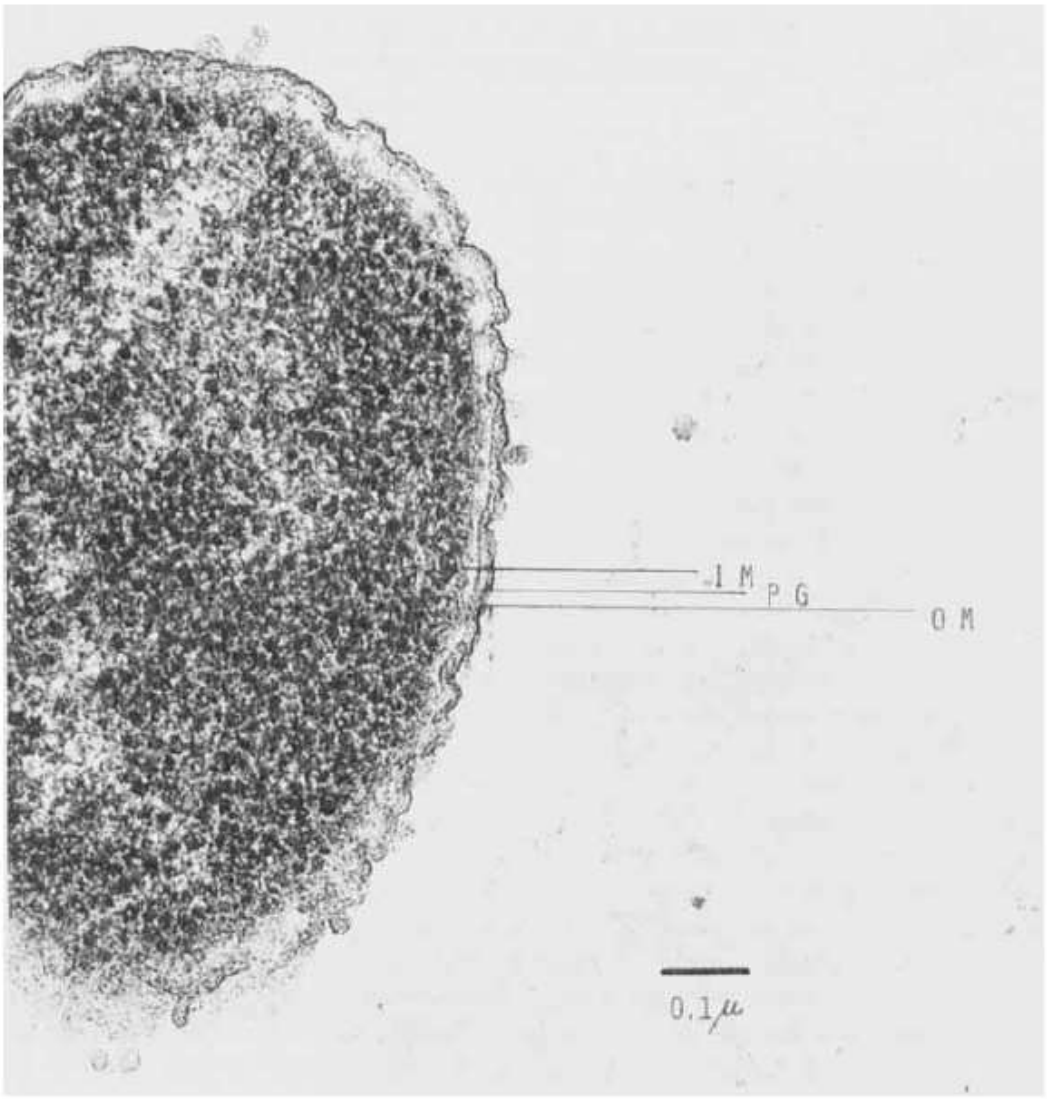

Figure 4: Electron microscopy micrograph of thin section of *E. coli J5*. OM: outer membrane; PG: peptidoglycan; IM: inner membrane.

Photography from (Costerton, Ingram et al. 1974)

Brucella outer membrane: a weapon for bacterial warfare

The envelope of Gram-negative bacteria contains three structures that can be visualized in the electron microscope (Figure 4): an inner (cytoplasmic) membrane, the periplasmic space enriched in peptidoglycan, and an outer membrane layer (OM) (Costerton, Ingram et al. 1974). The outer membrane (OM) of *Brucella* contains LPS, proteins and phospholipids. The OM plays an important role in the pathogenicity of the bacteria since it is in close contact with the host environment. The OM of *Brucella* contains: the LPS, proteins and phospholipids known as virulence factors (Lamontagne, Butler et al. 2007, Vermassen, Leroy et al. 2019) (Figure 5).

Lipopolysaccharide (LPS)

The LPS is a major component of the outer membrane of Gram-negative bacteria involved in maintaining its functional and structural integrity. *Brucella* LPS is composed of the lipid A connected to an oligosaccharide core which can itself be connected (smooth LPS) or not (rough LPS) to a hydrophilic O-polysaccharide chain (O-chain) as shown in Figure 3. Most of pathogenic *Brucella* possess a smooth LPS. The rough LPS strains are attenuated. However, in some *Brucella* species like *B. ovis* or *B. canis* their rough LPS is fully pathogenic for their primary host (Cardoso, Macedo et al. 2006).

Due to its peculiar structure *Brucella* smooth LPS is a poor agonist of TLR4. It inhibits the secretion of inflammatory cytokines by DCs or macrophages such as TNFα or type I or type II interferon (IFN) (Lapaque, Moriyon et al. 2005, Cardoso, Macedo et al. 2006). Moreover, *Brucella* LPS confers to bacteria the resistance against antimicrobial peptides and to other cationic bactericides. For instance, if *Brucella* LPS is transferred to the OM of *Salmonella* or *E. coli*, these enterobacteriea become resistance to cationic molecules such as Polymyxin B. Similarly, *Brucella* chimeras containing enterobacterial LPS become more sensitive (Detejada, Pizarrocerda et al. 1995, Freer, Moreno et al. 1996, Martirosyan, Moreno et al. 2011). Therefore, due its immunomodulatory properties *Brucell*a LPS is one of the most important virulence factors of *Brucella.*

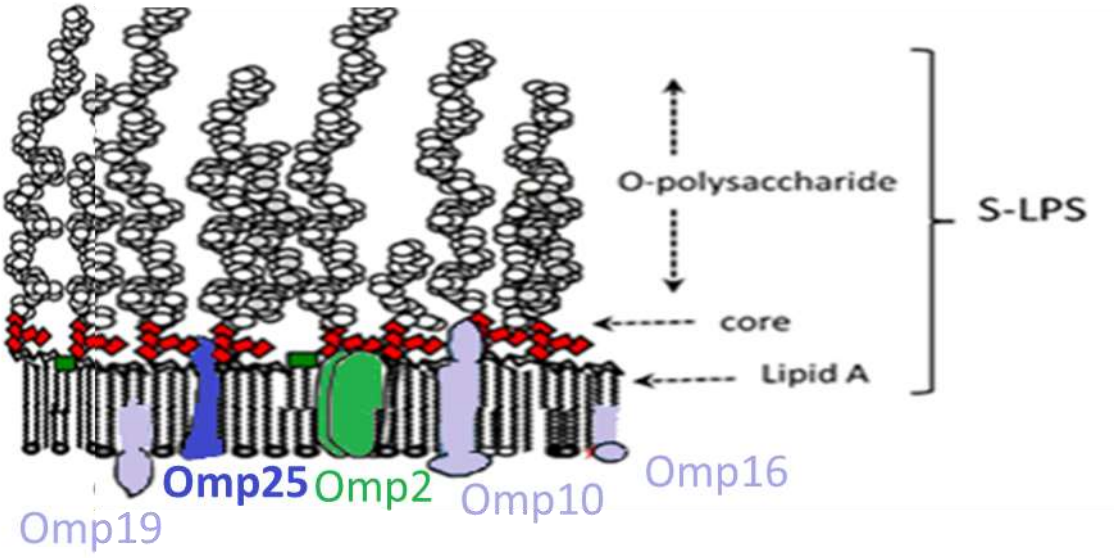

Figure 5: Diagram of the outer membrane of *Brucella*. The outer membrane (OM) of *Brucella* contains lipopolysaccharide (LPS), proteins and phospholipids. Based on their molecular weight *Brucella* outer membrane proteins are divided in 3 groups: **Group 1** (proteins from 94 to 96 kDa); **Group 2** (proteins from 36 to 48 kDa : Omp2a, Omp2b); **Group 3** (proteins from 25 to 31kDa : Omp25a, Omp25b, Omp25). **Lipoproteins** (Omp10, Omp16, Omp19).

Adapted from (Moriyón and López-Goñi 1998)

The outer membrane proteins

The outer membrane proteins (OMP) are separated in 3 groups based on their molecular weight: group 1 proteins weigh from 94 to 96kDa and participate in the biogenesis of the OM; proteins from the group 2 are very hydrophobic, form porins and participate in the OM hydrophobicity; group 3 contains proteins from 25 to 31kDa that are exposed to the OM surface such as Omp25 or Omp22 (Moriyón and López-Goñi 1998).

Many Omps seem to have functions in the immune response. For, instance Omp25 is one of the major proteins expressed In the *Brucella* OM. It has surface exposed epitopes and is expressed in all *Brucella* species (Jubier-Maurin, Boigegrain et al. 2001). Omp25 like Omp22 is regulated by BvR/BvRS two component sensory-regulatory system. *Brucella* lacking BvrR/BvrS sensory regulatory system fails to replicate intracellularly and is avirulent in the murine model due to the Omp modifications (Guzmán-Verri, Manterola et al. 2002). Surprisingly, mutant bacteria lacking Omp25 or Omp22 do not present any defect of replication in phagocytic and non-phagocytic cells infected *in vitro* (Manterola, Guzman-Verri et al. 2007). *In vivo*, Omp25-deficient *Brucella melitensis, Brucella abortus,* or *Brucella ovis* mutants showed attenuated virulence in the infected murine BALBc model and ruminant hosts 8 weeks after infection (Edmonds, Cloeckaert et al. 2002). Indeed, *B. suis Δomp25* does not show any attenuation in the spleen during the first 3 weeks of infection (Sidhu-Munoz, Sancho et al. 2018). In addition, Omp25 confers bacteria resistance to Polymixin B. However, lack of Omp25 makes *B. abortus* more resistant to human complement (Manterola, Guzmán-Verri et al. 2007). *In vitro* studies revealed that Omp25 inhibits pro inflammatory cytokine production in infected human and mice phagocytic cells (Billard, Dornand et al. 2007, Zhang, Zhang et al. 2017). Indeed, treatment of *B. suis* WT infected THP-1 human macrophages enhanced secretion of TNFα by these cells. The same results were observed during the infection of THP-1 macrophages with *B. abortus Δomp25.* These results suggest that Omp25 is released by the bacterium and acts on the extracellular cell membrane to inhibit inflammation (Jubier-Maurin, Boigegrain et al. 2001, Boigegrain, Salhi et al. 2004). The mechanism by which Omp25 inhibits TNFα extracellularly is unexplored.

Other Omps have been studied. Omp16 triggers the secretion of cytokines including TNF-α and IL-12, and over-expression of co-stimulatory molecules like CD80, CD86 and CD40 in mouse DCs (Pasquevich et al. 2009; Pasquevich et al. 2010; Ibanez et al. 2013). Omp19 -by its lipid moiety- dampens antigen presentation and MHC-II expression in IFN-γ-activated human monocytes (Barrionuevo et al. 2011). However, studies using recombinant proteins are very

controversial since the lipoproteins were cloned in *E. coli*. Acetylation will correspond to *E. coli* and not to *Brucella* fatty acids. Since the fatty acids are the relevant moieties recognized by TLR2, then it is likely that the biological effect reported for this chimeric Omp19 is due to *E. coli* fatty acids rather than to *Brucella*.

The outer membrane vesicles

It is important to mention that *Brucella* like other Gram-negative bacteria is able to release outer membrane vesicles (OMVs) (Boigegrain, Salhi et al. 2004, Pollak, Delpino et al. 2012). OMVs are closed spheroid vesicles between 10 and 300 nm in diameter that contain proteins, lipoproteins and other bacterial factors of the bacteria envelope by a process known as blebbing. Blebbing is a stress-induced adaption that bacteria have developed while confronted with environmental stresses such as nutrient deprivation, antimicrobial peptides, antibiotics, reactive oxygen species, etc. Table 3 shows host cell associated factors that have been shown to induce vesicle release. The exact process how membrane vesicles are generated is not fully understood. However three non-excluding models have been proposed for OMVs generation. Model A proposes that biogenesis of OMVs is induced when mechanisms for the maintained of the symmetry of the envelope is compromised. Model B argues that accumulation of misfolded proteins in the OM causes blebbing. Model C entails that OMVs are formed as the result of LPS modifications (Volgers, Savelkoul et al. 2018).

OMVs are implicated in many physiological process as they provide protection for bacteria against antibacterial compounds and also act as carriers of virulence factors. For instance, Omp25 of *Brucella* seems to be hijacked in the extracellular environment via OMVs during macrophage infection (Boigegrain, Salhi et al. 2004). In addition, treatment of monocytes by OMVs prior to infection leads to an increase of *Brucella* adhesion and internalization (Pollak, Delpino et al. 2012). However, it is important to note that blebbing can induce a modification of the acetylation sites of LPS or other lipoproteins, and therefore modulate their 3D structure and immunomodulatory properties. This can explain why unlike in the whole bacteria, OMVs from *Brucella melitensis* induce host inflammatory response and thus are targets for vaccine design (Dubray and Blzard 1980, Bowden, Cloeckaert et al. 1998, Avila-Calderon, Araiza-Villanueva et al. 2015).

However, the role of *Brucella* OMVs during *in vivo* infection has not been studied yet.

Category	Effector	Through	Induces	Stress response	Species
Nutrient deprivation and growth phase					
	Cysteine depletion	Growth phase transition	Oxidative stress	Putatively via through extracytoplasmic stress responses and lipid asymmetry (Vacj/Yrb ABC transporter)	N.meningitis
	Iron limitation	Iron uptake		Lipid asymmetry (Vacj/Yrb ABC transporter)	Vibrio cholerand H. influenzae
	Low cation levels	destabilization of cation-bridges		LPS modification	E. coli
Antimicrobial peptides and antibiotics					
Bacteria derived	Quinoline PQS	Destabilization of cation-bridges		LPS modification	P. aeruginosa
Host associated	Lysozyme	Muramidase activity	Acts on peptidoglycan	Unknown	P. aeruginosand Francisella novicida
		Cationic antimicrobial peptide	Putatively induces PhoPQ (Macfarlene et al. 2000 Bader et al. 2005 Kwon and Lu 2006 Gellatly et al. 2013)	Putatively LPS modification	
Antibiotics	Polymyxin B and colistin	Destabilization of cation-bridges		LPS modification	P. aeruginosand E. coli
	Gentamicin	Destabilization of cation-bridges		LPS modification	P. aeruginosa
	Amikacin	Destabilization of LPS-LPS bonds		LPS modification	P. aeruginosa
	Ciprofloxacin	DNA damage and SOS stress response	Oxidative stress	Putatively by inducing the and LPS modification	P. aeruginosa
	Mitomycin C	DNA damage and SOS stress response	Oxidative stress	Putatively through extracy-toplasmic stress responses and LPS modification	Shigella dysenteriae
	D-cycloserin		AlgU activation	Extracytoplasmic stress responses	P. aeruginosa
Physiological stressors					
	Oxidative stress			Putatively through extracy-toplasmic stress responses and LPS modification	P. aeruginosand N. meningitis
	Temperature			Putatively through accumu-lated misfolded proteins, lipid asymmetry, or altered LPS composition	P. aeruginosand E. coli
	Increased oxygen availability	Oxidative stress		Putatively through extracy-toplasmic stress responses and LPS modification	P. aeruginosa
Other host-associated environmental factors					
	Norepinephrine	Unknown		Unknown	Salmonella enterica serovar Typhi

Table 3: Host associated stress factors that have been shown to induce membrane vesicle release.

Adapted from (Volgers, Savelkoul et al. 2018)

Brucella-Dendritic cell interactions

Dendritic cells (DCs) serve as sentinels for the immune system. They scan the tissues for pathogens and initiate the adaptive immune response. DCs process antigens and present antigenic peptides at their plasma membrane via MHC (major histocompatibility complex). They present peptides to $CD4^+$ T helper cells via MHC of class II (classic presentation) and also present non-self-antigenic peptides via MHC class I to $CD8^+$ T cells (cross-presentation). For effective interactions with T cells and further release of cytokines, DCs regulate their co-stimulatory molecules like CD40, CD80, CD86, CD150 (SLAMF1), etc. (Platt, Ma et al. 2010). Spleen is a secondary lymphoid organ that serves as a platform to DCs for antigen presentation to T cells. Based on their origin, phenotype and function, mice splenic DCs are classified into 3 principal subsets: conventional DCs (cDCs) that encompass $CD11c^{high}$ $XCR1^+$ cDC1 and $CD11b^+$ $Sirp\alpha^+$ $CD24^-$ cDC2; plasmacytoid DCs (pDCs) and monocyte- derived DCs (MO-DCs), including splenic $iNOS^+$ DCs. Both cDC1 and cDC2 can present antigens to $CD4^+$ T cells. cDC1 serve as excellent antigen presenting cells for inducing a Th1 response and, through their cross-presenting capacity, for priming $CD8^+$ T cytotoxic lymphocytes response whereas cDC2 preferentially present exogenous antigens to prime $CD4^+$ T cells and to induce Th2 responses (Behboudi, Moore et al. 2004, Hey and O'Neill 2012, Alexandre, Ghilas et al. 2016). pDCs are the main resource of large amounts of type I interferon (IFN), pleiotropic cytokine with antiviral activity that enhances both innate and adaptive immune responses (Swiecki and Colonna 2015). MO-DCs have the capacity to induce Th1-polarized $CD4^+$ T cell response, prime antigen-specific $CD8^+$ T cells and exert a microbicidal action by producing TNF-α and iNOS, and regulate IgA production by B cells. Monocyte-derived DCs constitute an independent category of DCs recruited in the spleen during infection or inflammation (Leon and Ardavin 2008, Cheong, Matos et al. 2010, Qu, Brinck-Jensen et al. 2014).

In vitro, the most used models for generating DCs from bone marrow progenitors and hematopoietic stem cells are GMCSF bone marrow-derived DCs (GM-DCs) and Flt3 ligand bone marrow-derived DCs (Fl-DCs). GM-DCs give rise to monocyte-like DCs and FL-DCs give rise to spleen-like DCs such as cDC1, cDC2 and pDCs. Other cytokines can be added to GM-DCs in order to mimic different inflammation processes like IL-15 to match lung DCs. (Weigel, Nath et al. 2002).

Brucella is able to infect both human (Billard, Dornand et al. 2008) and mouse DCs (Salcedo, Chevrier et al. 2013, De Bolle, Crosson et al. 2015, Papadopoulos and Gorvel 2015,

Papadopoulos, Gagnaire et al. 2016, Zhao, Hanniffy et al. 2018) *in vitro* and *in vivo*. However, *Brucella* cannot replicate in all types of DCs (Terawaki, Camosseto et al. 2015). Indeed, *Brucella* is known as a weak activator of GM-DCs *in vitro*. BtpA, BtpB and the oligosaccharide core of *Brucella* LPS are responsible of preventing the DC maturation (Salcedo, Marchesini et al. 2008, Salcedo, Marchesini et al. 2013, Zhao, Hanniffy et al. 2018), that is translated by the inhibition of anti-inflammatory cytokine secretion and also by the incapacity to activate naïve T $CD4^+$ cells. Furthermore, *Brucella* infection limits signaling pathways involved in type I IFN production (Gorvel, Textoris et al. 2014).

In contrast, recent studies showed that *Brucella* is able to activate cDC generated from Fl-DCs or IL15-DCs *in vitro* leading to the production of IL-12 and IFNɣ (cytokines known to promote Th-1 response) at 8 and 12 h post-infection and IL-10 at 48 h post-infection. Cytokine response at early and late time points of *in vitro* DCs infection resembles to the profile observed during *Brucella* infection *in vivo*.

In vivo, inflammatory DCs ($CD11b^+$ $B220^-$ $Ly6^+$) are observed in the white pulp of the spleen during acute infection. But, the low profile of activation of $CD8^+$ cytotoxic T cells indicates that the capacity of DCs to prime T cells is affected. Indeed, *Brucella* inhibits $CD8^+$ T cell cytotoxic activity via Toll/Interleukin 1 receptor domain BtpA during chronic infection (Durward-Diioia, Harms et al. 2015). BtpA interferes with TL2/TLR4 signaling by interacting with Myd88, resulting in inhibition of pro-inflammatory cytokine secretion by DCs (Salcedo et al. 2008a). Moreover, contact of immature DCs with $CD4^+$ naïve T cells induces the generation of regulatory T-cells and prevents Th1 response in a TGFβ-dependent manner. Increase of TGFβ in the serum was also reported during human chronic brucellosis. T_{reg} cells produce IL-10. IL-10 is a crucial cytokine for *Brucella* chronicity since *Brucella* is cleared in mice lacking IL-10 (Elfaki and Al-Hokail 2009) .

To sum up, *Brucella* limits DCs activation to trigger a chronic infection by inhibiting the pro-inflammatory cytokine production, the expression of surface co-stimulatory molecules and participates in the generation of regulatory T cells to promote chronicity. However, *Brucella* uses several other unexplored mechanisms to limit DCs activation. Recent publications, showed that SLAMF1 (a costimulatory molecule expressed on activated DCs, monocytes and macrophages), can also modulate immune response during bacterial infection (van Driel, Liao et al. 2016). During my thesis I explored the contribution of this receptor in brucellosis.

Therefore, in the next chapter, I will sum up what we currently know about SLAMF1 as an immune modulator and bacterial sensor.

Chapter II: SLAMF1

SLAMF1 a signaling lymphocytic activation molecule family

SLAMF1 is a type one transmembrane protein belonging to signaling lymphocytic activation molecule family (SLAMF) expressed mostly by hematopoietic cells.

SLAM family includes nine members: SLAMF1 (CD150 or IPO-3 or SLAM) expressed on T, B lymphocytes, activated monocytes and dendritic cells (DCs) and by hematopoietic stem cells (HSCs); SLAMF2 (CD48 or BCM1, Blast-1, OX-45) expressed on B and T lymphocytes, DCs and endothelial cells; SLAMF3 (CD229 or Ly9) expressed on T and B cells, on monocytes, macrophages and HSCs; SLAMF4 (CD244 or 2B4, NAIL) expressed on NK cells T lymphocytes, dendritic cells, eosinophils, and mast cells; SLAMF5 (CD84 or Ly9B) expressed on T and B lymphocytes, platelets, mast cells and eosinophils; SLAMF6 (CD352 or NTB-A, SF2000, Ly108), expressed on NK cells, B and T lymphocytes, macrophages and plasmacytoid DCs (pDCs); SLAMF7(CD319 or CRACC, CS1, 19A24), expressed on B and T lymphocytes, monocytes, DCs and NK cells; SLAMF8 (CD353 or BLAME) expressed on $CD8^+$ T cells, monocytes, macrophages, DCs, neutrophils, endothelial cells and fibroblastic reticular cells, and SLAMF9 (SF2001 or CD84) expressed on monocytes and DCs (Naaman et al., 2018; Oguro et al., 2013; Sintes et al., 2008 (Aversa, Carballido et al. 1997, Veillette 2004, Kiel, Yilmaz et al. 2005, van Driel, Wang et al. 2015).

These receptors with the exception of SLAMF2 and SLAMF4 are self-ligands that participate in immune modulation in response to inflammation or infection. In addition, some of SLAMF receptors are engaged in heterophilic interactions with microbial compounds and serve as microbial sensors, which either positively or negatively modulate the function of macrophages (Detre, Keszei et al. 2010, Wang, Halibozek et al. 2015, van Driel, Liao et al. 2016).

SLAMF proteins are part of the CD2 Ig superfamily. With the exception of SLAMF 2,8,9, signaling lymphocytic family receptors are formed of an extracellular N-terminal variable (V) Ig domain lacking disulfide bonds followed by a truncated Ig constant 2 (C2) domain with two intra domain disulfide bonds, a transmembrane region and an intracellular region that contains at least two tyrosine-based switched motifs (ITSM) (Veillette 2010). ITSMs distinct from immune receptor tyrosine-based activation motifs (ITAM) and immune receptor tyrosine-based inhibition motif (ITIM) found in other activating or inhibitory immune receptors.

	Expression	SAP-dependent	EAT-2-dependent	Immune modulatory role
SLAMF1	act T, act B, monocytes, Møs, DCs, HSCs	T: ↑IL-4, ↑IL-13, proliferation, Th2/ Th17 polarization, NKT: development (with SLAMF6)	Unknown	T:↑IFNγ, B: ↑proliferation and activation, ↑apoptosis, Mø: ↑ROS, ↑IL-12, ↑TNFα,↑ NO, ↓ IL-6, ↑ myeloid cell migration, ↑ platelet aggregation, ↑phagocytosis , ↑Ig secretion
SLAMF2	lymphocytes	N/A	N/A	T:↑IL-2, proliferaton, B: ↑activation, ↓ apoptosis Mast: ↑ TNFα, eo: ↑activation, ↑mobilization, Mø: ↑ TNFα, ↑ IL-12, ↑ phagocytosis, DC: ↑ survival
SLAMF3	T,B,$CD8^+$, NKT, monocytes, Møs , DCs, HSCs	Unknown	Unknown	T: ↓IFNγ, ↑proliferation, ↑IL-2, ↑IL-4, ↑$iCD8^+$ T-cells, iNKT ↓development
SLAMF4	NK, NKT, T , DCs, eosinophils, mast cells, monocytes	T: (−) IFNγ, NK/ $CD8^+$ proliferation : ↑cytotoxicity,	NK: (−) Cytotoxicity of Slamf2-neg target cells, (−) IFNγ	eo: ↑ adhesion, ↑chemotaxis, ↑peroxidase, ↑IFNγ, ↑IL-4
SLAMF5	lymphocytes, platelets, mast cells, eosinophils	T-B: ↑ GC response	NK: ↑Cytotoxicity Mast: ↑ Degranulation	
SLAMF6	NK, NKT, T, B, Møs , pDCs	T-B: ↑ GC response, NK: ↑IFNγ, ↑NKT: development (with SLAMF1)	NK: ↑Cytotoxicity	T-B: ↓ GC response, Neutro: ↑ROS,↑IL-6, ↑TNFα
SLAMF7	T, B, mono, DCs, NK cells	Unknown/N/A	NK: ↑Cytotoxicity	NK: without EAT-2 ↓Cytotoxicity, B: ↑ proliferation
SLAMF8	iCD8, monocytes, DCs, Møs , neutrophils, endo, fiboblastic reticular cells	N/A	N/A	T: ↓ IFNγ, ↑ proliferation, IL-2, IL-4, iCD8+ T-cells, iNKT ↓development
SLAMF9	monocytes, DCs	N/A	N/A	N/A

Table 4. SLAM receptor expression and function.

T: T cells; B: B cells; act: activated; Mø: macrophage; DC: dendritic cells; plat: platelet; HSC: hematopoietic stem cell; mono: monocyte; NKT: natural killer T cell; eo: eosinophil; mast: mast cell; endo: endothelial cell; ROS: reactive oxygen species. These data are based on murine expression of SLAMF receptors.

Adapted from (van Driel, Liao et al. 2016)

These motifs can orchestrate a switch from the binding of a tyrosine phosphatase to binding of a tyrosine kinase depending on the presence or the absence of adaptor molecules. ITSMs bind to adaptor molecules that contain an SH2 domain. Indeed, the adaptor molecules recruited by the intracellular SLAMF ITSMs are SAP (SLAM associated protein) encoded by the *Sh2d1a* gene and EAT-2 (Ewing's sarcoma-associated transcript-2) encoded by the *Sh2d1b* gene and ERT (EAT-2-related transducer) encoded by the *Sh2d1b* gene. (Morra, Lu et al. 2001, Calpe, Erdos et al. 2006, Cannons, Tangye et al. 2011). SAP is expressed in T cells and natural killer (NK) cells, whereas EAT-2 was primarily found in NK and antigen presenting cells (APCs). Binding of SAP or EAT-2 blocks the recruitment of phosphatases such as SHP1 or SHP2.
The identification of specific physiological effects of each SLAMF receptor is difficult since SLAMF receptors share the same intracellular partners (Kiel, Yilmaz et al. 2005). Table 4 recapitulates the known function of each SLAM receptor.

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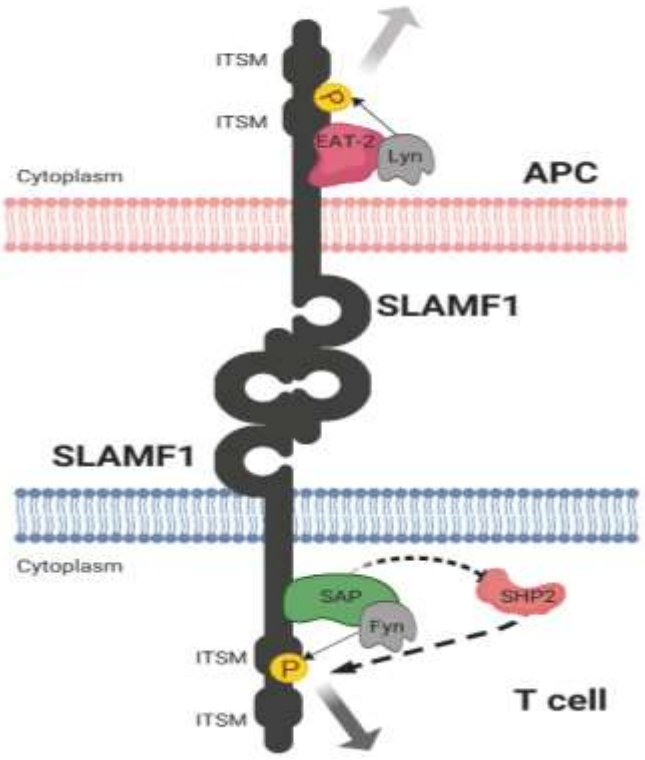

Fig. 6. SLAMF1 receptor modulates cell-cell communication between antigen presenting cells (APCs) and T cells.

Homophilic SLAMF1/SLAMF1 interaction induces the recruitment of SH2 domain-containing molecules (SAP or EAT-2) in the ITSM domain of intracellular SLAMF1. SAP is expressed in T and B cells and EAT-2 is expressed in APCs. SAP or EAT-2 binding to ITSM triggers the recruitment of Src kinases such as Lyn or Fyn. Src kinases recruit afterwards other cell partners that give rise to different immune signaling cascades. In addition the binding of SAP to ITSM blocks SHP2 binding

SLAMF1 (CD150) structure

SLAMF1 receptor was first described as 'IPO-3 antigen' (Pinchouk et al., 1988; Pinchouk et al., 1986; Sidorenko & Clark, 1993) on activated human B lymphocytes. In 1995, the cDNA for this receptor was cloned under the name signaling lymphocyte activation molecule (SLAM) (Cocks et al., 1995). In 1996 during the sixth workshop on Human Leukocyte Differentiation Antigens (HLDA). (Kobe, Japan) the IPO-3 antigen received international nomenclature CDw150 (Sidorenko, 1997) that was transformed into CD150 (SLAMF1) at the seventh HLDA workshop (Harrogate, Great Britain, 2000) (Sidorenko, 2002).

SLAMF1 receptors from human, mouse, dog, cow and marmoset have about 60–70% identity at the amino acid level, except human and marmoset SLAMF1, which have 86% identity (Ohno et al., 2003).

The gene encoding for SLAMF1 is localized on the long arm of human and mice chromosome 1 (Sidorenko and Clark 2003). *Slamf1* gene lacks the TATA-box and has binding sites for several transcription factors such as Sp1, Stat6, Irf4, NF-κB, ELF1,TCF3 and PU.1 and consist of eight exons separated by seven introns. Each of the Ig domains is encoded by separate exons (Vilagos, Hoffmann et al. 2012, Takeda, Kanbayashi et al. 2014). Moreover, thanks to the alternative splicing, several structurally different isoforms can be generated: the canonical transmembrane SLAMF1 isoform with two ITSM in cytoplasmic tail (mCD150), the secreted isoform without transmembrane region (sCD150), the novel SLAMF1 isoform (nCD150) with alternative cytoplasmic tail, the variable membrane SLAMF1 (vmCD150) isoform, which have truncated cytoplasmic tail, and the cytoplasmic SLAMF1 (cCD150) isoform lacking leader sequence (Romanets-Korbut, Najakshin et al. 2015). However, these isoforms have been detected on their messenger RNA level but have not been characterized at the protein level.

Currently, the only available data about SLAMF1-mediated signaling and function in hematopoietic cells concern the mCD150 isoform.

SLAMF1 homophilic interaction

Due to its 2 ITSM domains, SLAMF1 has docking sites for SH2-containing molecules and has been shown to interact with several components of the signaling machinery that directly and indirectly link this receptor to different signaling pathways (Fig.7).

In human T cells, the two intracellular ITSMs of SLAMF1 recruit SAP, SHP1 and SHP2, and also inositol phosphatase SHIP. Indeed, SAP, SHP1 and SHP2 phosphatases bind two the same domain of ITSM. (Sayos, Wu et al. 1998, Chan, Lanyi et al. 2003).

In murine thymocytes, after T cell receptor (TCR) activation and treatment with anti-SLAMF1 antibody, SLAMF1 binds to SAP, which recruits and activates the protein tyrosine kinase Fyn T. Phosphorylated SLAM triggers the activation of downstream substrates, such as the SH2 domain of inositol-containing phosphatase (SHIP), Dok (docking protein)-1, Dok-2 and Ras GTPase activating protein (RasGAP). Still, the interaction between TCR and the SLAM-SAP signaling pathway leads to the activation of substrates, such as protein kinase C-teta (PKC-θ), B cell lymphoma 10 (Bcl-10), nuclear factor-κB (NF-κB), and GATA-3. These signaling pathways increase the expression of Th-2 cytokines such as IL-4 (Cannons, Li et al. 2004, Cannons, Tangye et al. 2011). In contrast, stimulation of human Th-1 clones with anti-SLAM monoclonal antibodies increases proliferation, T cell toxicity and IFN-γ production, suggesting that SLAM/SAP signaling regulates T helper cell cytokine production (Cocks, Chang et al. 1995, Henning, Kraft et al. 2001).

SLAMF1 is also induced in antigen presenting cells (APCs) upon stimulation (Bleharski, Niazi et al. 2001). Indeed, treatment of pre-stimulated human mo-DCs with SLAMF1 monoclonal antibody increased the secretion of IL-12 and IL-8. But, when pre-stimulated mo-DCs were co-cultured with L929 cells or Jurkat T cells constitutively expressing SLAMF1, the level of IL-12, TNF-α, and IL-6 secretion was decreased (Rethi, Gogolak et al. 2006). This discrepancy suggests that SLAMF1 antibody blocks signaling induced by SLAMF1 homophilic interaction. Consistent with this hypothesis, treatment of systemic lupus erythematous with SLAMF1 antibody inhibits cytokine secretion by peripheral B cells (Karampetsou, Comte et al. 2019).

Moreover, the concomitant stimulation of B cells with CD40L or with *S. aureus* and the secreted SLAMF1 protein leads to B cell proliferation. Conversely, treatment of stimulated B cells with anti SLAMF1 antibody inhibits B cell proliferation during *S. aureus* infection (Mikhalap, Shlapatska et al. 2004).

SLAMF1-SLAMF1 ligation induces SHIP binding triggering MAPK pathway (ERK1/2, p38. MAPK and JNK1/2) in a SAP-independent manner in activated B cells (Yurchenko, Shlapatska

et al. 2011, Gordiienko, Shlapatska et al. 2018). In addition, in B cells from chronic lymphocytic leukemia (CLL) patients SLAMF1 binds directly beclin-1,Vsp34 and UVRAG, and contributes thus to autophagosome generation (Bologna, Buonincontri et al. 2016).

Moreover, SLAMF1 is also suggested to be involved in Natural Killer T (NKT) cell development. SLAMF1 role in NKT development is redundant with the role of SLAMF4. Neither *Slamf4*$^{-/-}$, neither *Slamf1*$^{-/-}$ mice presented any defect in NKT development. However *Samf1*$^{-/-}$ *Slamf4*$^{-/-}$ mice lack NKT cells (Sintes, Cuenca et al. 2013).

SLAMF1 is also involved in phagocyte migration. Phagocytic cells in *Slamf1*$^{-/-}$ *Rag*$^{-/-}$ mice are unable to infiltrate the lamina propria of the colon during colitis (Wang, van Driel et al. 2015).

To conclude, all these result reveal that downstream signaling due to SLAMF1 homophilic engagement could be important for immune modulation, hematopoietic development and cell motility.

SLAMF1 heterophilic interaction

SLAMF1 is known as the entry receptor for Measles Virus in human macrophages. (Yurchenko, Shlapatska et al. 2011, Romanets-Korbut, Kovalevska et al. 2016, van Driel, Liao et al. 2016). Study of SLAMF1 in mice showed that the role of this receptor in microbial pathogenesis depends on the pathogen. *Slamf1*$^{-/-}$ mice are protected from *T. cruzi* acute lethal challenge (Calderon, Maganto-Garcia et al. 2012) but are sensitive to *Leishmania major* and to the attenuated *Salmonella* strain *SseB*$^{-}$ (Wang, Satoskar et al. 2004, Calderon, Maganto-Garcia et al. 2012).

In vitro studies have shed light on the way SLAMF1 protects host from *E. coli* or *Salmonella* infection.

For instance, infection of macrophages with *E. coli* showed that the extracellular SLAMF1 domain binds the *E. coli* outer membrane protein (OmpC and OmpF) leading to the formation of the Beclin1/Vps34/Vps15/UVRAG complex. Active Beclin-1/VpS34/UVRAG enzyme complex mediates generation of phosphatidylinositol-3-phosphate, which in turn recruits EEA-1 for phagosome maturation, NOX-2 activation and reactive oxygen species (ROS). Hence, SLAMF1 participates in bacterial clearance (Berger, Romero et al. 2010).

In addition, SLAMF1 localizes in the *bacterial* phagosome during *E. coli* infection of human macrophages. Indeed, SLAMF1 binds TRIF in the phagosome and regulates TLR4-TRAM-

TRIF complex formation. This complex enhances IFNβ production facilitating therefore bacterial clearance. (Yurchenko, Skjesol et al. 2018).

These two studies revealed that SLAMF1 plays an intracellular and extracellular role in microbial pathogenesis.

In the case of brucellosis, SLAMF1 is a strongly upregulated in human DCs stimulated with *Brucella* cyclic β1,2-glucan (CβG) (Martirosyan, Perez-Gutierrez et al. 2012, Degos, Gagnaire et al. 2015). However, its role in pathogenesis remains to be deciphered. Indeed, this receptor has been shown to have a role in innate immunity (microbial sensing), adaptive immunity and hematopoietic cell development. Furthermore, SLAMF1 is also expressed on HSCs. *Slamf1*$^{-/-}$ mice do not present any defect on hematopoiesis in steady state (Kiel, Yilmaz et al. 2005). However, SLAMF1 homophilic interaction between bone marrow regulatory T cells expressing SLAMF1 and HSCs is claimed to be important for maintenance of the HSCs in the BM niche. During my thesis I focused on the bone marrow niche, and the role of SLAMF1 in hematopoiesis during *Brucella* infection.

Chapter III: Hematopoiesis

Brucella and hematopoiesis

Around 40% of brucellosis patients suffer from osteo-articular complications such as osteomyelitis and arthritis as shown in Figure 7 (Aydin et al., 2005; Mutolo et al., 2012; Skyberg et al., 2012). Moreover, clinical studies showed that *Brucella* can be transmitted from bone marrow (BM) transplantation (Ertem et al., 2000; Naparstek et al., 1982; Ruben et al., 1991). Such is the case of a 8 years old boy suffering from Fanconi anemia that received an allogenic bone marrow transplantation from one of his siblings. High doses of doxycycline and gentamycin were administrated to this patient to clear *Brucella abortus* infection. Thus, a growing number of clinical data suggests that *Brucella* infects human bone marrow and bone.

First observations in the murine model, showed that bioluminescent *B. abortus* is also found in mouse bones (Rajashekara et al., 2005). Several in vitro experiments confirmed that *Brucella* can infect human and mouse osteoblasts (Delpino et al., 2009; Scian et al., 2012) and inhibit bone formation (Giambartolomei et al., 2017).

Bone provides fundamental factors that regulate and support hematopoiesis (Reddi, 1992). Hematopoiesis is the vital process allowing formation of blood cells and also osteoclasts from hematopoietic stem cells (HSCs) (Fantuzzi and Faggioni, 2000). Thus, bone tissues and bone marrow are tightly associated structurally and functionally (Del Fattore et al., 2010). In addition, infection induces changes in bone and bone marrow environment. These changes interfere with HSCs function and cause hematological abnormalities such as anemia, pancytopenia, etc. (Crosby et al., 1984; Glatman Zaretsky et al., 2014). Understanding the impact of *Brucella* infection in hematopoiesis, and in particular on HSCs homeostasis, could provide key knowledge necessary to develop new therapeutic strategies

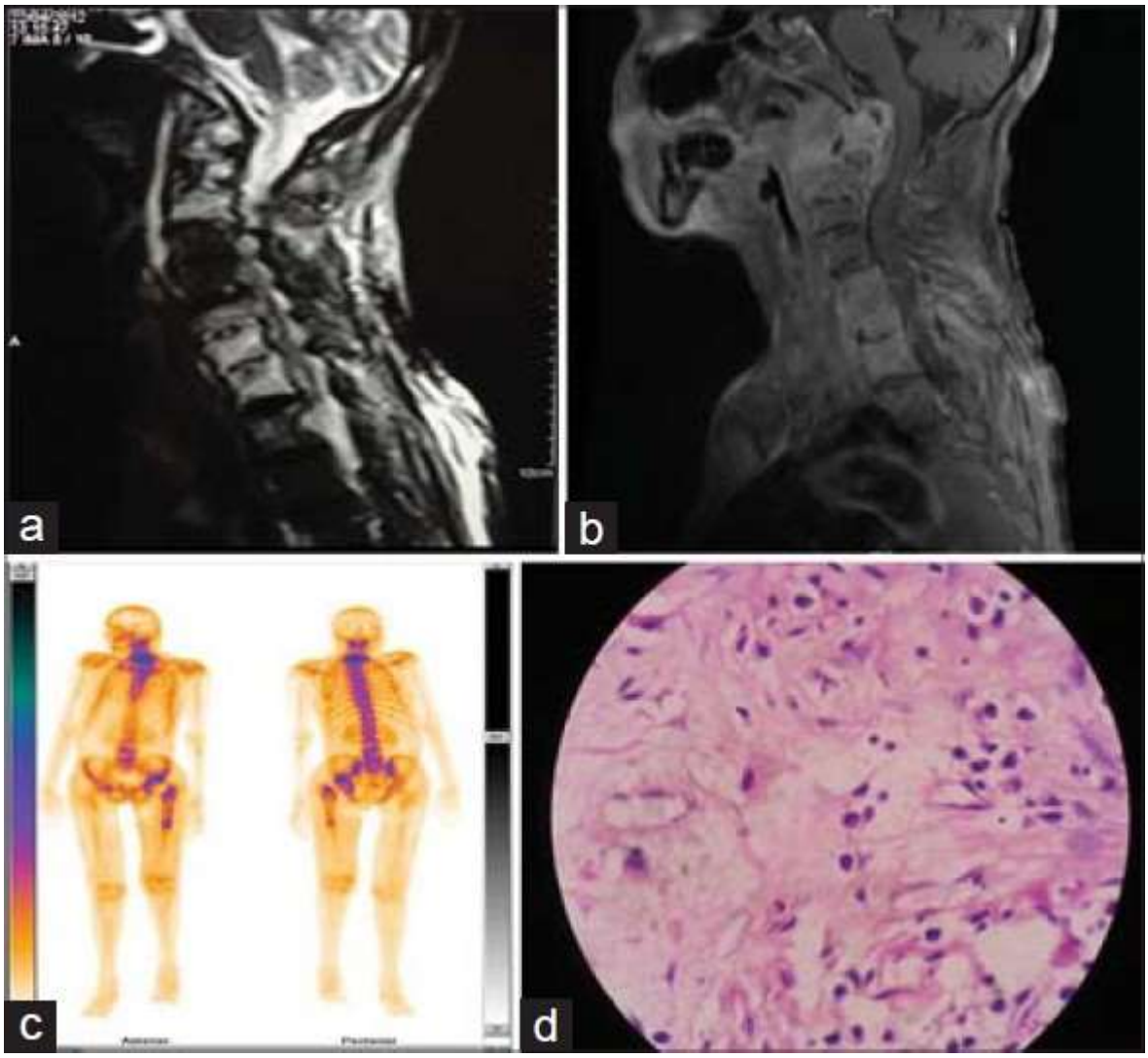

Figure 7: Osteo-articular complication in brucellosis patient

Magnetic Resonance Imaging showing altered marrow signal in cervical vertebrae and diffuse disc bulges. b) Magnetic Resonance Imaging of cervical spine on subsequent admission showing progression of lesion with altered marrow signal c) bone scan showing increased radionucleotide uptake in cervical region. (d) vertebra biopsy showing chronic nonspecific inflammation.

Adapted from (Aydin, Yapar et al. 2005)

Hematopoiesis and bone marrow architecture

The bone

The bone is a compact and solid organ that is formed during embryogenesis. However this organ is in continuous remodeling in adults. Three major actors participate in bone remodeling: osteoblasts, osteocytes and osteoclasts. Osteoblasts and osteocytes originate from embryonic cartilage cells (ECCs) and adult mesenchymal stem cells (MSCs) and osteoclasts originate from erythro-myeloid progenitors (EMPs) during embryonic development and adult HSCs that reside in the adult BM (Dar, Azam et al. 2018, Jacome-Galarza, Percin et al. 2019, Verbeeck, Geris et al. 2019).

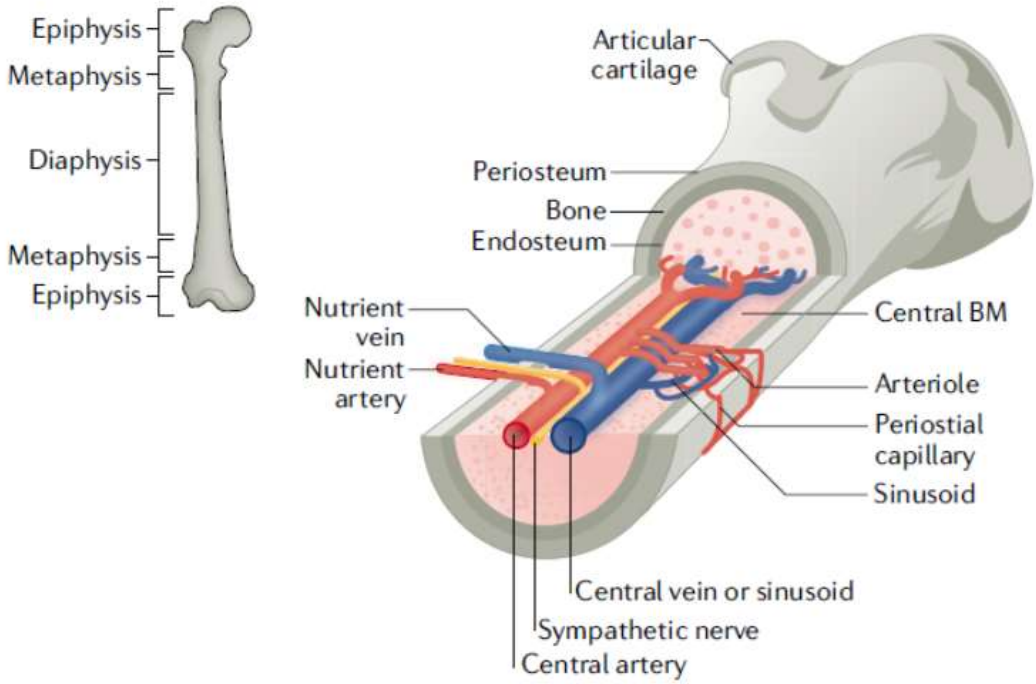

Figure 8: Architecture of adult mouse femur.

Adapted from (Pinho and Frenette 2019)

Osteoblasts and osteocytes are known as bone forming cells. **Osteoblasts** produce collagenous and non-collagenous proteins that form a rigid scaffold structure called osteoid. Osteoid differentiate thereafter into **osteocytes**. Hence, osteocytes are composed of osteoids but have a different gene profiling compared to osteoblasts. These cells build the bone structure and are able to sense mechanical stimuli. Inversely, **osteoclasts** are bone-destroying cells. This process of bone formation and destruction is called bone remodeling (Figure 8).

Indeed, osteoblasts produce a factor called RANKL (receptor activator of nuclear factor NκB ligand), protein of TNF family that binds to RANK receptor expressed in monocytes progenitors. RANKL/RANK interaction induces fusion of monocyte progenitors and differentiation of the fused cells into osteoclasts. This process is called osteoclastogenesis. Once formed, osteoclasts are recruited to the bone sites and initiate bone destruction and the release of minerals like calcium and phosphate (Clarke 2008). Osteoblasts are also able to secrete a soluble molecule called osteoprotegerin (OPG). Indeed, OPG binds to RANKL and antagonizes its binding to RANK. The OPG/RANKL bond inhibits osteoclastogenesis and induce osteoclast death (Liu and Zhang 2015). Bone remodeling is a physiological process that ensures the renewal of the bone matrix and regulation of calcium and phosphate levels in the organism.

As already mentioned, changes in bone homeostasis have profound repercussion in hematopoiesis. Indeed, Osteoblasts are very important for HSCs maintenance in the niche.VCAM1, ICAM1, Annexin II, N-cadherin, CD44 and CD164 expressed by osteoblasts bind to HSCs integrins and maintain them in the niche (Li and Xie 2005) . Depletion of osteoblasts causes HSC loss in the BM and HSCs mobilization. On the other hand, in vitro studies have shown that osteoblast can secrete cytokines such as G-CSF, M-CSF, GM-CSF, IL-1, IL-6, IL-7 CXCL12. These cytokines affect not only bone remodeling but also the hematopoiesis process (Taichman 2005, Mercier, Ragu et al. 2011, Baschuk, Rautela et al. 2015, Pesce Viglietti, Arriola Benitez et al. 2016).

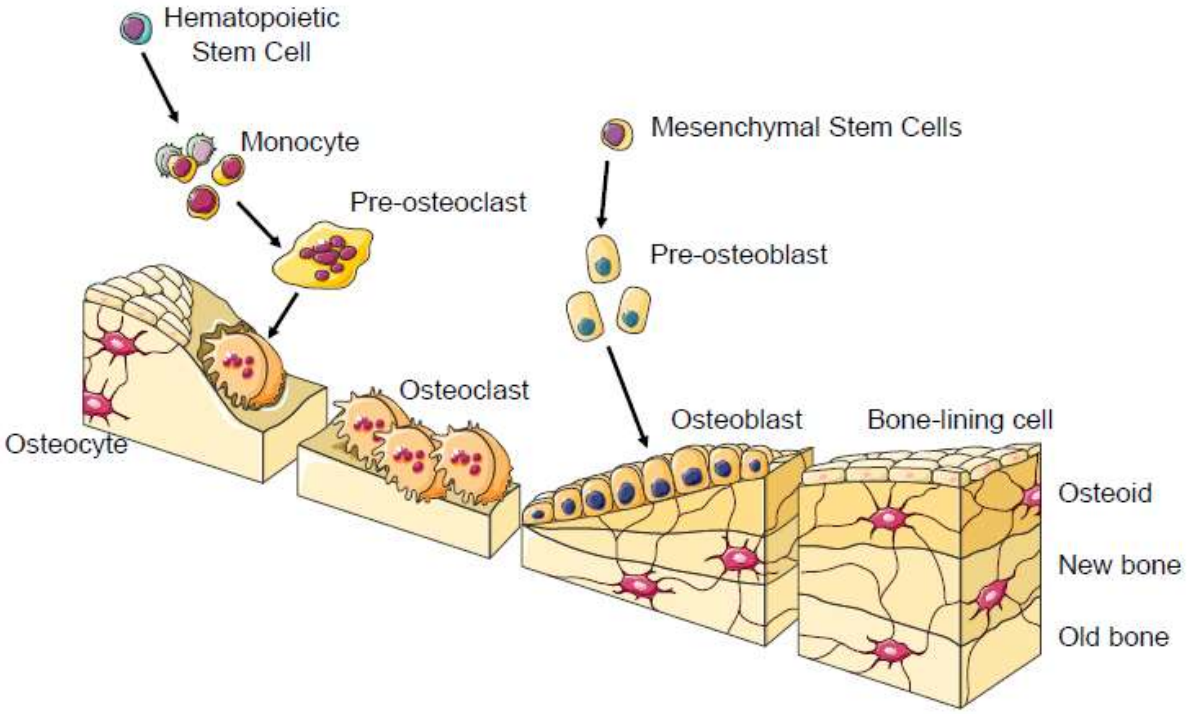

Figure 9: Bone Remodeling

During bone remodeling osteoclasts, derived from hematopoietic stem cells, resorb old and damaged bone (in the right). At the end of this phase, osteoblast recruited to the resorbed area form a new bone. Osteoblasts that get trapped in the bone matrix during bone formation become osteocytes (in the left).

The bone marrow

The BM is an intricate organ that encompasses several hematopoietic and non-hematopoietic cell types that are interconnected by a vascular and innervated network. The bone marrow is composed of hematopoietic cells (red marrow) and non-hematopoietic cells (yellow marrow). **The yellow marrow** is composed of MSC derived cells like adipocytes and osteoblasts and **the red marrow** is composed of hematopoietic stem cells (HSCs), plasma B cells, megakaryocytes, T cells and also myeloid cells (Morrison and Scadden 2014). The main role of the BM in post-natal life is to generate blood cells and also osteoclasts for bone remodeling from HSCs. (Schofield 1978, Spradling, Drummond-Barbosa et al. 2001). HSCs are multipotent cells able to self-renew and to give rise to the whole hematopoietic system ((McCulloch and Till 1960). HSCs are developed during embryogenesis and migrate to the BM just before birth.

Embryonic hematopoiesis

In vertebrates, the hematopoietic system develops from three distinct waves and takes place in different organs throughout the embryonic development (Al-Drees, Yeo et al. 2015, Nandakumar, Ulirsch et al. 2016, Yumine, Fraser et al. 2017, Hoeffel and al, 2015).

The first or the primitive wave of hematopoiesis emerges at embryonic day E7 in mice and generates primitive hematopoietic progenitors in the extra-embryonic yolk sac blood islands. Primitive Hematopoietic progenitors seem to be unipotent cells that give rise either to primitive nucleated erythrocyte progenitors, or primitive macrophages (Moore and Metcalf 1970, Haar and Ackerman 1971, Kyba, Perlingeiro et al. 2002).

The second wave or the pro-definitive wave of hematopoiesis starts at E8.25 in the first formed hemogenic endothelium of the YS vasculature, and potentially the placenta and the intraembryonic aorta-gonad-mesonephros (AGM) regions. This second wave is characterized by the appearance of multipotent progenitors with restricted lineage potentials. First, erythro-myeloid progenitors (EMPs) that produce erythrocytes capable of synthesizing adult hemoglobin, myeloid cells such as neutrophils, mast cells, fetal monocytes and osteoclasts (Hoeffel and Ginhoux 2015, McGrath, Frame et al. 2015, Gentek, Ghigo et al. 2018) Then the lymphoid potential emerges and provide B1 B cells and T cells that derived from distinct progenitors called lympho-myeloid progenitors (LMPs) (Dzierzak and Speck 2008, Hoeffel and Ginhoux 2015, Hoeffel and Ginhoux 2018).

During **the third or definitive wave,** more sophisticated hematopoietic progenitors emerge from hemogenic endothelium after E9.5, which ultimately give rise to pre-HSCs and then HSCs. Pre-HSCs are multipotent progenitors able to give rise to all hematopoietic cells but cannot reconstitute the hematopoietic system of an irradiated recipient. pre-HSC migrate in the fetal liver where they undergo maturation and massive expansion and form the definitive pool of HSCs. Definitive HSCs are pluripotent and will migrate just before birth in the BM. (Orkin and Zon 2008) .

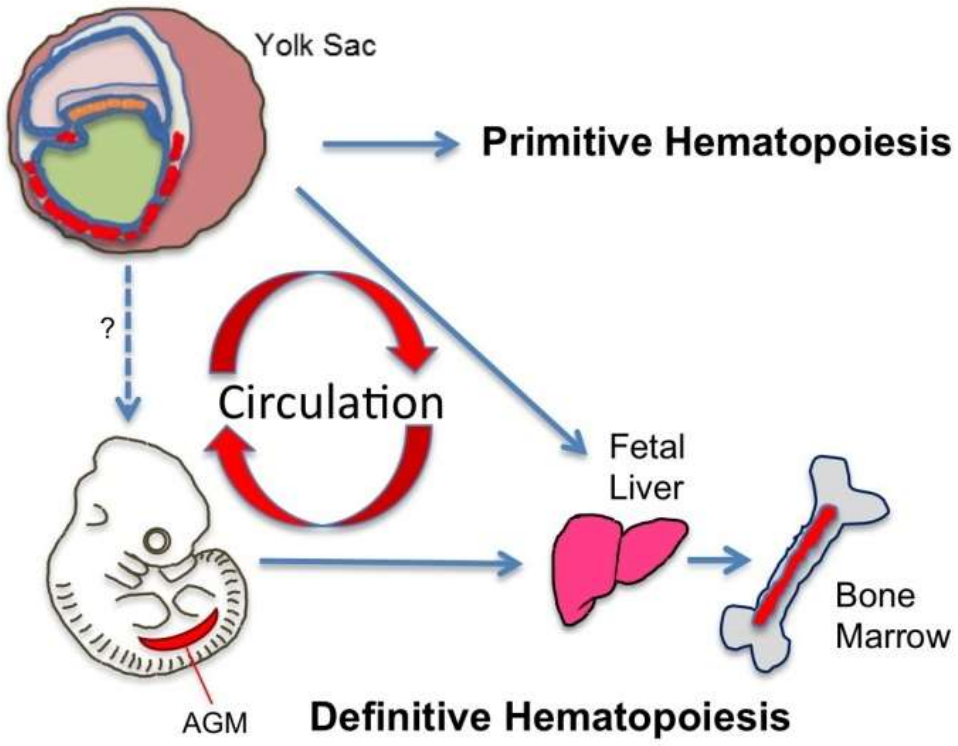

Figure 10: Primitive and definitive hematopoiesis

In vertebrates, the hematopoietic system develops from three distinct waves and takes place in yolk sac, fetal liver during embryonic development and in bone marrow after birth.

Adapted from (Chen, Mao et al. 2014)

Adult hematopoiesis

In adults, the principal niche of HSCs is the bone marrow (BM). A small number of HSCs are also found in secondary lymphoid organs such as spleen and liver. Adult hematopoietic stem and progenitor cells (HSPCs) are divided in different groups based on their reconstitution capacity and lineage bias commitment: Long term HSCs (HSC^{LT}), short term HSCs (HSC^{ST}) and multipotent progenitors (MPPs) Table 6. **HSC^{LT}** are called long term because they have a reconstitution capacity of more than six months and can be transplanted into secondary or even tertiary hosts (Domen and Weissman 1999). **HSC^{ST}** can reconstitute only for four to twelve weeks after transplantation. Indeed, during transplantation assays in mice it is considered that hematopoietic cells generated 12 weeks after transplantation originate from HSC^{LT}. Both HSC^{LT} and HSC^{ST} are pluripotent cells (Doulatov, Notta et al. 2012). Different genes have been identified as specific to HSCs and are used to establish mice model for HSC biology as shown in Table 5 (Cabezas-Wallscheid, Klimmeck et al. 2014, Pietras, Reynaud et al. 2015). MPPs are multipotent progenitors and they are already biased to myeloid (MPP3), myeloid-erythroid-megakaryocyte (MPP2) or lymphoid lineage (MPP4)

Dormancy and self-renewal of hematopoietic stem cells (HSCs)

While in mammals, most cells are in phase G1 phase of the cycle, stem cells are mostly in phase G0. During this phase, the cell metabolism slows down. Indeed, the analysis of gene expression from quiescent and active HSCs showed a decreased RNA transcription and decreased metabolism in quiescent HSCs (Venezia, Merchant et al. 2004). Many factors were identified as regulators of dormancy and HSC exit from dormancy and entry in G1 phase as shown in Table 7 (Pietras, Warr et al. 2011). p53, p21, p57, p27 and c-myc as well as cyclins and their associated kinases (CDK, Cycline Dependent Kinase) are critical for maintaining HSCs quiescence, but are also involved in regulating cell cycle. They are strongly expressed in HSC^{LT} compared to progenitors. Their deletion causes an increase in the proliferation of HSCs and a decrease in their ability to replenish the host immune system(Cheng, Rodrigues et al. 2000, Dumble, Moore et al. 2007, Liu, Elf et al. 2009) Conversely, c-myc is poorly expressed in quiescent cells but its expression increases during differentiation (Wilson, Laurenti et al. 2008).In HSCs, c-myc regulates negatively p21 (Wu, Cetinkaya et al. 2003). An overexpression of c-myc leads to a loss of HSCs due to extensive proliferation (Wilson, Murphy et al. 2004).

Mouse strains	Genetic modification	Specificity within the adult hematopoiesis	References
Hoxb5-Tri-Cherry	Knock-in	Specific to HSC^{LT}	Chen et al, Nature 2016
Ctnnal1–GFP	Knock-in	Restricted to HSPCs	Acar et al Nature 2015
Fgd5–mCherry	Knock-in	Restricted to HSCs, low expression in hematopoietic progenitors	Gazit et al, 2014
Vwf–GFP	Transgenic	Labels platelet-biased and myeloid-biased HSCs, megakaryocyte progenitors, megakaryocyte and platelets	Sanjuan-Pla et al, Nature 2013
Msi2–GFP	Knock-in	Labels hematopoietic progenitors	Koechlein et al, 2016
Pdzk1ip1–GFP	Transgenic	Enriches for highly purified HSCs but also labels a small subpopulation of hematopoietic progenitors and mature granulocytes	Sawai et al, Immunity 2016
Evi1–GFP	Knock-in	Labels hematopoietic progenitors	Kataoka et al, J.exp med
Scl–tTA-H2B–GFP	Transgenic	Labels HSPCs	Wilson et al Cell 2008 ; Sugimura et al, Cell 2012
Tie2–GFP	Transgenic	Labels HSPCs	Ito et al, Science 2016
Gprc5c–GFP	Transgenic	Labels HSPCs	Cabezas-Wallscheid, Cell 2017
Hdc–GFP	Transgenic	Labels HSPCs	Chen et al, Cell Stem Cell 2017
Krt7–GFP	Knock-in	Specific to HSCs	Tajima et al, Sci Rep,2017
Gata2–GFP	Knock-in	Enriches for HSPCs	Suzuki et al Proc natl 2006

Table 5: Mouse strains available for specific labeling of Hematopoietic Stem Cells

Adapted from (Pinho and Frenette 2019)

Different strategies are used to discriminate quiescence (G0 phase) from the other phases of cell cycle. Most of them are based on the fact that quiescent cells have a very low transcriptomic and metabolic activity. For instance, these cells do not express Ki67 (Rossi, Seita et al. 2007). Ki67 is present during all active phases of the cycle (G1, S, G2 and mitosis), but is absent in quiescent cells (G0) (Scholzen and Gerdes 2000). In addition, quiescent HSCs are not positive for pyronine Y or orange acridine which stains RNA (Hayashi, Morita et al. 1990, Gordon, Bocharova et al. 1997). *In vivo*, BrdU or EdUs are used to discriminate between cyclic and quiescent cells. These molecules that are incorporated into the DNA of cells undergoing replication and can be detected by specific antibodies, allows to discriminate between cyclic and quiescent cells. Indeed, about 70% of HSC^{LT}, 60% of HSC^{ST} and 50% of MPPs seem to be in G0 phase *in vivo* (Passegué, Wagers et al. 2005). The quiescence is a form of protection that HSCs develop to reduce the risk of DNA mutations and oncogenic transformations. As such, quiescent HSCs are protected from genotoxic drugs such as 5-Fluorouracil (5-FU) (Harrison and Lerner 1991, Randall and Weissman 1997, Venezia, Merchant et al. 2004, Jang and Sharkis 2007). A mouse model expressing the fusion protein Histone H2B-GFP has been generated to monitor cell proliferation (Foudi, Hochedlinger et al. 2009). This model allows to measure the number of times a HSC have been divided by quantifying the GFP intensity.

The analysis of the HSC compartment by this technic showed that HSCs that divide less than five times during the life of an organism present a significant potential for recovery and are suggested to be reserve HSCs (rHSCs). Indeed active HSCs are responsible of replenishment of hematopoietic system during homeostasis while rHSCs are required to restore HSC pool after stress such as chemotherapy (Zhao et al., 2019).

	Surface Markers	Lineage Bias Commitment
HSC^{LT}	lin- SCa1+ c-Kit+ CD48- CD150+ CD34- Flt3-	multipotent, long term self-renewal capacity
HSC^{ST}	lin- SCa1+ c-Kit+ CD48- CD150- CD34- Flt3-	multipotent, short term self-renewal capacity
MPP2	lin- SCa1+ c-Kit+ CD48+ CD150+ Flt3+	erythroid-megakaryocyte bias
MPP3	lin- SCa1+ c-Kit+ CD48+ CD150- Flt3-	myeloid bias
MPP4	lin- SCa1+ c-Kit+ CD48+ CD150- Flt3+	lymphoid bias

Table 6: Hematopoietic Stem and Progenitor Cell Phenotype

Adapted from (Pietras, Reynaud et al. 2015)

Types of factors	Effect	Factors requlating quiescence	Type of factor
Extrinsic Factors	activatory	SCF/KIT,Tie2/Ang1,TPO/Mpl, CXCL12/CXCR4, Hypoxie, Ca2+, N-cadherin, Integrins, OSP, Wnt	Cytokines/Receptors Chemical conditions Extracellular matrix Signaling pathways
	inhibitory	Hedgehog	Signaling pathways
Intrinsic factors	activatory	Gfi1,p53,Pbx1,Bmi1, p21,p57,p27,p18,Rb,CD4/6,CDK2, CyclinD1/D2/D3	Transcription factors Cell cycle molecules
		PTEN, Akt, FoxO	PI3K signaling pathway
	inhibitory	MEF, Lnk, Myc,Evi1	Transcription factors

Table 7: Hematopoietic Stem and Progenitor Cell Phenotype

Adapted from de LAVAL thesis, 2013

Differentiation of hematopoietic stem cells

In theory HSCs have an unlimited self-renewal capacity. However, serial transplantation of HSCs in the murine model showed that HSCs lose their reconstitution capacity after several transplantations (Orford and Scadden 2008). In fact HSCs self-renewal is inversely related to their proliferation and differentiation capacity. Indeed, under stress conditions like reconstitution of an irradiated recipient or infection HSCs exit from quiescence and enter in cell cycle (Walter, Lier et al. 2015). In general, entry in cell cycle and cell division gives rise to two identical cells. HSCs division gives one HSC and one progenitor cell. This phenomenon is called asymmetric division. Two non-exclusive hypotheses have been proposed to explain this asymmetry: divisional asymmetry and environmental asymmetry (Figure 11). During the divisional asymmetry, intrinsic stem cell fate determinants will locate in only one side of the cell during mitosis. Therefore, two non-identical cells will be produced: one that retains the stem cell fate and the other that initiates differentiation. The environmental asymmetry theory suggests that external clues can impact HSCs fate and specific microenvironmental signals dictate HSCs way of differentiation. For instance, after mitosis, the two daughter cells will end up in different microenvironments within the BM. One cell will stay in contact with the niche that provide stem cell identity, and the other cell will receive new signals triggering its differentiation (Wilson and Trumpp 2006) into MPPs Therefore the bone marrow environment is extremely important for HSCs maintenance and function. (Schofield 1978, Spradling, Drummond-Barbosa et al. 2001, Li and Xie 2005, Scadden 2014).

Many intrinsic and extrinsic factors have been identified for their role in balancing the dormancy, self-renewal, differentiation and survival of HSCs.

Most of the extrinsic factors are produced by BM niche cells, including osteoblasts, osteomacs, endothelial cells, megakaryocytes, nerves, etc(Calvi, Adams et al. 2003, Li and Xie 2005, Taichman 2005, Fujisaki, Wu et al. 2011, Casanova-Acebes, Pitaval et al. 2013, Hirata, Furuhashi et al. 2018).

The exchange between the niche and HSCs is necessary for many functions such as migration or membership, quiescence or proliferation. Many molecules and signaling pathways are involved in this exchange: cadherins, integrins, chemokines, cytokines, signaling molecules and their receptors (Figure 12).

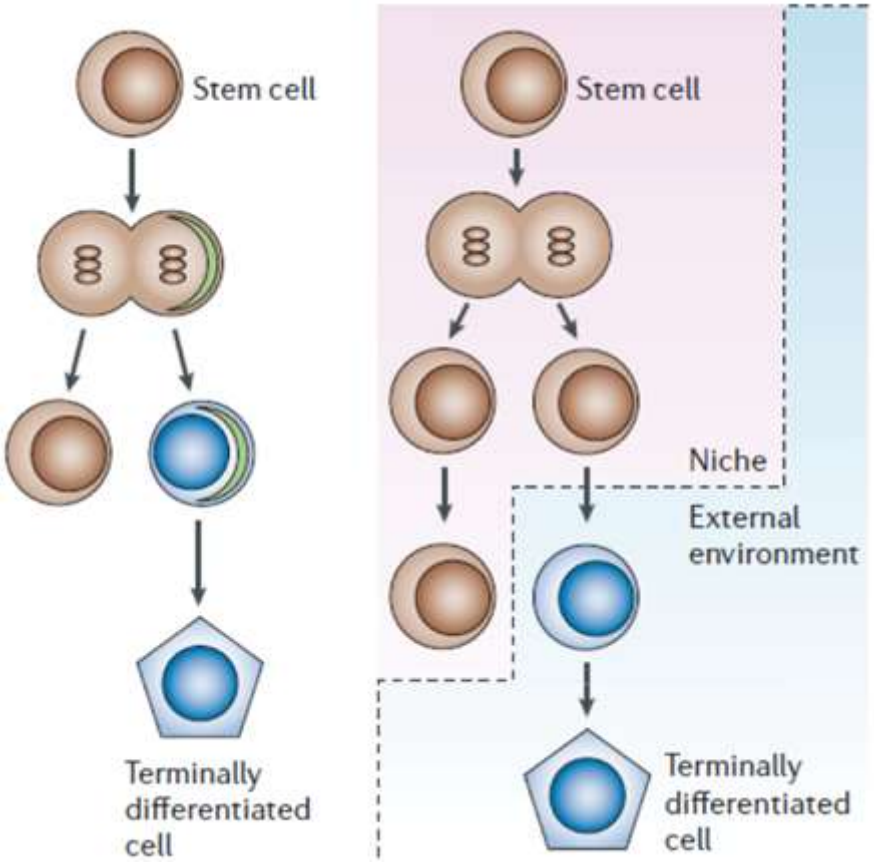

Figure 11: Models of HSC division

On the left is represented a symmetrical model of HSCs division and on the right an asymmetric model of HSCs division.

Adapted from (Wilson and Trumpp 2006)

The hematopoietic stem cell niche

Multiple hematopoietic stem cell niches have been identified based on different methodologies or surface markers (Figure 11).

The endosteal niche (enriched in N-Cadherin$^+$ (N-Cad$^+$) stromal cells), has a predominance of BMP, non-canonical Wnt, Jagged1, Angiopoietin 1 and Osteopontin signaling, suggesting a role in maintaining deep-quiescent HSCs, while Col2.3$^+$ mature osteoblasts have a supportive role. HSCs isolated from the endosteal region in both mouse and human BM have a higher engraftment capacity than those isolated from the central marrow (Grassinger, Haylock et al. 2010, Guezguez, Campbell et al. 2013). The mechanism behind this geographic specificity of quiescent HSCs involve Flamingo (Fmi) and Frizzled 8 (Fzd8)-mediated non-canonical Wnt signaling, which maintains deep-quiescent (or dormant) HSCs at the endosteal region of the trabecular area. Loss of either Fmi or Fzd8 leads to a loss of HSCs coupled with a ~70% reduction in reconstitution capacity (Sugimura et al, 2012). Altogether, these studies show that endosteal stromal cells present in trabecular bone maintain quiescent HSCs. Moreover, a pool of the endosteal HSCs support BM regeneration after chemotherapy (Zhao, Tao et al. 2019).

(2) **The perivascular niche (**enriched in Nestin$^+$ and Ng2$^+$ cells) provides inhibitors for BMP signaling and activators of Hedgehog, canonical and non-canonical Wnt signaling, suggesting a role for maintaining primed (poised for action) HSCs, while Chemokine 12 (***Cxcl12)*** abundant in reticular (CAR) cells provide signals for HSC retention. Ablation of Cxcl12 abundant reticular cells (CAR) or Nestin$^+$ cells present in the perivascular region reduces the number of phenotypic HSCs by 50-60% from the bone marrow (Sugiyama, Kohara et al. 2006, Mendez-Ferrer, Michurina et al. 2010, Omatsu, Sugiyama et al. 2010) and results in their mobilization to the spleen (Mendez-Ferrer, Michurina et al. 2010). Also, deletion of Cxcl12 (encoding stromal derived factor 1 [SDF1]) from perivascular cells results in a 50% reduction of functional HSCs in primary transplant recipients (Ding and Morrison 2013, Greenbaum, Hsu et al. 2013).

(3) **The sinusoidal niche** (enriched with Pecam$^+$ endothelial cells) shows predominantly Notch, SCF and Wnt signaling, with megakaryocytes having predominantly TGF-β (an inhibitory signaling for balancing proliferation) and FGF signals, suggesting a role for supporting active HSC self-renewal and progenitor cell proliferation. The specific deletion of the Notch ligand Jag1 from endothelial cells using VE-cadherin-Cre mice resulted in lower HSC numbers in steady state and reduced hematopoietic recovery after sublethal irradiation (Poulos et al., 2013).

Deletion of SCF from endothelial cells using Tie2-Cre mice also led to a dramatic reduction in HSC numbers in the bone marrow and spleen (Ding et al., 2012). Deletion of stem cell factor (SCF) (required for HSC proliferation and survival), from perivascular stromal cells and/or from sinusoids, results in 90% loss of phenotypic HSC number, functional HSCs are only partly (~50%) compromised (Ding et al., 2012).

However, with the improvement of microscopy techniques the concept of HSCs niche is starting to be controversial. Acar *et al.,* observed HSCs that do not express ki67 in the sinusoidal zone suggesting that quiescent HSCs can be found everywhere, regardless their specific location in the BM (Acar, Kocherlakota et al. 2015).

Moreover, the composition and the cellularity of BM niche changes during stress conditions such as inflammation or infection. In the next section I will sum up the recent findings about the changes in hematopoiesis during infection.

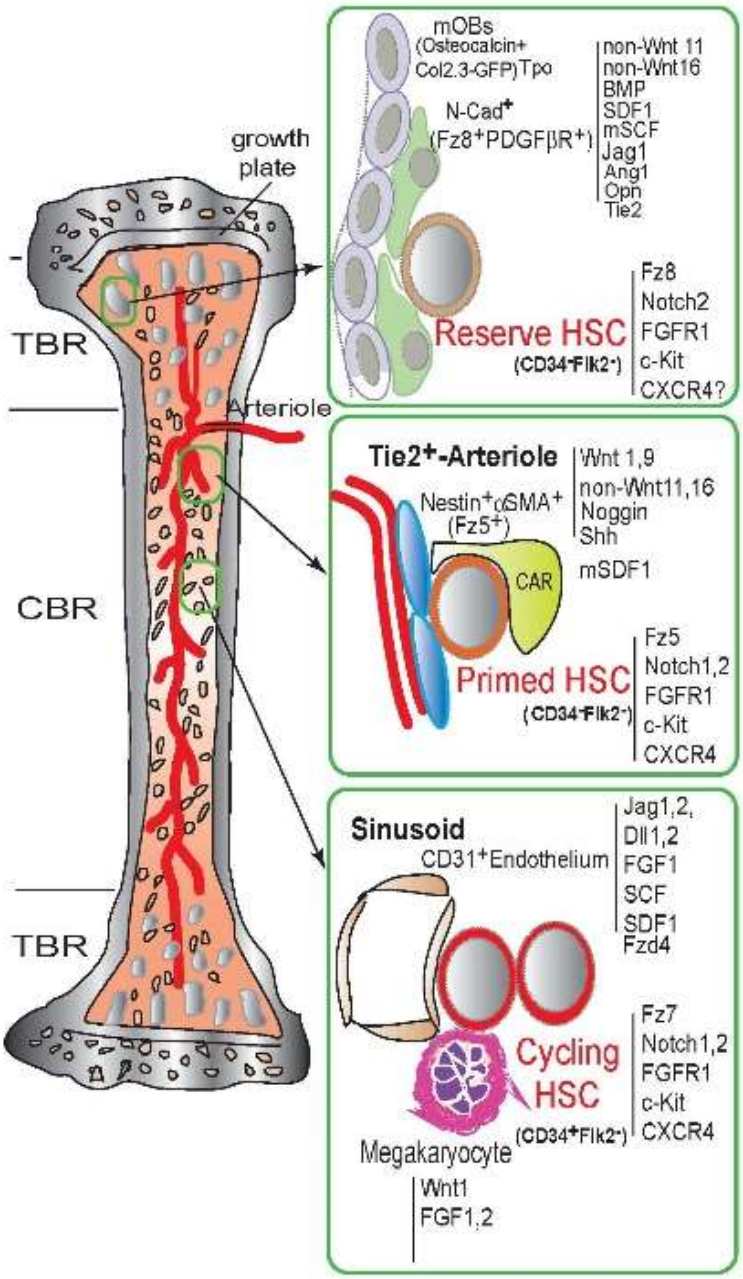

Figure 12: Bone marrow niches

On the left is represented a symmetrical model of HSCs division and on the right a asymmetric model of HSCs division.

Adapted from HYSENAJ et al, poster in Stowers Summer School Poster day ,2014

Courtesy of Linheng Li laboratory

Infection induces changes in hematopoiesis

Infectious disease are associated with hematological abnormalities such as anemia or the increase of myeloid progenitors in the blood. In fact, during systemic inflammation HSCs enhance myeloid cells production. This phenomena is called emergency myelopoiesis (Boettcher, Ziegler et al. 2012, Takizawa, Boettcher et al. 2012).

In fact, during microbial infection mature hematopoietic cells in the periphery recognize pathogens via pattern recognition receptors (PRRs) and produce humoral mediators (cytokines and chemokines) to induce immune response. Some of these humoral mediators will circulate through blood and reach the HSCs in the BM niche (King and Goodell 2011). Factors such as G-CSF will lead to egress of neutrophils from the BM, and secondarily result in HSC enhanced proliferation or/and mobilization (Semerad, Liu et al. 2002, Burberry, Zeng et al. 2014). In the periphery HSCs can detect microbial factors such as the LPS. Recognition of LPS by HSCs triggers HSC expansion and is responsible for the extramedullary hematopoiesis (Massberg, Schaerli et al. 2007). In addition, HSCs communicate with the surrounding cells. The interaction can be direct via adhesion molecules or via secreted factors. With advancements in genomics and proteomics, we already know that HSCPs express not only PRRs but also cytokine receptors such as M-CSF, IL-1β or TNFα stimulation. These cytokines cause enhanced HSC myeloid commitment (Mossadegh-Keller, Sarrazin et al. 2013, Haas, Hansson et al. 2015, Pietras, Mirantes-Barbeito et al. 2016).

To sum up, multiple nonexclusive scenarios can affect HSC function during infection: cell-cell communication, cytokine mediated signaling and direct pathogen detection.

In this section I will focus on the effect of cytokines and direct pathogen interaction in HSCs biology.

Bacteria	BM infection	Infected cells	BM HSPCs	Clinical manifestations	References
Chlamydophila pneumoniae	+	MO, Mϕ, MC	unknown	chronic anemia	(Nebe, Rother et al. 2005)
Clostridium perfringens	(in case of septic schoc)	Mϕ		unknown	(Lazarescu, Kimmoun et al. 2012)
C. burnetii	+	Mϕ		neutropenia in few cases	(Baumgärtner, Boyce et al. 1995, Eldin, Melenotte et al. 2017, Cherla, Zhang et al. 2018)
E coli	+	Mϕ	HSPC +++ HSPC dysfnction NOD-like and TLR 4 dependent		(Burberry, Zeng et al. 2014)
Ehrlichia ewingii	+	Ly, MegaK, plathelets	HSCs exhaustion dependent on IFNɣ↑	Pancytopenia	(Allen, Pritt et al. 2014)
Listeria monocyotogenes	+	MO, Neu	unknown		(Hardy, Chu et al. 2009, Witter, Okunnu et al. 2016)
Legionella pneumonia	+	MO, Mϕ	unknown	anemia	(De Truchis, Dournon et al. 1988)
Mycobacterium tuberculosis	+	HSCs , MSC , Mϕ	unknown	pancytopenia	(Kashyap and Forman 1998, Reece, Vogelzang et al. 2018)
Mycobacterium avium-intracellulare	-	MO,Mϕ s	HSPC +++, HSC echaustion due to IFNɣ↑	pancytopenia	(Baldridge, King et al. 2010, Matatall, Jeong et al. 2016)
Propionibacterium acnes	+	extracellular		unknown	(Waitzkin 1969)
Salmonella enterica serovar typhimurium	+	Plasma B, DCs , MO	HSPCs+++; HSC dysfunction dependent on TLR4-TRIF-ROS-p38, HSC increase their capacity to generate B1a cells	anemia, left shift	(Wain, Pham et al. 2001, Castro-Eguiluz, Pelayo et al. 2009, Takizawa, Fritsch et al. 2017)
Staphylococcus aureus	+	extracellular MSCs	TLR2-dependent HSPC expansion	anemia, left shift	(Granick, Falahee et al. 2013, Witter, Okunnu et al. 2016)
Staphylococcus epidermidis	+	extracellular	Acute arrest of hematopoiesis (cessation of red blood cell production by HSCs)	anemia	(Bi, Li et al. 2016)
Streptoccocal sepsis	+	extracellular		anemia	(Greenthal 1938)

Table: List of bacteria that infect bone marrow

HSCs and cytokines

Cytokines play a key role in guiding HSC fate. For instance, IL-6, TNF-α, type I IFN, type II IFN, TGFβ, and M-CSF, have been identified as involved in HSC proliferation and differentiation (Baldridge et al. 2010; Challen et al. 2010; Essers et al. 2009; Mossadegh-Keller et al. 2013; Pronk et al. 2011).

Interferon: Interferon are split into type I interferon (INFα/β) that signals through the IFNAR receptor and also type II interferon (IFNγ). IFNα is highly produced during viral infection. In mouse treatment with the double stranded RNA (dsRNA) polynosinic-polycytidylic acid (PolyI-C) mimics viral RNA and leads to IFNα/β signaling activation (Darnell, Kerr et al. 1994). *In vivo* treatment with poly I:C in mice induces HSCs proliferation and loss of self-renewal capacity through INFα/IFNAR/Stat1 signaling (Essers, Offner et al. 2009). This exit from quiescence is followed by mitochondrial ROS production that leads to DNA damage in HSCs (Walter, Lier et al. 2015). In contrast, an *ex vivo* study showed that during chronic poly I:C stimulation, HSCs return to quiescent due to the increase in Irf2 (transcription factor that negatively regulates IFNα signaling) (Sato, Onai et al. 2009, Pietras, Lakshminarasimhan et al. 2014). Protection of HSC from replicative stress induced by IFNα can be assured also by other factors like retinoic acid (Cabezas-Wallscheid, Buettner et al. 2017).

Type II interferon (IFNγ) also modulates proliferation and maintenance of HSCs. During *Mycobacterium avium* and *Mycobacterial Bacillus Calmette Guerin* (BCG) infection, an increase of proliferating HSC pool is observed and this is dependent to IFNγ and not to IFNα production (Baldridge, King et al. 2010, Kaufmann, Sanz et al. 2018). Consistent with this data, Matatall *et al,* showed that during *M. avium* infection, Batf2 (a mediator of IFNγ) induces HSPC myeloid differentiation. In the same study, the authors showed that chronic *M. avium* infection leads to depletion and exhaustion of HSCs in the BM. In this study, no mobilization of HSCs to the spleen was observed. However, it is also possible that HSCs migrate in another secondary lymphoid organ (Matatall, Jeong et al. 2016).

Interleukin β (IL-1β) is part of a family of 11 cytokine. IL-1 consist of 2 related genes IL-1α and IL-1β with distinct regulation but similar biological activities, which bind IL1R receptor and trigger downstream transcriptional response through adaptor MyD88 and a vast range of signaling pathways like NFκβ, P38, MAPK, JNK and AP-1 (Martin and Wesche 2002). Interleukin-1 (IL-1) is released during infection and it is a highly active pro-inflammatory cytokine that lowers pain thresholds and enhances tissue damage (Dinarello, Simon et al. 2012).

Treatment of HSCs with IL-1β *in vitro* induces PU.1 expression via NFκβ signaling downstream of IL-1R. PU.1 is a crucial transcription factor for myeloid differentiation. PU.1. Its upregulation in HSCs is associated with myeloid commitment (Mossadegh-Keller, Sarrazin et al. 2013, Giladi, Paul et al. 2018). *In vivo,* chronic IL-1β administration leads to impairment of HSCs function. However, IL-1β withdrawal, reestablish HSCs function (Pietras, Mirantes-Barbeito et al. 2016).

Tumor Necrosis Family α (TNFα) is a pleiotropic cytokine that participates in several cellular processes. Aberrant expression of TNF leads to inflammatory disease. TNF signals through different receptors: Tnfrs1a (TNF receptor super family 1a or TNFR-p55) and Tnfrs1b (TNF receptor super family 1b or TNFR-p75). TNFRS1a is involved in apoptosis signals whether TNFRS1a is implicated in the promotion of cellular proliferation (Bradley 2008). TNFα administration in mice induces myelopoiesis through PU.1 upregulation (Pronk, Veiby et al. 2011, Etzrodt, Ahmed et al. 2019).

Interleukin 6 (IL-6) is a pro-inflammatory cytokine produced in the same time as IL-1. IL-6 affects HSCs proliferation and differentiation. Indeed, IFNγ produced by cytotoxic CD8 T cells, induces the production of IL-6 by mesenchymal stem cells (MSCs). In addition, Baltimore's team showed that HSPCs are able to produce IL-6 by themselves in response to LPS challenge. This cytokine acts in a paracrine fashion to induce HSPCs myeloid differentiation (Schurch, Riether et al. 2014, Zhao, Ma et al. 2014). IL-6 is also involved in promoting pre-leukemic HSCs survival via activation of signaling through SHP2 STAT3 (Cai, Kotzin et al. 2018)

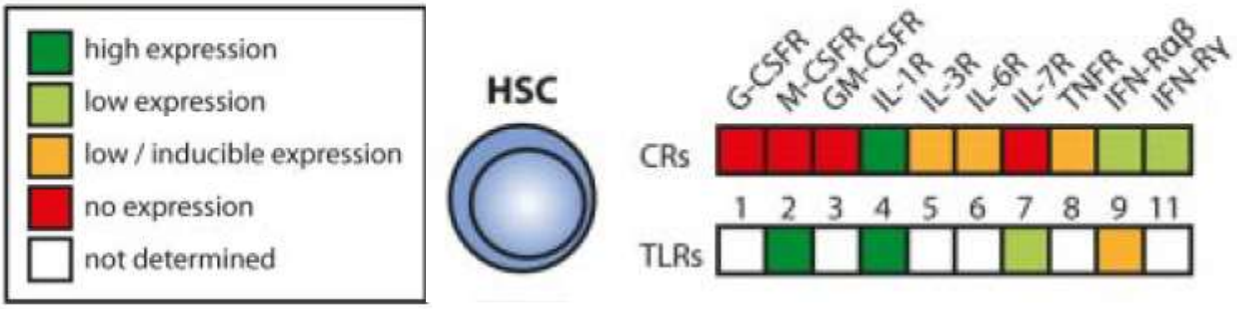

Figure 14: Cytokine receptor and Toll like receptor in HSPCs.

Direct sensing of pathogens by HSCs

As shown in Table 6 HSCs can encounter pathogens in the BM or in the periphery. HSCs express the same PRRs as phagocytic cells. However the intracellular machinery of myeloid cells and HSCs is not the same, making difficult to predict the effect of pathogen pattern recognition in HSC function. Recently, pioneering work studied the effect of Toll-like receptors (TLRs) and stimulator of interferon genes (STING) pathway in HSC function.

Toll-like receptors (TLRs) are cell receptors that detect microbial cell wall components, non-self-nucleic acids or danger associated self-molecules (Poltorak, He et al. 1998). The most studied TLR is TLR4. TLR4 is an extracellular receptor. This receptor forms a complex with the myeloid differentiation factor-2 (MD2) on the cell surface, and together they serve as the main LPS-binding component. (Gutsmann, Müller et al. 2001). Upon stimulation in macrophages TLRs activate two types of pathways. One that involves myeloid differentiation primary response protein 88 (Myd88) adaptor and leads to pro-inflammatory cytokine production and the other that involves TIR domain-containing adaptor inducing IFNβ (TRIF) and the TRIF related adaptor molecules (TRAM) that leads to the activation of IFN related pathways. (Akira and Akeda, 2004).

In HSPCs, *in vitro* stimulation with LPS induces HSPC expansion (Zhao, Ma et al. 2014). When the pre-stimulated HSCs are transplanted into the renal capsule they are able to expand and generate myeloid clusters (Massberg, Schaerli et al. 2007). LPS administration in mice induces emergency myelopoiesis and extra medullary hematopoiesis in the spleen upon acute stimulation. In addition, chronic stimulation with LPS causes increased genotoxic stress in HSCs and the impairment of self-renewal capacity (Esplin, Shimazu et al. 2011, Takizawa, Regoes et al. 2011). LPS administration in vivo induces a strong inflammatory response. Hematopoietic and non-hematopoietic cells such as mesenchymal cells possess TLR receptors and respond to LPS stimulation. Therefore, it is very difficult to distinguish the direct effect of LPS *in vivo*.

However, experimental work demonstrated that HSPC dysfunction induced by LPS is TLR4-TRIF-ROS-PR38. Indeed, WT:TLR4$^{-/-}$ BM chimeric mice TLR4$^{-/-}$ were treated with ROS/P38 inhibitors and afterward injected with LPS. BM analyses showed that inhibitor treatment abolished the LPS induced increase in proliferation.

The same authors showed that conversely emergency granulopoeisis induced by LPS stimulation is not dependent on TLR4-TRIF but on TLR4-Myd88 pathway (Takizawa, Boettcher et al. 2012, Takizawa, Fritsch et al. 2017). Moreover, it was shown that TLR4 works

on synergy with NOD-like receptors to induce HSC mobilization in the spleen. NOD-like Receptors (NLRs) are a subset of pattern recognition receptors (PRRs) found in the cytosol that detect bacterial flagellin or peptidoglycan and initiate the innate immune response. NLRs are divided into subfamilies based on their N-terminal protein-interacting domains: NLRC and NLRP. NOD1 and NOD2 are two well-characterized NLRs belonging to the NLRC subfamily. Upon activation, they homo-oligomerize and recruit signaling molecules that drive the NF-κB/AP-1-dependent expression of pro-inflammatory cytokines. In mice, *E. coli* infection activates NOD1, NOD2, and TLR4 signaling. Concomitant activation of TLR4 and NOD1 NOD2 triggers the decrease of CXCL12 levels in bone marrow and induces the release of G-CSF. The latter causes HSC mobilization in the spleen. HSCs mobilized in the spleen generate more myeloid cells then lymphoid or erythroid cells suggesting that extra medullary hematopoiesis enhances antimicrobial immunity (Burberry, Zeng et al. 2014).

The stimulator of interferon genes (STING): The cyclic GMP-AMP synthase cGAS is able to detect DNA in the cytoplasm. Upon DNA binding cGAS catalyzes DNA in cyclic dinucleotides (cDNs) such as c-diGMP and c-diAMPs. cDNs are directly sensed by the stimulator of interferon genes STING that induces downstream TBK1/IRF3/IFN or TBK1/ NF-κB (Burdette and Vance, 2013; Watson et al., 2015; Zhang et al., 2014)

Bis-3'-5'-cyclic dimeric guanosine monophosphate (c-di-GMP) is a second messenger used by bacteria for complex biological processes such as biofilm formation. c-di GMP is an agonist of STING pathway.

In vivo, c-di-GMP administration activates STING pathway in hematopoietic and non-hematopoietic cells. Therefore, administration of c-di-GMP in mice displayed MPP expansion in the BM, decreased number of HSCs in the BM and loss of their reconstitution capacity. Moreover, the mobilization of MPPs were observed in the spleen. Interestingly, while stimulated *in vitro* with c-di-GMP, HSCs and MPPs presented impaired capacity to form hematopoietic colonies. No HSCPC expansion were observed during *in vitro* stimulation. Hence, these findings argued that MPP mobilization in the spleen is a secondary effect of STING signaling activation. The decrease in number of PDGFRα^{+} CD51^{+} mesenchymal stem cells in the bone marrow can be one of the causes of MPP mobilization. Moreover authors showed that the mobilization was induced by TGFβ and G-CSF signaling but was inhibited type I interferon signaling. (Kobayashi, Kobayashi et al. 2015). G-CSF signaling was also activated during TLR4 and NOD1 NOD2 signaling suggesting that PRRs in HSCs share common downstream pathways.

To conclude HSCs can respond to infection by mobilizing in the spleen and by inducing an enhanced myeloid expansion. The increase of myeloid differentiation and the extramedullary hematopoiesis augments the number of downstream mature cells fighting the pathogen. Therefore, in order to better fight against infection, it is important to decipher the mechanisms by which HSCs sense pathogens or how pathogens manipulate HSCs fates to produce pathogen permissive cells.

Aim of the thesis

If we knew what we were doing, it would not be called research, would it?

Albert Einstein

Dendritic cells (DC) play an important function in *Brucell*a host immune responses and also provide a safe replication niche for the bacterium (Copin, De Baetselier et al. 2007, Macedo, Magnani et al. 2008, Salcedo, Marchesini et al. 2008, Papadopoulos and Gorvel 2015, Papadopoulos, Gagnaire et al. 2016, Zhao, Hanniffy et al. 2018, Avila-Calderon, Flores-Romo et al. 2019). *Brucella* controls DC maturation and TLR signaling through its effector proteins, BtpA and BtpB, and as such counteracts the protective Th1 immune response (Salcedo, Marchesini et al. 2008, Salcedo, Marchesini et al. 2013). In addition, the non-canonical LPS of *Brucella* is a low agonist of TLR4 and limits DC maturation. (Conde-Alvarez, Arce-Gorvel et al. 2012, Fontana, Conde-Alvarez et al. 2016, Zhao, Hanniffy et al. 2018). *In vitro*, infection with *Brucella* leads to moderate activation of mouse DCs as illustrated by intermediate expression levels of co-stimulatory molecules and cytokine secretion (Billard, Dornand et al. 2007, Billard, Dornand et al. 2007, Salcedo, Marchesini et al. 2008).

Brucella outer membrane proteins (Omp) are also implicated in the modulation of host immunity and in particular by Omp25. Recombinant Omp25 has been considered as a potential vaccine for *Brucella* (Bowden, Cloeckaert et al. 1998, Commander, Spencer et al. 2007, Commander, Brewer et al. 2010, Goel and Bhatnagar 2012, Goel, Rajendran et al. 2013). Further work is thus needed to clarify the role of this outer membrane protein in the regulation of host immune responses.

Our recent transcriptomics study identified *SLAMF1* as a strongly upregulated gene in human DCs stimulated with the *Brucella* cyclic β1,2 glucan (CβG) . SLAMF1 plays a dual role as both an activator and an inhibitor of the immune system during infection. The role of SLAMF1 in brucellosis has not been investigated so far.

Therefore, the first aim of my thesis was to determine the role of SLAMF1 during *Brucella* infection.

DCs are the key players in induction of both innate and adaptive immunity. Thus, studying the role of SLAMF1 in DCs could shed light on how the bacterium persist in the organism. In *vitro* the role of SLAMF1 as a bacterial sensor was explored by using the GM-CSF-derived BMDCs. Cells generated in this model resemble more to the inflammatory DCs recruited in the spleen during infection (Helft, Bottcher et al. 2015) and the human monocyte-derived DCs (Zhao, Hanniffy et al. 2018). *Slamf1*$^{-/-}$ mice were used to further investigate the *in vivo* function of

SLAMF1 as an immune co-receptor and bacterial sensor in the establishment of host immune responses.

In addition, case reports showed that *Brucella* infects the niche of the HSCs: the BM (Ertem et al., 2000; Naparstek et al., 1982; Ruben et al., 1991). SLAMF1 is also expressed in murine HSCs (Kiel, Yilmaz et al. 2005). The role of this receptor in HSCs during infection has not been explored yet. When HSC function is disrupted, the whole immune system is perturbed. Other pathogens such as *Escherichia Coli*, *Salmonella* Typhimurium or *Mycobacterium avium* interfere with HSC function (Massberg, Schaerli et al. 2007, Kobayashi, Kobayashi et al. 2015), In the case of brucellosis, the impact of the infection on HSC biology have not been examined yet. The second aim of my study was to study the effect of *Brucella* infection in the HSC niche (BM) and decipher the consequence of a potential Omp25-SLAMF1 interaction in HSC function.

Contribution to this work

Manuscript 1: I designed, performed the experiments and analyzed the data for Figure 2 and Figure 4 ; performed statistical analyses and made the figures for all data in the paper apart Figure 1*.

Manuscript 2: I designed and performed the experiments for Figure 1 and Figure 3 and participated in manuscript writing.

Manuscript 3: I designed, performed the experiments, analyzed the data and made the all the figures with the exception of Figure 2e. I also wrote the first draft of the paper.

*Figure 1 experiments were designed, performed and analyzed by Matthew J. Dufort, Sean Hannify, Matthew C. Altman, Prasong Khaenam from Benaroya Research Institute at Virginia Mason, Seattle, USA using a UO1 NIH grant.

Do not try to predict the results. Just do it!

Jean-Pierre Gorvel

Results

On doit exiger de moi que je cherche la vérité mais pas que je la trouve.

Denis Diderot

Manuscript 1: The host SLAMF1-Brucella Omp25 interaction restricts inflammation and nurtures chronic infection in brucellosis (Degos, **Hysenaj** et al, *in preparation)*

The host SLAMF1-*Brucella* Omp25 interaction restricts inflammation and nurtures chronic infection in brucellosis

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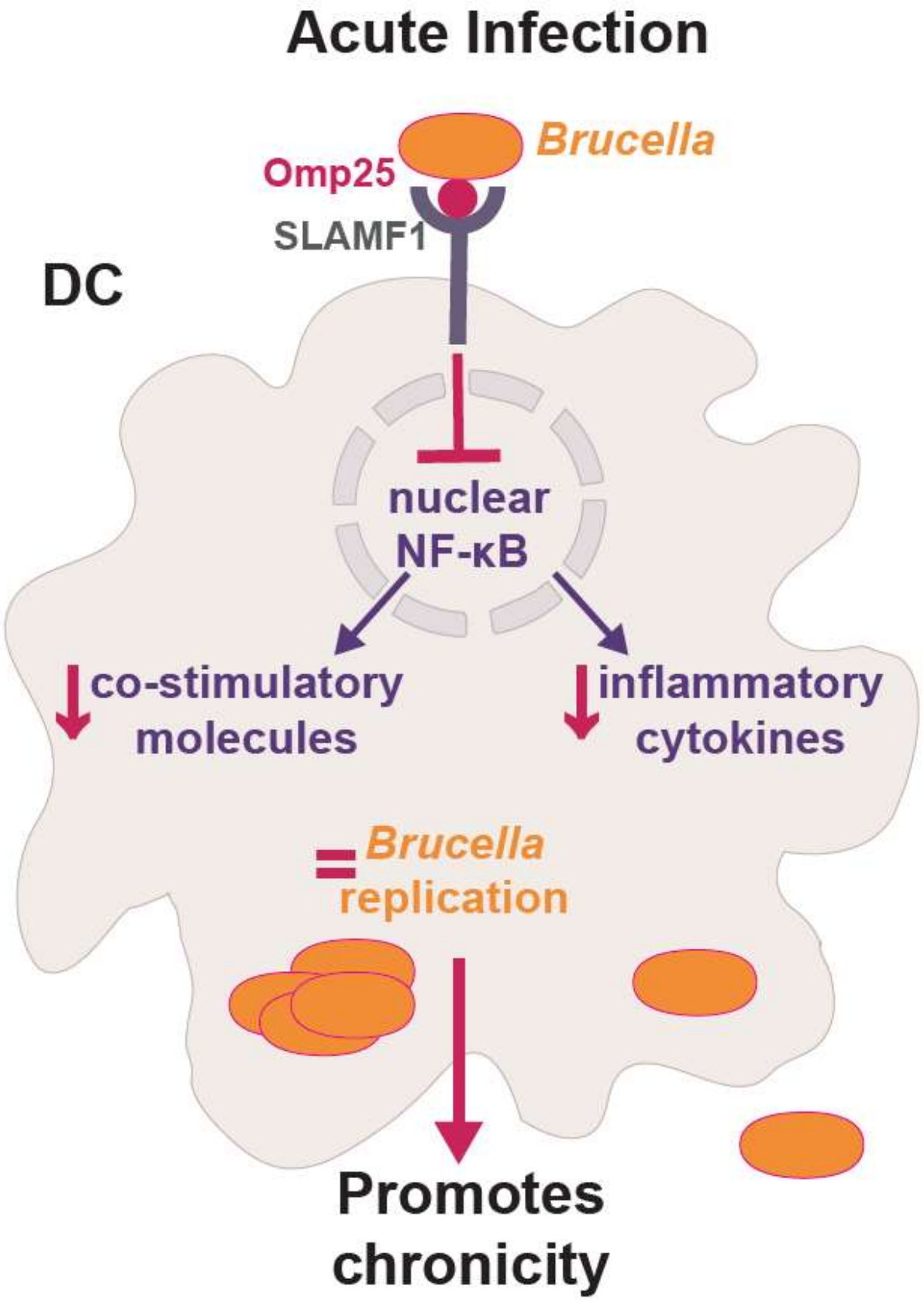
Running title: The SLAMF1/omp25 axis restricts *Brucella*-induced inflammation

Keywords: *Brucella*, Omp25, SLAMF1, dendritic cells, NF-κB, inflammation

Abstract:

Brucella is a facultative intracellular pathogenic bacterium responsible for brucellosis, a worldwide re-emerging zoonotic disease affecting wildlife and livestock. *Brucella* can be transmitted to humans via contaminated food or infected aerosol particles, thereby leading to undulant fever followed by chronic devastating multi-organ inflammation if untreated. By RNA-seq analysis of blood of patient cohorts from Spain and Macedonia, we have identified SLAMF1 as a biomarker of the acute phase of *Brucella* infection in humans. In addition, cytokine analysis led us to define a set of four serum biomarkers (IL-10, IL1RA, CXCL1 and IL-7) for *Brucella* chronicity. SLAMF1 is an immune cell receptor expressed notably at the surface of dendritic cells (DC), lymphocytes and haematopoietic stem cells and involved in the control of immune responses. We investigated its function in a murine model of *Brucella* infection. We showed that *B. abortus* drove SLAMF1 upregulation in bone marrow-derived DCs (BMDCs) independently of its outer membrane protein Omp25. However, SLAMF1 bound specifically *B. abortus* Omp25 and not the related protein Omp19. By using *B. abortus* Δ*omp25* mutant and its complemented version as well as *Slamf1*$^{-/-}$ mice, we provided evidence for the functional relevance of this binding both *in vitro* and *in vivo*. The SLAMF1-Omp25 interaction limited NF-κB translocation, subsequently decreasing pro-inflammatory cytokine secretion and impairing DC activation *in vitro* with no impact on *Brucella* intracellular trafficking or replication. *In vivo*, the SLAMF1-Omp25 interaction dampened the immune response during the acute phase of brucellosis, as illustrated by higher cDC1, cDC2, pDC and activated CD4^{+} and CD8^{+} T cell numbers in animals infected with the Δ*omp25* mutant strain. In contrast, bacterial replication in acutely infected mice was stable under all conditions but altered significantly in a SLAMF1-Omp25-dependent fashion at the chronic phase of infection, as a consequence of the hitherto modulated inflammation at the acute stage. Together these findings shed light on a yet unrecognised mechanism by which *Brucella* subverts the immune system. They suggest that the Omp25-SLAMF1interaction is essential for *Brucella* to establish a low inflammatory profile during the acute phase of infection in order to support bacterium settling in its furtive replicative niche and progression to chronicity.

Graphical Abstract



Introduction

*Brucell*a spp. are Gram-negative facultative intracellular coccobacilli. They are the causative agent of brucellosis, a worldwide re-emerging zoonosis, affecting livestock (bovine for *B. abortus*, ovine and caprine for *B. melitensis*, swine *for B. suis*) and wildlife with serious economic losses, as *Brucella* infected animals undergo spontaneous abortion and infertility(Corbel 1997, Cutler, Whatmore et al. 2005, Moreno 2014). Brucellosis can be transmitted to humans via contaminated food or infected aerosol particles and discloses a broad spectrum of clinical manifestations (Pappas, Akritidis et al. 2005, Dean, Crump et al. 2012). Acute human brucellosis is characterized by undulant febrile illness, which, if left untreated as often the case, leads to spreading of bacteria in various tissues. This ends up in a chronic disease with severe symptoms, such as osteoarthritis, spondylitis, endocarditis and neurological disorders (Corbel 1997, Cutler, Whatmore et al. 2005). *B. melitensis* is the most frequent cause of human brucellosis(Corbel 1997). Due to its high infectivity, *Brucella* has been classified as a potential warfare agent (Robinson-Dunn 2002) and is manipulated in BSL3 only. *Brucella* spp. have the ability to enter, survive and replicate within phagocytic and non-phagocytic cells and as such evade host immune system defence mechanisms (Gorvel and Moreno 2002, Robinson-Dunn 2002, Martirosyan, Moreno et al. 2011, von Bargen, Gorvel et al. 2012, Byndloss and Tsolis 2016). These properties may account for human brucellosis particularities(Skendros, Pappas et al. 2011).

Dendritic cells (DC) have been widely studied in brucellosis and demonstrated to be important for the induction of immune responses as well as providing a safe replication niche for the bacterium (Copin, De Baetselier et al. 2007, Macedo, Magnani et al. 2008, Salcedo, Marchesini et al. 2008, Papadopoulos and Gorvel 2015, Papadopoulos, Gagnaire et al. 2016, Zhao, Hanniffy et al. 2018, Avila-Calderon, Flores-Romo et al. 2019). DCs play a central role in the induction of both innate and adaptive immune responses by activating T cells during the course of *Brucella* infection(Copin, De Baetselier et al. 2007, Macedo, Magnani et al. 2008, Salcedo, Marchesini et al. 2008, Avila-Calderon, Flores-Romo et al. 2019). However, *Brucella* controls DC maturation and TLR signalling by amongst others expressing two *Brucella* effector proteins, BtpA and BtpB, to counteract the protective Th1 immune response (Salcedo, Marchesini et al. 2008, Salcedo, Marchesini et al. 2013) and harbouring a non-canonical LPS(Conde-Alvarez, Arce-Gorvel et al. 2012, Fontana, Conde-Alvarez et al. 2016, Zhao, Hanniffy et al. 2018). *In vitro*, infection with *Brucella* leads to moderate activation of mouse DCs as illustrated by intermediate expression levels of co-stimulatory molecules and cytokine

secretion(Billard, Dornand et al. 2007, Billard, Dornand et al. 2007, Salcedo, Marchesini et al. 2008). *In vivo*, DCs promote *Brucella* dissemination when macrophages are depleted(Archambaud, Salcedo et al. 2010). DCs are also required to control bacterial growth through secretion of pro-inflammatory cytokines such as interleukin-12 (IL-12) or enzyme like iNOS (Copin, De Baetselier et al. 2007, Macedo, Magnani et al. 2008).

Brucella outer membrane proteins (Omp) are also implicated in the modulation of host immunity. As such, recombinant Omp16 triggers secretion of cytokines including tumour necrosis factor alpha (TNF-α) and interleukin (IL)-12, and over-expression of co-stimulatory molecules like CD80, CD86 and CD40 in mouse DCs(Pasquevich, Garcia Samartino et al. 2010, Ibanez, Smaldini et al. 2013). The lipoprotein Omp19 -by its lipid moiety- dampens antigen presentation and MHC-II expression in IFN-γ-activated human monocytes (Barrionuevo, Cassataro et al. 2008). DC immune response against *Brucella* is also controlled by Omp25, a transmembrane protein which is one of the most abundant members of the Omp family. *In vitro*, *Brucella* Omp25-defective mutants induce human DC and macrophage activation, which results in IL-12 and TNF-α secretion(Jubier-Maurin, Boigegrain et al. 2001, Billard, Dornand et al. 2007), without any effect on bacterial intracellular replication in these cells(Billard, Dornand et al. 2007, Manterola, Guzman-Verri et al. 2007). *In vivo*, the role of *Brucella* Omp25 is more controversial since Omp25-defective *B. abortus* or *B. melitensis* mutants (Δomp25) have been reported as being attenuated (Edmonds, Cloeckaert et al. 2002) or as virulent as the wild-type (WT) parental strains (Manterola, Guzman-Verri et al. 2007). Even though Omp25 has been considered as a potential vaccine (Bowden, Cloeckaert et al. 1998, Commander, Spencer et al. 2007, Commander, Brewer et al. 2010, Goel and Bhatnagar 2012, Goel, Rajendran et al. 2013), further work is needed to clarify the role of this outer membrane protein in the regulation of host immune responses.

Our recent transcriptomics study identified *SLAMF1* as a strongly upregulated gene in human DCs stimulated with the *Brucella* cyclic β1,2 glucan (CβG) (Martirosyan, Perez-Gutierrez et al. 2012, Degos, Gagnaire et al. 2015). SLAMF1 (or SLAM, CD150) is a cell surface receptor belonging to the signalling lymphocyte activation molecule family (SLAMF) of receptors, itself a member of the Immunoglobulin (Ig) superfamily (Cannons, Tangye et al. 2011, Fouquet, Marcq et al. 2018). SLAMF1 is expressed on haematopoietic cells only and constitutes a self-ligand that triggers T cell activation (Cocks, Chang et al. 1995, Aversa, Carballido et al. 1997, Detre, Keszei et al. 2010). SLAMF1 has been involved in immune responses against various pathogens (van Driel, Liao et al. 2016). SLAMF1, identified as the entry receptor for Measles

virus, controls DC and T cell responses during the course of viral infection (Tatsuo, Ono et al. 2000, Hahm, Arbour et al. 2004). In macrophages, SLAMF1 also binds *E. coli* outer membrane proteins OmpC and OmpF, thus regulating bacterial phagosome function and killing bacteria (Berger, Romero et al. 2010, Ma, Wang et al. 2012, Yurchenko, Skjesol et al. 2018). SLAMF1 plays a dual role as both an activator and an inhibitor of the immune system during *M. tuberculosis* and *T. cruzi* infections (Pasquinelli, Quiroga et al. 2004, Watanabe, Numata et al. 2004, Calderon, Maganto-Garcia et al. 2012, Avila-Calderon, Araiza-Villanueva et al. 2015). The role of SLAMF1 in brucellosis has not been investigated thus far.

Here, we show that in human brucellosis expression of SLAMF1 is specifically upregulated in blood leukocytes of acute phase patients only, whereas other serum biomarkers define the chronic stage of the disease. Overexpression of SLAMF1 on the surface of DCs is also triggered in murine BMDC upon infection with *Brucella* or contact with its CβG, independently of Omp25. We further demonstrate a specific direct interaction of *Brucella* Omp25 with the SLAMF1 receptor. We show that this interaction functionally limits the pro-inflammatory DC response *in vitro* as well as the expansion of splenic DC subsets and T cell activation early on *in vivo*, without affecting bacterial replication. This highlights the importance of the Omp25-SLAMF1 interaction during *Brucella* acute infection. Together our results unmask components of human brucellosis pathophysiology and bring to light a new avoidance strategy employed by *Brucella* to prevent recognition and elimination by the immune system thus disseminating and establishing its replication niche.

Results

SLAMF1 is a marker of the acute phase of *Brucella* infection in humans

We postulated that human patients infected with *Brucella* would elicit distinct patterns of gene expression in their peripheral blood according to their clinical outcome and that these gene expression profiles might be used to define immune responses associated with this pathogen and disease stages. To test this assumption, we performed RNA sequencing (RNA-seq) on whole blood samples collected from adult patients exhibiting at their first visit acute infection (n = 54), acute infection with relapse (i.e. patients who developed disease relapse while treated) (n = 6), chronic infection (n = 12) as well as from healthy control subjects (n = 36). Transcript profiling by RNA-seq of blood peripheral leukocytes of this patient cohort allowed for quantitative unbiased analysis of all expressed genes. To identify infection-specific effects on patient gene expression, we performed differential gene expression analysis and assigned genes to gene network modules using weighted gene correlation network analysis (WGCNA) (**Figure 1a**). Expression of individual genes clearly separated clinical groups: Chronic patients displayed an overall gene expression similar to healthy controls with no significant difference; acute patients and acute patients with relapse presented numerous transcriptional changes relative to healthy controls. Interestingly acute patients with relapse showed larger magnitude modifications than acute ones. Up-regulated genes were enriched for response to Interferon (IFN) gamma, cytolysis, T cell proliferation and cell cycle; down-regulated genes included genes involved in B cell proliferation. The Volcano plot in **Figure 1b** highlights the enhanced phenotype of the acute with relapse patient group versus the chronic one. **Figure 1c** and **Table S1** detail significant variations in expression of selected genes. *SLAMF1* gene expression was significantly upregulated in peripheral blood leukocytes of acute patients compared to healthy controls or to chronic group; this rise in *SLAMF1* expression was further exacerbated in the acute with relapse group. Again no significant differences in *SLAMF1* expression were observed between chronic group and healthy controls. The *SH2D1A* gene which encodes the cytoplasmic adapter for SLAM family members followed the same pattern of expression, as did *C1QC*, encoding the first component of serum complement, or genes coding for proteins involved in T_H1 response (such as those of the transcription factors *EOMES*, *TBX21/T-BET*), innate immune response like granzymes, *GZMK* and *GZMH*, inflammasome component AIM2, and *NKG7*, expressed in T cells and NK cells. Expression profiles of the *CD19* and *SSH2* genes were shown as examples of respectively invariant and down-regulated genes upon human *Brucella* acute or acute with relapse infection.

We also quantified in patient sera the levels of cytokines/chemokines potentially related to their immune response to *Brucella* infection. Then, we tested the correlations between cytokine and gene expression levels together with their variations amongst patient groups by using the OPLS-DA (Orthogonal Partial Least-Squares Discriminant Analysis) model created from the entire cohort by SIMCA (**Figure 1e**). A number of serum cytokines appeared to be responsible for the discrimination between groups (P=5.34218e-006). In particular, acute patients secreted significantly more IL-9, IL-31 and TNFβ, all pro-inflammatory cytokines, acute with relapse patients, IL-22, IL-1α, VEGF-D, CXCL10/IP-10, βNGF; and chronic patients, IL-10, CXCL1/GROα, IL1RA, IL-7. Overall, variations in levels of secreted cytokines between groups diverge from that of differentially leukocyte expressed genes. In conclusion, we characterized for the first time the transcript pattern of peripheral blood leukocytes from brucellosis patients and correlated it to the levels of cytokines in their serum. Determinants that discriminate between acute and chronic patients were also identified, amongst which SLAMF1 appeared as a biomarker of acute *Brucella* infection in humans.

Brucella infection of murine BMDCs induces SLAMF1 expression without any impact on its replication and persistence

In addition to the above described identification of SLAMF1 as a marker of acute infection in human brucellosis, we also noted a strong up-regulation of *SLAMF1* gene expression in human monocyte (mo)-DCs treated with *B. abortus* cyclic β1,2 glucan (CβG) in one of our previous transcriptomic studies(Martirosyan, Perez-Gutierrez et al. 2012) (**Table S2**). Since we showed earlier that murine granulocyte macrophage colony-stimulated factor (GM-CSF)-derived DCs data could be translated to human mo-DCs derived with GM-CSF and IL-4 (Zhao, Hanniffy et al. 2018), we decided to investigate whether *B. abortus* also modulated murine SLAMF1 protein levels in DC *in vitro*. After 5 days of culture, mouse bone marrow GM-CSF-derived dendritic cells (BMDC) were infected with the wild-type strain of *B. abortus* (Ba WT) at a multiplicity of infection (M.O.I.) of 30 for 16 h, mock-treated or treated with *E. coli* LPS at 100 ng/mL or CβG at 10 μg/ml as reported (Martirosyan, Perez-Gutierrez et al. 2012, Zhao, Hanniffy et al. 2018). Non-adherent cells were used for phenotypic investigation by flow cytometry and analysed as described in **Supplementary Figure S1**. A marked increase in the expression of SLAMFI protein levels was observed with infected samples compared to that of mock-treated BMDCs, which was much stronger than those obtained in *E. coli* LPS- or *Brucella*

CβG-treated cells (**Figure 2a**). Such a rise in SLAMF1 expression has been reported following binding of *E. coli* outer membrane proteins OmpC and OmpF to macrophages(Berger, Romero et al. 2010). Since Omp25, a major outer membrane protein of *Brucella*, has been shown to modulate human DC activation upon infection (Billard, Dornand et al. 2007), we further evaluated the impact of *Brucella* Omp25 on SLAMF1 expression in BMDCs by infecting them in parallel with the *B. abortus* mutant defective in Omp25 expression *(*Ba Δ*omp25*) or its complemented mutant strain (Ba Δ*omp25c*), which stably re-expressed Omp25 (**Supplementary Figure S2a)**. Both mutant strains induced SLAMF1 expression similarly to the WT strain, suggesting a *Brucella* Omp25-independent control of SLAMF1 regulation. We then analysed the intracellular replication of the Ba Δ*omp25* mutant within BMDCs and compared it with that of Ba WT, Δ*omp25c* or Δ*virB* strains (**Figure 2b**). The Ba Δ*omp25* mutant replicated at a rate comparable to that of the WT or Δ*omp25c* strains while a *virB* mutant was not able to persist for more than 48 h as we reported earlier (Sieira, Comerci et al. 2000). These data revealed that Omp25 did not impact *Brucella* replication in BMDCs. In addition, the Δomp25 strain replicated within the endoplasmic reticulum (ER) 24 h post-infection (p.i.) similarly to the WT strain, showing that intracellular trafficking in BMDCs is equivalent in Ba WT and Δ*omp25* mutant strains (Salcedo, Marchesini et al. 2008) (**Supplementary Figure S2b**). Next we examined *Brucella* replication in wild-type BMDCs or in BMDCs from knock-out mice devoid of SLAMF1 (*Slamf1*$^{-/-}$) infected with the WT, Δ*omp25* or Δo*mp25c* strains (**Supplementary Figure S2c)**. Equivalent replication rates were obtained in all strains or DC genetic backgrounds, indicating that SLAMF1 engagement does not affect *Brucella* replication.

BMDC activation is restrained in a SLAMF1/Omp25-dependent manner upon *Brucella* infection

Omp25 has been reported to prevent human DC activation upon *Brucella* infection(Billard, Dornand et al. 2007). We thus checked the phenotype of BMDCs infected with WT, Δ*omp25* or Δo*mp25c* strains and compared it with that of BMDCs treated as a positive control with *E. coli* LPS for 24 h. CD80, CD86, CD40 co-stimulatory molecules were analysed by flow cytometry as described in **Supplementary Figure 1**. Expression levels of these co-stimulatory molecules and thus DC activation reached in Ba WT-infected BMDC were low compared to those observed in LPS-treated cells, as we previously reported (Salcedo, Marchesini et al. 2008, Gorvel, Textoris et al. 2014) (**Figure 2c**). However, Ba Δ*omp25*-infected BMDCs exhibited significantly a higher expression of CD80, CD40 and CD86 than that of WT-infected strain,

although the expression levels were still below those achieved in *E. coli* LPS-treated cells. The rescue of the Δ*omp25* mutant phenotype by the complemented strain (Δ*omp25c)* further demonstrated a direct role of Omp25 in controlling DC surface marker expression. MHCII expression was stable whatever *Brucella* strain considered and below that obtained in LPS-treated cells (**Supplementary Figure S3**), supporting the notion that intermediate level of DC activation were reached by the Ba Δ*Omp25* strain. When using *Slamf1*$^{-/-}$ BMDCs, surface activation markers were expressed at similar levels regardless of the stimulus (LPS, wt or mutant bacteria) (**Figure 2c** and **Supplementary Figure S3**). Collectively these results indicated a SLAMF1/Omp25-mediated decrease of BMDC activation upon *Brucella* infection. We next measured by cytometric bead assay the secretion of inflammatory cytokines in culture supernatants of wt or *Slamf1*$^{-/-}$ BMDCs infected with Ba WT or Δ*omp25* strains at 24 h post-infection (p.i.) (**Figure 2d**). In wt BMDCs, WT *Brucella* led to a significant increase of the secretion of TNF-α, IFN-γ and IL-6 secretion in mock cells. These levels were further increased by Δ*omp25* Ba mutant infection.. In *Slamf1*$^{-/-}$ BMDC cytokine secretion elicited by *Brucella* WT strain reached equivalent levels to those obtained with Δ*omp25* strain. The higher cytokine secretion of the Δ*omp25* mutant phenotype in wt BMDCs and loss of difference between the *Brucella* strains in the SLAMF1-deficient BMDCs demonstrated the Omp25/SLAMF1-mediated control of DC activation. Overall these data revealed that the Omp25/SLAMF1 axis plays an important role in restricting the host response and inflammation triggered by *Brucella* infection *in vitro*.

Purified recombinant *Brucella* Omp25 protein binds SLAMF1 directly

To determine whether Omp25 is a ligand for the SLAMF1 receptor, we constructed a plasmid encoding the N-terminal myc-tagged extracellular domain of SLAMF1. A similar construct containing the extracellular domain of another member of the Ig receptor family, CD90/Thy1 also fused to N-terminal myc tag was used as a negative control, as constructs made with the extracellular domain of another SLAMF family member, i.e. SLAMF4, were not expressed at sufficient levels. After transfection of each of these plasmids in COS-7 cells, total proteins were extracted and a pull-down assay was performed with an anti-myc antibody. Immunoprecipitated complexes were then incubated with purified recombinant *Brucella* Omp25 and analysed by Western blot (**Figure 3a**). SLAMF1 extracellular domain pulled-down purified recombinant Omp25, in contrast to that of CD90, which was unable to do so in the presence of 0.1 ng of Omp25, although a faint non-specific interaction was detected with 1 ng of this recombinant

protein. We concluded that purified *B. abortus* recombinant Omp25 protein interacted specifically with ectopically expressed SLAMF1 extracellular domain since it did not occur with an unrelated member of the super Ig family (CD90). We then tested if such interaction was unique to this Omp or a feature shared by other Omps of *Brucella*. The same pull-down approach was followed but immunoprecipitated complexes were incubated here with 0.1 ng of Omp19 (**Figure 3b**). The purified recombinant *B. abortus* Omp19 protein did not interact with ectopically expressed SLAMF1 neither with CD90 extracellular domain. This demonstrated that the interaction of SLAMF1 with *Brucella* Omp25 was specific to this Omp. These data provide the first direct evidence for a specific binding of *Brucella* Omp25 protein to the SLAMF1 receptor.

The Omp25-SLAMF1 interaction controls NF-κB nuclear translocation in BMDC upon *Brucella* infection

To assess the impact of Omp25 binding to the SLAMF1 receptor at an early stage of BMDC activation *in vitro*, we then monitored by immunofluorescence microscopy the translocation of NF-κB to the nucleus 2 h p.i. upon BMDC infection with *B. abortus* WT, Δ*omp25* or Δ*omp25c* (**Figure 3c**). BMDCs infected with Ba Δ*omp25* displayed a significantly higher level of nuclear NF-κB than that observed in cells infected with Ba WT, which showed a trend for an increase of nuclear p65 compared to mock-treated cells. The rescue of the Δ*omp25* mutant phenotype by the complemented strain, Δ*omp25c*, confirmed the role of Omp25 in restricting NF-κB translocation to the nucleus of *Brucella*-infected BMDCs. The same experiment in *Slamf1*$^{-/-}$ BMDCs (**Figure 3b**) or in WT BMDCs in the presence of a SLAMF1 blocking peptide (**Supplementary Figure S4**) led to significant and comparable rises of NF-κB nuclear translocation upon infection with Ba WT or Δ*omp25* relative to those obtained in WT BMDC or BMDC treated with a control scramble peptide. Collectively, these results suggested that the binding of Omp25 to the SLAMF1 receptor in BMDCs limited translocation of NF-κB to the nucleus, a feature shown to be essential for pro-inflammatory gene transcription (Salcedo, Marchesini et al. 2013). We thus examined the gene expression profile of BMDC upon infection by either *B. abortus* WT or the Δ*omp25* mutant strain (**Supplementary Figure S5**). BMDC infection with *Brucella* WT resulted in an over-expression of *Il6*, *Il12b*, *Ccl2*, *Il1b*, *Tnfa*, *Cxcl1/Kc*, *Nos2* and *Ptsg*2 mRNA levels at both 6 h and 24 h p.i.. In accordance with enhanced nuclear NF-κB translocation, Δ*omp25* infection led to a further increase of *Il6*, *Il12b*, *Ccl2*, *Cxcl1/Kc*, *Nos2* and *Ptsg*2 mRNAs notably at 24 h p.i.. In conclusion, these results suggested

that the Omp25-SLAMF1 interaction restricted the inflammatory response to early stages of *Brucella* infection *in vitro*.

The Omp25-SLAMF1 interaction promotes survival of *Ifng*$^{-/-}$ mice upon *Brucella* infection *in vivo*

To further investigate the role of the Omp25-SLAMF1 interaction during *Brucella* infection we carried out *in vivo* studies in the C57BL/6 mouse model (Grillo, Blasco et al. 2012). C57BL/6J wt or *Slamf1*$^{-/-}$ mice were inoculated intraperitoneally with either *Brucella* WT, Δ*omp25* or Δ*omp25c* mutants (the latter for the wt mice only) or PBSx1 injected (Mock) and assessed 8 days later for spleen weight and bacterial burden (**Figure 4a**). This unique time-point of investigation was chosen as it corresponds to the acute phase of infection (Grillo, Blasco et al. 2012). A significant increase in spleen weight of Δ*omp25*-infected wt mice or *Brucella* infected *slamf1*$^{-/-}$ mice compared to that of Ba WT-infected C57BL/6J mice suggested an increased inflammatory response in the absence of Omp25 or SLAMF1, corroborating the above *in vitro* data. Interestingly, equivalent bacterial counts in spleen at day 8 p.i. were enumerated whatever *Brucella* strain or mice considered.

In order to quantify granuloma formation, thought to represent the host's ability to develop a protective immune response, we also performed histological examination of spleens from wild-type C57BL/6J or *Slamf1*$^{-/-}$ mice at day 8 p.i. infected with Ba WT or Δ*omp25* strains. No significant difference in granuloma formation was detected between the Ba WT-infected C57BL/6J mice and any other infected animals, although a trend for an increase in the Ba Δ*omp25*-infected spleens versus the Ba WT ones was pointed out (**Supplementary Figure S6**). Together, these results suggested that Omp25-SLAMF1 interaction finely tuned the inflammatory response early on *in vivo* during *Brucella* infection without affecting bacterial replication itself.

To test such a hypothesis, we then used a lethal model of mouse brucellosis (Brandao, Oliveira et al. 2012) by infecting immuno-compromised *Ifng*$^{-/-}$ mice with *B abortus* WT or Δ*omp25* strains (**Figure 4b**). Mice infected with the Ba WT strain survived significantly longer (until day 30) than those infected with the Δ*omp25* strain (all dead by day 25) despite equivalent bacterial CFU counts in the spleen at day 20 p.i. (median of 5.08 ± 0.3 logCFU/spleen for Ba WT versus 5.27± 0.37 logCFU/spleen for the Δ*omp25* mutant) (**Figure 4b**). The same infection experiment in an *Ifng*$^{+/+}$ background showed no effect on mouse viability, as expected(Grillo, Blasco et al. 2012). We concluded that the reduced survival of *Ifng*$^{-/-}$ mice infected with the

Δ*omp25* mutant strain might be due to a precocious hyper-inflammation triggered by the loss of Omp25, and illustrated by the augmented weight of the spleen of infected animals at day 8 p.i.. Together, these data suggested that Omp25 limited inflammation at early stages of *Brucella* infection *in vivo* without affecting the bacterial burden itself.

The Omp25-SLAMF1 interaction prevents splenic expansion of all DC subsets, and inhibits downstream CD4$^+$ and CD8$^+$ T cell activation during the acute phase of *Brucella* infection *in vivo*

The fact that spleen of wt mice infected by *Brucella* Δ*omp25* mutant were significantly heavier than that of mice infected with Ba WT strain and also similar to that of WT or Δ*omp25 Brucella* infected *Slamf1*$^{-/-}$ mice, raised the possibility of *in viv*o Omp25/SLAMF1-dependent changes in cellularity of immune cells upon infection. To determine if it was the case, we infected wild-type C57BL/6J (wt) or *Slamf1*$^{-/-}$ mice with *B abortus* WT, Δ*omp25* or Δ*omp25c* strains (the latter for the wt mice only). At 8 days p.i., cell suspensions were prepared after enzymatic digestion and gentle dissociation of the spleens. After exclusion of neutrophils, NK cells, B cells, and T cells, the remaining lineage negative cells were analysed by flow cytometry for CD11c expression and identification of DC splenic subsets. CD11c^{pos} cells were divided into CD11c^{int}CD11b$^-$Bst2$^+$SiglecH$^+$ plasmacytoid DC (pDC) and CD11c^{hi} cells, further identified as XCR1$^+$SIRPα$^-$ conventional DC type (cDC)1 and XCR1$^-$SIRPα$^+$CD24$^-$ cDC2 cells as described(Schlitzer, Sivakamasundari et al. 2015) (**Supplementary Figure S7**). Absolute numbers of cDC2 were significantly increased upon infection with Ba WT or Δ*omp25c* strains compared to those detected in Mock PBSx1 injected mice (**Figure 4c**). Loss of Omp25 led to a further rise of this cDC2 population to levels akin to those seen upon infection with Ba WT or Δ*omp25* of *Slamf1*$^{-/-}$ mice in the same conditions. An equivalent increase of the cDC1 and pDC subsets were also observed, although numbers of these subset cells obtained in infected *Slamf1*$^{-/-}$ mice were lower than those seen in wt mice infected with the Δ*omp25* mutant strain.

Next, we asked whether this *in vivo* modulation of cDC1, cDC2 and pDC splenic subpopulations by *Brucella* Omp25 protein interaction with the SLAMF1 receptor impacted downstream T cell activation. We thus examined in dissociated spleens the CD3$^+$ positive fraction in detail (**Supplementary Figure S7**) and looked at the CD4$^+$ and CD8$^+$ cells, and in particular at the percentages and absolute numbers of the related activated CD69$^+$ T cell subsets (**Supplementary Figure S8** and **Figure 4d**). Percentages and absolute numbers of CD4$^+$CD69$^+$ T cells varied positively with the lack of CD25 in *Brucella* or of SLAMF1 in the host. Here

again in *wt* mice, the Δ*omp25* mutant phenotype was rescued by the complemented strain, Δ*omp25c*. Absolute numbers of $CD8^+CD69^+$ T cells fluctuated the same way, but their percentages did not significantly differ upon infection. The rescue of the Δ*omp25* mutant phenotype by the complemented strain, Δ*omp25c*, further demonstrated a direct role of Omp25 in controlling expansion of cDC1, cDC2 and pDC, and subsequently increasing $CD4^+$ and $CD8^+$ $CD69^+$ activated T cells.

The Omp25-SLAMF1 interaction facilitates settlement of *Brucella* at the chronic phase of infection *in vivo*

Finally, we explored the impact of the Omp25-SLAMF1 interaction in the chronic phase of mouse infection. As described above, C57BL/6J wt or *Slamf1*$^{-/-}$ mice were inoculated intraperitoneally with either Ba WT, or Δ*omp25* or Δ*omp25c* mutants (the latter for the wt mice only) and evaluated 30 days later for spleen weight and bacterial burden (**Figure 4e**). During the chronic phase of infection, the situation as regards these two parameters was just the opposite of the acute phase. Indeed, spleen weights of all infected mice were similar at day 30 p.i.. However, a significant reduction in bacterial CFUs was observed in the spleens of wt mice infected with the Ba Δ*omp25* mutant or in the spleens of *Slamf1*$^{-/-}$ mice infected with either Ba strains compared to those enumerated in the spleens of wt mice infected with Ba WT. Here again the Δ*omp25* mutant phenotype was rescued by the complemented strain, Δ*omp25c*, unquestionably demonstrating the requirement of Omp25 for an optimal settlement of *Brucella* at the chronic phase. The comparable behaviour of Δ*omp25* mutant in wt mice and of the Ba WT and mutant strains in the absence of SLAMF1 further supported the hypothesis that Omp25 operates through its binding to the SLAMF1 receptor. Collectively, these results are consistent with a role for the Omp25-SLAMF1 interaction in fine-tuning of the inflammatory response early during the acute phase of *Brucella* infection to favour settlement of *Brucella* at the chronic phase *in vivo*.

Discussion

Here, we have recapitulated the first blood transcriptional signature of human brucellosis using RNA seq that discriminates acute brucellosis from chronic brucellosis and healthy individuals. We show that this unbiased sequencing approach is robust in distinguishing in patients the acute phase, treated or with relapse, from the chronic phase of brucellosis, and that this latter presents an overall gene expression very close to that of uninfected controls. This situation explains why,

if acute brucellosis is not diagnosed but left untreated as often the case, it is hard to recognise chronic *Brucella* infection when peripheral inflammatory symptoms appear. However, thanks to the comparative analysis of blood transcriptome and cytokine profiling in our clinical cohort, we have identified a number of molecules specific to each disease stage. As such, SLAMF1, a molecule overexpressed in several inflammation-related diseases (including infection, arthritis and pulmonary allergy), has been identified as a marker of the acute phase of brucellosis in humans together with other classical markers of infection. This is in accordance with previous reports indicating SLAMF1 upregulation at phagocyte cell surface during the initial phase of infectious inflammation, then followed by that of SLAMF8(Kingsbury, Feeney et al. 2001, Wang, Abadia-Molina et al. 2012, Wang, van Driel et al. 2015). The *SH2D1A* gene, which encodes the SLAMF1 adaptor protein (SAP), varies accordingly to SLAMF1. A series of genes encoding other molecules shown to be involved in brucellosis were also overexpressed in *Brucella*-infected human blood such as C1QC, the first component of serum complement(Gonzalez-Espinoza, Barquero-Calvo et al. 2018), or genes encoding proteins involved in T_H1 response(Dornand, Gross et al. 2002, Billard, Cazevieille et al. 2005) (such as those of the transcription factors EOMES, TBX21/T-BET), innate response like Granzymes, GZMK and GZMH(Martirosyan, Ohne et al. 2013) or an inflammasome component AIM2(Costa Franco, Marim et al. 2018, Costa Franco, Marim et al. 2019). As expected, acute patients secreted in their serum significantly more pro-inflammatory cytokines, in particular TNF-α, IL-9 and IL-31, while acute with relapse patients presented an exacerbated phenotype with more serum IL-1β together with IL-22, VEGF-D, CXCL10/IP-10 and βNGF. IL-22, a T-cell derived cytokine structurally related to IL-10, which protects against tissue destruction caused by exacerbated immune response, has been reported to be induced in murine T cells by IL-9, which itself is involved in the induction of T_H2 cell type immune response(Dumoutier, Louahed et al. 2000). The upregulation of this cytokine in acute blood patients might explain the rise in IL-22 when relapse occurs. Moreover, the upregulation of these cytokines indicates an important contribution of the T_H2 response in human acute brucellosis, as confirmed by that of VEGF-D, shown to elicit T_H2 responses in DC through binding to its receptor VEGFR3(Lee, Link et al. 2004), and of NGF, which inhibits TLR-mediated inflammation in human monocytes(Prencipe, Minnone et al. 2014). CXCL10/IP-10, a pan-marker of viral or bacterial infection(Oved, Cohen et al. 2015) and an IFN-stimulated gene, like AIM2, is induced in bone marrow-derived murine macrophages via the STING pathway upon *Brucella* infection(Khan, Harms et al. 2016, Costa Franco, Marim et al. 2018), suggesting an important role of the IFN-

type I in human brucellosis. Chronic brucellosis, although silent at the transcriptional level, is marked by the presence in patient serum of classical anti-T_H1 cytokines like IL-10 and IL1RA, as well as of two molecules known as a neutrophil and pro-angiogenic chemokine for CXCL1/KC, and as a key pro-B and T cell differentiating cytokine for IL-7. Since *Brucella* elicits premature cell death of human neutrophils without inducing proinflammatory phenotypic changes(Barquero-Calvo, Martirosyan et al. 2013, Barquero-Calvo, Mora-Cartin et al. 2015), elevated blood CXCL1 levels might account for the neutropenia described in chronically-infected brucellosis patients(Crosby, Llosa et al. 1984). High levels of both IL-10 and IL1RA explain the low activation transcriptional signature of chronic patients and are thought to trigger an anergic state supporting persistence of bacteria(Mege, Meghari et al. 2006). As regards *Brucella*, IL-10 plays indeed an essential role in its maintenance *in vivo* notably by favouring escape of *Brucella* from the late endosome compartment in macrophages(Corsetti, de Almeida et al. 2013, Xavier, Winter et al. 2013). Interestingly, genetic polymorphism in the IL1RA gene has been linked to susceptibility to brucellosis(Hajilooi, Rafiei et al. 2006). High serum levels of IL-7 in the Chronic patient group likely reflected an impaired T cell sensitivity to IL-7 as recently reported for tuberculosis patients(Lundtoft, Afum-Adjei Awuah et al. 2017) and is not related to IL-7 function itself. These data highlight specific features of the chronic phase of human brucellosis and infer that combined detection of IL-10, IL1RA, CXCL1 and IL-7 should form a unique and promising biomarker serum signature for identifying *Brucella* chronicity.

Collectively, these results disclose distinctive hallmarks of human brucellosis that are of the utmost importance for improving its diagnosis. They open up new avenues of detection of all phases of this disease with blood identification of SLAMF1 as an acute brucellosis biomarker and a set of four serum biomarkers (IL-10, IL1RA, CXCL1 and IL-7) for chronic brucellosis.

We turned to the mouse to unravel the function of SLAMF1 in an animal model of brucellosis. We first looked at SLAMF1 expression in murine DC and could confirm that similarly to human blood leukocytes, *Brucella* drove SLAMF1 upregulation in BMDCs. This control was Omp25-independent. In addition, CβG triggered overexpression of SLAMF1 in both BMDCs and human mo-DCs. This adds to the list of molecules, including TLR-2, -4, -5 ligands (LTA, peptidoglycan, *E. coli* LPS, flagellin)(Farina, Theil et al. 2004) and IL-10(Williams, Jarai et al. 2002), reported to upregulate SLAMF1 in human monocytes *in vitro*. Identification of the signalling pathway(s) involved will require further investigation.

In this study, we identify a role of SLAMF1 in *Brucella* infection and provide direct evidence for its association with Omp25, a major outer membrane protein of *Brucella*. SLAMF1 has first

been recognised as the Measles virus entry receptor and was later reported to interact with several *E. coli* outer membrane porins OmpC and OmpF(Berger, Romero et al. 2010). We show here that SLAMF1 binds *B. abortus* Omp25 and not the related *B. abortus* outer membrane protein Omp19. Other members of the SLAM receptor family have been shown to sense various microbial components(van Driel, Liao et al. 2016). As such, SLAMF6 interacts with *E. coli* and *Citrobacter rodentium(van Driel, Wang et al. 2015)* while SLAMF2 binds FimH, a lectin from *E. coli(Baorto, Gao et al. 1997, Malaviya, Gao et al. 1999, Cannons, Tangye et al. 2011)*. More studies will be needed to determine whether beside SLAMF1 other SLAM members exhibit any role in brucellosis.

The binding of OmpC and OmpF to SLAMF1 leads to macrophage activation and efficient killing of *E. coli* during infection, through phagolysosome maturation(Berger, Romero et al. 2010, Yurchenko, Skjesol et al. 2018). In the case of *Brucella*, we present here multiple lines of evidence indicating that the interaction of SLAMF1 with Omp25 results in a totally different outcome. In BMDCs, we show that neither host's SLAMF1 deletion nor lack of Omp25 affected *Brucella* intracellular trafficking and phagosome escape from the endocytic pathway, proving normal bacterial replication. This is in sharp contrast to what happens upon *E. coli* infection of macrophages where intracellular trafficking requires SLAMF1[45]. Consistent with our *in vitro* data, *Brucella* replication is independent of Omp25 and SLAMF1 in mice during the acute phase of infection *in vivo*. However, when Omp25 or SLAMF1 are missing, the bacterial burden is significantly diminished during the chronic phase of infection. This indicates that by itself the SLAMF1-Omp25 axis does not directly control bacterial replication during early infection but alters the host's response by tuning down inflammation, which as a secondary consequence influences bacterial load at the chronic stage. Removing the SLAMF1-Omp25 axis then allows the host to better control bacteria burden at chronic phase. These data are in agreement with previous reports indicating that Omp25-deficient *Brucella* strains do not trigger any bacterial attenuation in human DC, neutrophils, epithelial cell line and murine and human macrophage cell lines(Billard, Dornand et al. 2007, Manterola, Guzman-Verri et al. 2007) and that *Brucella* growth in DC is restrained through secretion of pro-inflammatory cytokines such as interleukin-12 (IL-12)(Manterola, Guzman-Verri et al. 2007) or enzyme like iNOS(Copin, De Baetselier et al. 2007). Other mechanisms contribute to *B. abortus* persistence *in vivo*, such as the early production of IL-10 by $CD4^+CD25^+$ T cells upon infection, which down-regulates production of pro-inflammatory cytokines by macrophages and allows them to escape the late endosome compartment(Xavier, Winter et al. 2013).

Several studies highlighted a versatile role of SLAMF1 after various engagement signals resulting in either pro- or anti-inflammatory outcomes(Bleharski, Niazi et al. 2001, Rethi, Gogolak et al. 2006). *B. suis* Omp25 was previously proposed to modulate immune response in human monocyte-derived cells(Jubier-Maurin, Boigegrain et al. 2001, Billard, Dornand et al. 2007). We therefore focused on the analysis of the role of the SLAMF1-Omp25 interaction during *B. abortus* infection of murine DCs. Our *in vitro* data indicated that the SLAMF1-Omp25 axis limited nuclear translocation of NF-κB, subsequently muting pro-inflammatory gene transcription, cytokine and chemokine secretion and co-stimulatory molecule expression without affecting MHCII levels. *In vivo* the Omp25-SLAMF1 interaction restricted the inflammatory response early on during *Brucella* infection. It promoted survival of *Ifng*-deficient mice upon *Brucella* infection, in contrast to mice infected with a mutant strain devoid of Omp25, thus revealing a SLAMF1/Omp25 down-regulated inflammation, corroborated by changes in cellularity of immune cells. Indeed the SLAMF1-Omp25 axis prevented expansion of all types of splenic DCs (i.e. cDC1, cDC2 and pDCs), and inhibited downstream $CD4^+$ and $CD8^+$ T cell activation. Rise of activated $CD69^+$ T cells might augment tissue damage and explain the earlier death of Ba Δ*omp25*-infected mice compared to WT-infected ones in the lethal model of brucellosis. SLAMF1 was indeed shown to increase TCR-mediated cytotoxicity(Henning, Kraft et al. 2001). cDC1 cells are known to preferentially activate $CD8^+$ T cells and cDC2 $CD4^+$ T cells(Lewis, Williams et al. 2019). The current model for *in vivo* DC-T cell activation in the spleen is that cDC2 cells first activate $CD4^+$ T cells, which then licence $XCR1^+$ cDC1 cells to prime $CD8^+$ T cells. cDC1 cells are thought to act as a platform to convey $CD4^+$ T cell help to $CD8^+$ cells(Chudnovskiy, Pasqual et al. 2019). Splenic pDCs are known as high producers of interferon upon activation and drivers of enhanced cross-priming(Bauer, Dress et al. 2016). In this context, our results suggest that the binding of Omp25 to SLAMF1 *in vivo* might target these critical steps of DC-T cell interactions. Accordingly, SLAMF1 is recognised as a co-stimulatory molecule between DC and T cells(Cocks, Chang et al. 1995). Since in the absence of Omp25, *Brucella* was more potent in inducing all DC subset expansion and T cell activation *in vivo*, a possible mechanism might be that binding of Omp25 to SLAMF1 impedes SLAMF1 dimerization and subsequent transduction of danger signals to the host cell. As such, Omp25 might be used by the bacterium to control SLAMF1 signalling and consequently T cell activation as an immune response escape mechanism. SLAMF1, as other SLAMF receptors, associates via its cytoplasmic tail, which contains immunoreceptor tyrosine-based switch motifs (ITSM), with the SLAM Adaptor Protein (SAP) in a phospho-tyrosine

dependent mode. The ITSM are used by phosphatases, such as SHP-2, and kinases, like FynT, recruited through SAP to SLAMF1 cytoplasmic domain, to control T cell activation(Detre, Keszei et al. 2010, Cannons, Tangye et al. 2011). The recruitment of the SAP/Fyn cascade may be impaired by the SLAMF1-Omp25 interaction. As SLAMF1 has recently been shown to be essential for TLR4-mediated TRAM-TRIF-dependent signalling in human macrophages(Yurchenko, Skjesol et al. 2018), another possibility is that this interaction affects the recruitment of other signalling receptor(s) or adapter protein(s). Further investigations are clearly needed to understand how the interaction of *Brucella* Omp25 with the SLAMF1 receptor controls NF-κB nuclear translocation and to define the transduction pathways involved and their interplay.

Our data support an anti-inflammatory role of the SLAMF1-Omp25 interaction during the infection process early on *in vivo*, which mirrors what was seen in BMDC *in vitro*. Confirming prior reports(Jubier-Maurin, Boigegrain et al. 2001, Billard, Dornand et al. 2007), Omp25 stands as an important antigen synthesised by *Brucella* to control immune responses, and adds to the long list of *Brucella* proteins devoted to restrain DC immune responses, including BtpA, BtpB, PrpA, WadC or WboA(McQuiston, Vemulapalli et al. 1999, Fernandez-Prada, Nikolich et al. 2001, Salcedo, Marchesini et al. 2008, Conde-Alvarez, Arce-Gorvel et al. 2012, Salcedo, Marchesini et al. 2013, Spera, Comerci et al. 2014). The enhanced inflammation triggered by the Δ*omp25* mutant strain *in vitro* in BMDC and *in vivo* in wild-type mice or by the *Slamf1*$^{-/-}$ cells/mice infected by all *Brucella* strains tested, suggests that Omp25 plays a role in virulence *in vivo* via its association with SLAMF1. Altogether, the subtle SLAMF1-Omp25-dependent downsizing of the inflammatory response early on during the acute phase of *Brucella* infection *in vivo* promotes *Brucella* settling to proceed to chronic phase.

In conclusion, this study has shed the first light on the blood gene expression signature of human brucellosis, demonstrating that chronic phase is globally indistinguishable from healthy donors at the transcriptomic level and identifying SLAMF1 as the first significant blood marker for human acute brucellosis. In addition, we propose a set of four serum biomarkers for detection of chronic brucellosis. On a mechanistic point of view, we have demonstrated in the mouse that SLAMF1 interacts specifically with *Brucella* omp25. This interaction does not affect *Brucella* replication but is essential to finely tune inflammation down during the acute phase of infection in order to allow *Brucella* to settle in its furtive replicative niche, the endoplasmic reticulum, and for progression to the chronic phase. The SLAMF1-Omp25 interaction therefore illustrates a novel discrete evasion strategy exploited by *Brucella* within infected DCs to mediate its

dissemination *in vivo* by controlling innate immune responses in a timely manner thus fostering chronicity. *Brucella* targeting SLAMF1 may have broader consequences given that, on one hand, *Brucella* can be transmitted from bone marrow transplantation in humans(Naparstek, Block et al. 1982, Tuon, Gondolfo et al. 2017) and is able to colonise and persist in murine bone marrow(Tuon, Gondolfo et al. 2017) and that, on the other hand, SLAMF1 is a key cell surface receptor of stem cells regulating lineage-commitment steps of haematopoiesis(Oguro, Ding et al. 2013). Investigating the impact of the SLAMF1-Omp25 interaction on haematopoietic stem cell maintenance and differentiation during and after *Brucella* infection should open promising research avenues.

Materials and Methods

Ethics

Animal experimentation was conducted in strict compliance with good animal practice as defined by the French animal welfare bodies (Law 87–848 dated 19 October 1987 modified by Decree 2001-464 and Decree 2001-131 relative to European Convention, EEC Directive 86/609). INSERM guidelines have been followed regarding animal experimentation (authorization No. 02875 for mouse experimentation). All animal work was approved by the Direction Départementale Des Services Vétérinaires des Bouches du Rhône and the Regional Ethic Committee (authorization number 13.118). Authorization of *Brucella* experimentation in BSL3 facility was given under the numbers: AMO-076712016-5, AMO-076712016-6 and AMO-076712016-7. All efforts were made to minimise suffering during animal handling and experimentation. As regards human PBMCs, blood was taken from healthy volunteers acquired under protocols approved by the Institutional Review Board (IRB) of the University General Hospital of Albacete, Albacete, Spain, the University Clinic for Infectious Diseases and Febrile Conditions, Skopje, Macedonia and the Benaroya Research Institute, Seattle, USA.

Human study design

Patients were enrolled for clinical studies from 2010 to 2015 at the University General Hospital of Albacete, Albacete, Spain and the University Clinic for Infectious Diseases and Febrile Conditions, Skopje, Macedonia, following a procedure established by the Benaroya Research Institute, Seattle, USA. On admission, a trained nurse applied a standardized questionnaire to gather demographic and clinical data. Whole blood samples were collected in EDTA and Tempus tubes from each adult, on or shortly after admission. One set of EDTA tubes were sent to the routine clinical laboratory for complete blood counts and the other set was used to prepare serum, which was partly frozen at -80°C and sent to the CIML on dry ice for cytokine dosage. Tempus tubes were frozen at -20°C and shipped to the Benaroya Research Institute on dry ice. Blood samples were transported to the laboratory within 1 hour. In total data were collected from 56 healthy controls and 145 brucellosis patients with a range of treatment outcomes. Patient disease was classified based on previous history of *Brucella* infection and progression of symptoms during treatment. Gene expression data was collected via RNAseq at initial visits.

RNA sequencing

Genes were assigned to modules using weighted gene correlation network analysis (WGCNA)

Bacterial strains

B. abortus smooth virulent strain 2308 and derived strains devoid of Omp25, the Δ*omp25* strain, and its complemented version, Δ*omp25c*, have been described(Manterola, Guzman-Verri et al. 2007). *Brucella* strains were grown on Tryptone Soya Agar (TSA) plates (Sigma Aldrich), supplemented with kanamycin 30 µg/mL for the Δ*omp25* strain or kanamycin and ampicillin 50 µg/mL for the Δ*omp25c* strain. For infection, strains were grown for 16 h approximately at 37°C under shaking in Tryptic Soy Broth (TSB) (Sigma Aldrich), in presence of the appropriate antibiotics when required, until the OD at 600nm reached 1.8. All *Brucella* were kept, grown and used under strict biosafety containment conditions all along experiments in the BSL3 facility, Marseille. For sub-cloning of SLAMF1 and CD90 constructs, Top10 thermo-competent *E. coli* bacteria were used. Liquid cultures were incubated for 16h in Luria Broth (LB) at 37°C under constant shaking and in presence of the adequate antibiotics. Solid cultures were grown onto LB agar.

Mice

6-10 week-old female C57BL/6J mice from Charles River, *Slamf1*$^{-/-}$ mice (kindly provided by Yusuke Yanagi)(Davidson, Shi et al. 2004) or *Ifng*$^{-/-}$ mice, both on a C57BL/6J background, were used. Animals were housed in cages with water and food ad libitum in the CIPHE animal house facility, Marseille. Two weeks before the start of experiments, mice were transferred to the BSL3, CIPHE, and kept under strict biosafety containment conditions all along infection with live bacteria.

Reagents

Antibodies used in flow cytometry are the following, F4/80-BV785 (cloneBM8), CD86-FITC (clone GL-1), CD150/SLAMF1-PE/Cy7 (clone TC15-12F12.2), CD80-PE/Cy5 (clone 16-10A1), CD40-Alexa 647 (clone 3/23), MHC II (I-A/I-E)-AF700 (clone M5/114.15.2), CD11c-APC/ Cy7 (clone M1/70), CD19-APC-Cy7 (clone N418), NK1.1-APC-Cy7 (clone PK136), CD3-BV650 (clone17A2), CD8-BV711 (clone 53-6.7), CD69PE-Cy7 (clone H1.2F3), XCR1-Bv421 (clone ZET), CD317/Bst2, CD11c-PE-Cy7 (clone N418), CD172a-SIRPα-APC (clone 84) were all purchased from BioLegend. CD4-eFluor450 (clone RM4-5), CD3 eFluor450, CD317/Bst2-PE-eFluor610 (clone eBio927) were purchased from eBioscience. CD11b-PE (clone M1/70) was purchased from BD Biosciences. Antibodies used in immunoprecipation assays were home-made anti-c-myc Tag monoclonal antibody (9E10) and anti-Omp25 or -

Omp19 antibodies (A595F1C9 and A765C10A8 respectively; kindly given by Axel Cloeckaert, INRA, Tours, France). *E. coli* LPS, ATCC 35218, was extracted and purified as described previously(Velasco, Bengoechea et al. 2000). For immunofluorescence studies, primary antibodies were rabbit anti-p65 (1/200, Santa Cruz), hamster anti-CD11c (1/100, BioLegend) cow anti-*Brucella* LPS antibody (1/2000, I. Moriyón), rabbit anti-mouse calnexin (1/200, Abcam), rat anti-mouse Lamp1 (clone 1D4B, 1/200, Santa Cruz) and phalloïdin coupled to Alexa546 (1/1000, Invitrogen); Secondary antibodies were goat anti-hamster-Alexa594, donkey anti-rabbit-Alexa546, goat anti-cow-FITC from Jackson Immunoresearch and anti-rabbit Ig-Pacific Blue, anti-goat Ig-Alexa 546 and anti-rat Ig-Alexa 647 from Life Technologies (Invitrogen). Nuclei were stained with TOPRO-3 (Thermofisher). Blocking and control peptides to SLAMF1 were synthetized by ThermoFisher Scientific following published sequences(Jordan, Fletcher et al. 2007). Type II collagenase was from Worthington Biochemical Corporation) and DNase I from Sigma.

Cell Culture

BMDC were prepared from 6-8 week-old C57BL/6J or *Slamf1*$^{-/-}$ female femurs and tibias as previously described(Papadopoulos, Gagnaire et al. 2016). Briefly, bone ends were cut off and bone marrow was flushed with RPMI 1640 (Gibco, Life Technologies) supplemented with 5% FCS, 100 IU/mL Penicillin, 100 µg/mL Streptomycin and 50 µM 2-mercaptoethanol. Red blood cells were removed by 1 min exposure to 1xRBC lysis buffer solution (eBioscience). $3x10^6$ cells were seeded onto 6-well plates in 5 mL medium containing 0.8 % supernatant of the J558L GM-CSF producing cell line. Medium was changed at day 2.5 and GM-DCs were ready to use at day 5. Cells were mock-treated or stimulated with *E. coli* LPS (100 ng/mL), *B. abortus* CβG (10 µg/mL) or infected for 16 h. Cells were collected for flow cytometry and supernatant was kept at -80°C for cytokine dosage.

Construction of myc-SLAMF1 exon 2-3 and myc-CD90 exon 2-3:

cDNA of mouse *slamf1* gene was obtained from Origene and cDNA of mouse *thy1* gene was a kind gift from Claude Grégoire (CIML). The two first coding exons of each of these cDNA, which encoded the extracellular domain of SLAMF1 or CD90/Thy-1, were amplified by PCR using the following primers, SLAMF1-Forward: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACAGGTGGAGGTGTGATGGAT-3'; SLAMF1-Reverse: 5'-

GGGGACCACTTTGTACAAGAAAGCTGGGTCCTACTGAGGAGGATTCCTGCTTGC-3'. and the two first coding exons were amplified by PCR using the following primers, CD90-Forward: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCCAGAAGGTGACCAGCCTGACA-3'; CD90-Reverse 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCCTAACACTTGACCAgctTGTCTCTATACACACTGA-3' and cloned into a pCMV-myc vector using the Gateway Technology (Invitrogen, Life Technologies). Plasmids were transformed into thermo-competent *E. coli* Top10 for amplification and then purified with endotoxin-free MaxiPrep Plasmid Kit (Qiagen). Clones were checked by sequencing.

Expression and purification of myc-SLAMF1 exon2-3 or myc-CD90 exon 2-3:

10 µg of myc-SLAMF1 or myc-CD90 expression vectors were transfected into COS-7 cells (from the American Type Culture Collection, ATCC CRL-1651) using Fugene (Promega) according to manufacturer's instructions. 48 h after transfection, cells were harvested and lysed into 1xPBS, 1 % Triton X-100 in presence of a protease inhibitor cocktail (Roche). Expression of the transfected proteins was confirmed by western blot against the myc epitope.

Immunoprecipitation of myc-SLAMF1 and myc-CD90

50 µl of protein G Dynabeads (Life Technologies) were coupled to 1 µg of anti-c-myc antibody (9E10) during 1 h at 4°C and then incubated with whole transfected COS-7 cell extracts in 1xPBS, 0.1 % Triton X-100 and protease inhibitors for 1 h at 4°C. After washes in 1xPBS, 0.1 % NP-40, 0.1 % Fetal Bovine Serum and protease inhibitors, myc-SLAMF1 and myc-CD90 immunoprecipitated complexes were incubated 1 h with 1 ng or 0.1 ng of purified recombinant *B. abortus* Omp25 or Omp19 at 4°C. Washes in 0.5 M NaCl, 0.001 % SDS were then performed to eliminate non-specific interactions. Samples were heated at 70°C in Laemmli buffer and centrifuged for 10 min at 14,000 g. Supernatants were denaturated at 95°C for 5 min, prior to loading on 12% SDS PAGE. Western blot against Omp25 or Omp19 was then performed using Mouse IgG True Blot HRP (eBioscience) as a secondary antibody to avoid unspecific Ig binding.

Expression of recombinant Omp25 and Omp19 *Brucella* proteins Recombinant U-Omp19 was produced as previously described(Pasquevich, Estein et al. 2009) and finally stored in following buffer: 50 mM NaH2PO4, 300 mM NaCl pH 7.4. To produce Omp25, the complete sequence of *B. abortus* omp25 gene (GenBank_X79284.1)(de Wergifosse, Lintermans et al. 1995) was synthetized and subcloned into pET22(b)+ (Novagen) in frame with 6×His-tag (Genscript). Expression and purification was performed as described in(Goel and Bhatnagar 2012). Recombinant purified Omp25 was finally refolded and stored at -20 ºC in refolding buffer (50 mM TrisHCl, 1 M NaCl, 0.2 mM DTT, 0.1 mM EDTA, 0.5 M L-Arginine-HCl, 10% Glycerol, 0.15 M Urea, 3.6 mM Imidazole).

Cell culture infection

BMDC were infected at a multiplicity of infection (M.O.I.) of 30:1. Bacteria were centrifuged onto cells at 400 g for 10 min at 4 ºC and then incubated for 30 min at 37 ºC with 5% CO_2. Cells were washed twice with medium and incubated for 1 h in medium containing 100 μg/ml gentamicin (Sigma Aldrich) to kill extracellular bacteria. Thereafter, antibiotics concentration was decreased to 20 μg/ml. To monitor bacterial intracellular survival, infected cells were washed 3 times in 1xPBS and lysed with 0.1% Triton X-100 in H_2O. Serial dilutions were plated in triplicates onto TSB agar to enumerate CFUs after 3 days at 37°C.

RNA extraction and RT

Total RNA were extracted from infected BMDC using RNeasy Mini Kit (Qiagen) following manufacturer's instructions. cDNAs were generated by using Quantitech Reverse Transcription Kit (Qiagen) according to manufacturer's recommendations using 300 ng of RNA as a template.

qPCR

2 μl of cDNA corresponding to 6 ng of starting total RNA were used for qPCR. Amplification reactions were performed with SYBR Green (Takara) in an ABI PRISM 7500 Fast sequence detector (Applied Biosystem). HPRT was used as a housekeeping gene to normalise expression among samples and determine ΔCt. Data are presented as fold increase versus Mock treated cells, put arbitrarily at 1. Primers used to amplify mouse cDNA are listed in Table S3.

Cytokine dosage

Cytokines and chemokines from patient sera were analysed by Luminex. Cytokine profiles (TNFα, IFNγ, and IL-6) in BMDC culture supernatants were analysed by cytometric beads assay (BD Biosciences, Mouse Inflammation kit).

Flow cytometry

Cells were stained for 20 min at 4°C with the antibodies listed above, then washed once in 2% FCS in 1xPBS and once in 1xPBS. Infected cells were then fixed for 20 min in 3 % PFA at 22°C. Events were collected on flow cytometry using a or FACS LSRII UV or Fortessa (BD Biosciences) and analysis was performed on FACS DIVA and FlowJo softwares (BD Biosciences).

Immunofluorescence microscopy

Cells were fixed in 3 % paraformaldehyde, pH 7.4, at 22°C for 20 min. For NF-κB localisation studies, cells were then permeabilized for 10 min with 0.1 % saponin in 1xPBS, followed by 1h blocking with 2 % BSA in PBS. Primary antibodies were incubated for 1 h followed by 2 washes in 1xPBS, 45 min incubation with secondary antibodies, 2 washes in 1xPBS and 1 wash in water before mounting with Prolong Gold (Life technologies). For the other immunofluorescence labelling, 2% BSA in 1xPBS was used for 1 h to block non-specific interactions. Then primary antibodies were incubated for 30 min in 1xPBS, 0.1 % saponin, 0.1 % horse serum. Coverslips with cells were then washed twice in 1xPBS, 0.1 % saponin before 30 min incubation with secondary antibodies and were finally mounted in Prolong Gold (Invitrogen). Samples were examined on a Leica SP5 laser scanning confocal microscope for image acquisition. Images of 1024x1024 pixels were then assembled using Adobe Photoshop 7.0 or ImageJ. In all experiments, an anti-CD11c antibody was used to restrict analysis to DC only. Quantifications were achieved by counting at least 50 DCs from 5 independent experiments, for a total of at least 250 DC analysed.

Mouse infection

6-8 week-old female C57BL/6J (wild-type), *Slamf1*$^{-/-}$ or *Ifng*$^{-/-}$ mice were infected in the BSL3 facility by intraperitoneal (IP) injection. 1x10^{6} CFU were injected into 200 µl of sterile endotoxin-free PBS for each mouse. Organs were harvested at 8 or 30 days p.i, weighted and then dissociated into sterile 0.1 % Triton X-100 diluted in H2O. Serial dilutions in sterile 1xPBS were used to count CFU. For cytometry analyses, spleen were harvested, digested for 20 min at

37°C with type II collagenase and DNase I and then treated with 10 mM EDTA to stop digestion. Cut pieces of spleen crushed with a syringe plunger in a 70-µm nylon strainer cell strainer were filtered in 1xPBS, 5 mM EDTA, 2 % FCS. After removal of red cells by the Red blood cell lysis buffer (eBioscience) single splenic cell suspensions were proceeded for FACS analysis. For histology studies, organs were harvested and placed into 10 % formalin for 24 h at 22°C before inclusion in paraffin. Slides were then stained with hematoxylin and eosin.

Statistical analysis

Statistical analyses were done using with the GraphPad Prism software. When comparing groups of our human cohort, Kruskal-Wallis rank sum test was performed followed by variance analysis with the Post-hoc Dunn test on group comparisons. For mouse data, Brown-Forsythe ANOVA test followed by variance analysis with the Welch's test were performed, otherwise indicated in the figure legend. Mantel Cox test was used for survival curve. All values are expressed as mean ± standard deviation. Differences between values were considered significant at $P<0.05$,* ($P<0.05$; **, $P<0.001$; ***, $P<0.0001$). All experiments were performed at least three times in triplicate otherwise indicated.

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Supplementary material

Table S1. *P* values of different clinical group comparisons for main regulated genes in our human brucellosis cohort

	P values amongst clinical groups					
Gene	**Control-Acute**	**Control-Acute relapse**	**Acute-Acute relapse**	**Control-Chronic**	**Acute-chronic**	**Acute relapse-Chronic**
SLAMF1	1.03e-8	8.65e-7	0.021	*0.143*	0.00165	0.000155
SH2D1A	6.4e-9	3.1e-7	0.0139	*0.121*	0.00194	0.0000994
TBX21	1.12e-7	0.0000014	0.0156	*0.225*	0.00152	0.0000964
EOMES	1.96e-7	3.3e-7	0.00605	*0.34*	0.000576	0.0000124
C1QC	0.00000243	0.000475	0.149	*0.151*	0.0131	0.0108
GZMH	1.88e-7	0.0000066	0.0311	*0.19*	0.00289	0.000405
GZMK	9.63e-8	0.00000105	0.0138	*0.124*	0.00532	0.000221
IFI35	0.00000143	0.000103	*0.08*	*0.228*	0.00453	0.00215
NKG7	8.31e-8	0.00000255	0.0229	*0.176*	0.00245	0.000234
CD19	0.00434	0.0244	*0.251*	*0.143*	*0.221*	*0.143*
SSH2	2.86e-8	0.00617	*0.405*	*0.0602*	0.0111	*0.111*

PBMC were isolated using Ficoll from blood of healthy volunteers or brucellosis patients, and RNA sequencing performed as described in Materials and Methods. Number of patients enrolled in each clinical group,: Control, n = 36; Acute treated, n = 54; Acute with relapse, n = 6; Chronic, n = 12. On selected genes, groups were analysed with the non-parametric Krukal Wallis test followed by variance analysis with the Post-hoc Dunn test. *P* values <0.05 were considered as significant. *P* values ≥0.05 are shown in italics.

Table S2. List of main up-regulated genes in CβG-stimulated human mo-DCs

Fold Increase in Gene Expression	Gene Name	Genbank Accession Number
39.91	TNFAIP6	NM_007115.3
36.98	IL6	NM_000600.3
32.35	SLAMF1	NM_003037.3
31.55	TNIP3	NM_001244764.1
28.47	BATF	NM_006399.3
27.12	IL1A	NM_000575.3
18.91	CCL20	NM_004591.2

Human mo-DCs were extracted from PBMC using Ficoll from healthy volunteers. Cells were then mock-treated or stimulated for 6 h with 0.25 μM CβG before RNA extraction as previously described[37]. Fold increase in gene expression levels of CβG-stimulated cells compared to mRNA expression levels in mock-treated cells is shown.

Table S3. qRT PCR Primers used in this study

Gene	Sequence (Forward/Reverse)
Hprt	Fw 5'-AGCCCTCTGTGTGCTCAAGG-3' Rv 5'-CTGATAAAATCTACAGTCATAGGAATGGA-3'
Ptgs2	Fw 5'-ACCTCTGCGATGCTCTTCC-3' Rv 5'-TCATACATTCCCCACGGTTT-3'
Tnfa	Fw 5'-CATCTTCTCAAAATTCGAGTGACAA-3' Rv 5'-TGGGAGTAGACAAGGTACAACCC-3'
Nos2	Fw 5'-CAGCTGGGCTGTACAAACCTT-3' Rv 5'-CATTGGAAGTGAAGCGTTTCG-3'
Il12b	Fw 5'-AAATTACTCCGGACGGTTCA-3' Rv 5'-ACAGAGACGCCATTCCACAT-3'
Il6	Fw 5'-GAGGATACCACTCCCAACAGACC-3' Rv 5'-AAGTGCATCATCGTTGTTCATACA-3'
Il1b	Fw 5'-TCCAGGATGAGGACATGAGCAC-3' Rv 5'-GAACGTCACACACCAGCAGGTTA-3'
Cxcl1	Fw 5'-CAGCCACCCGCTCGCTTCTC-3' Rv 5'-TCAAGGCAAGCCTCGCGACCAT-3'
Ccl2	Fw 5'-GCCTGCTGTTCACAGTTGC-3' Rv 5'-ATTGGGATCATCTTGCTGGT-3'

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Legends to Figures

Fig 1. Expression of SLAMF1 marks acute phase patients.

(A) Global expression of selected genes versus controls. Colours reflect membership of genes in Weighted Gene Correlation Network (WGCNA) analysis modules on RNA seq samples from controls and primary brucellosis patients in Acute, Acute with relapse or Chronic phase, at initial visit. Code colour for WGCNA modules are the following: Blue, B cell activation and B cell receptor signalling pathway; Grey, Leukocyte, cytolysis and T cell activation; Yellow, Inflammatory response to bacteria Brown, Type I and II IFN response; Green, Mitotic cell signalling; Red, Cell signalling, protein kinases. Only modules that vary among groups and only genes with connectivity > 0.75 to those modules are shown. Each circle represents a gene. Filled circles correspond to a false discovery rate (FDR) of < 0.01 and open circles to FDR ≥ 0.01.

(b) Volcano plots representing overall gene expression changes observed in Acute relapse (top) and Chronic (bottom) infected groups. Each point represents a gene, the dashed vertical lines indicate log2 fold-changes of -1 and +1, and the dashed horizontal line indicates a false discovery rate (FDR) of 0.01. Genes displaying statistically significant differences in expression compared with Controls are marked in black. The numbers of differentially expressed genes (DEGs) that are either upregulated (Up) or downregulated (Down) are indicated.

(c) The median expression of selected differentially expressed genes is shown. X-axis: control (n = 36; Red), Acute treated (n = 54; Yellow), Acute with relapse (n = 6; Light Green), Chronic (n = 12; Dark green). Y-axis: log2 residual gene expression counts after regressing out the effect of leukocyte proportions.

(**d**) Multivariate analysis (OPLS-DA) loading plot of cytokine and chemokine secretion (green) measured by Luminex in the sera of the three different groups of patients, Acute (n = 54), Acute with relapse (n = 6) and Chronic (n = 12), together with Controls (n = 36) (Blue). Variables near each other are positively correlated; variables opposite to each other are negatively correlated. Variables closer to dots corresponding to “Acute, Acute relapse, Chronic” or “Controls” dots (i.e. with the largest absolute loading values) are higher in the corresponding populations. *P*=5.34218e

Figure 2: *B. abortus* inhibits BMDC activation in a SLAMF1-Omp25 dependent fashion.

(**a**) C57BL/6J BMDCs were non-treated (Mock), stimulated with 0.1 μg/ml of *E. coli* LPS or 10 μg/ml of *B. abortus* CβG, or infected at a multiplicity of infection (M.O.I.) of 30 with wild-type *B. abortus* (Ba WT) for 16 h. SLAMF1 expression levels (MFI, Median of Fluorescence Intensity) in DC were then measured by flow cytometry. Data obtained from at least 4 independent experiments are shown. All error bars are standard deviations obtained from pooled data. Significant differences from Ba WT-infected DCs are presented; **, $P < 0.01$; ***, $P < 0.001$.

(**b**) C57BL/6J BMDCs were infected with wild-type *B. abortus* (Ba WT), or Ba Δ*omp25*, Δ*omp25* complemented (Δ*omp25c*) or Δ*virB* (non-replicative strain) mutants at a M.O.I. of 30. At different time-points post-infection (p.i.), as indicated on the x axis, cells were harvested and lysed in 0.1% Triton-X100 to assess bacterial replication. CFU were counted and the mean ± SD of at least 4 independent experiments is shown. Significant differences from Ba WT infected DCs are presented; ****, $P<0.0001$.

(**c**) C57BL/6J wild-type (wt, left column) or *Slamf1*$^{-/-}$ (right column) BMDCs were non-treated (Mock), stimulated with 0.1 μg/ml of *E. coli* LPS (positive control) or infected with *B. abortus* (Ba) WT, Δ*omp25* or Δ*omp25c* at a M.O.I. of 30 for 24 h. Co-stimulatory molecule (CD40, CD80 and CD86) levels of expression (MFI) were measured by flow cytometry. Data are expressed as MFI fold change versus MFI levels of one set of mock-treated cells put arbitrarily at 1. At least 100,000 CD11c^{+} (DC marker) cells were analysed. Graphs show combined data from at least four independent experiments. All error bars are standard deviations obtained from pooled data. Significant differences from Ba WT-infected DCs are shown. *, $P< 0.05$; **, $P< 0.001$; ***, $P<0.0001$; ****, $P<0.00001$. ns, non-significant.

(**d**) Wild-type (wt, top line) or *Slamf1*$^{-/-}$ (bottom line) BMDCs were non-treated (Mock) or infected with *B. abortus* (Ba) WT or Δ*omp25* at a M.O.I. of 30 for 16 h. Culture supernatants were harvested 24h p.i. and cytokine secretion (μg/ml) was assessed by cytometric bead assay (IL-6, TNF-α and IFN-γ). Graphs show combined data from three independent experiments. All error bars are standard deviations obtained from pooled data. For TNF-α data, statistical analysis was performed with the multiple comparison Kruskal Wallis ANOVA test, followed by variance analysis with the Dunn's test. Significant differences from Ba WT-infected DCs are shown. *, $P< 0.05$; ***, $P<0.0001$. ns, non-significant.

Figure 3: SLAMF1 interacts with Omp25 thus limiting NF-κB translocation & subsequent cytokine secretion in *B. abortus* infected BMDCs.

(**a**) COS-7 cells were transfected with 10 μg of plasmid expressing myc-SLAMF1(exons 2-3) or myc-CD90(exons 2-3). 48 h after transfection, cells were harvested and proteins extracted. After myc-tag pull-down and incubation with 1 or 0.1 ng of purified recombinant Omp25, the binding of Omp25 to SLAMF1 was assessed by western blot. One representative experiment out of 3 independent ones is shown.

(**b**) COS-7 cells were transfected with 10 μg of plasmid expressing myc-SLAMF1(exons 2-3) or myc-CD90(exons 2-3). 48 h after transfection, cells were harvested and proteins extracted. After myc-tag pull-down and incubation with 0.1 ng of purified recombinant Omp19, the binding of Omp19 to SLAMF1 was assessed by western blot. One representative experiment out of 3 independent ones is shown.

(**c**) Representative confocal microscopy images of wild-type (wt, left panels) or *Slamf1*$^{-/-}$ (right panels) BMDC non-treated (Mock) or infected with *B. abortus* (Ba) WT, Δ*omp25* or Δ*omp25c* at a M.O.I. of 30. Two hours p.i., cells were fixed and labelled with TOPRO-3 for nucleus (yellow), anti-p65 (NF-κB, red) and anti-CD11c for DC (cyan). Scale bar 10 μm. At the right bottom of the panel, quantification of NF-κB translocation in the nucleus of infected cells is shown. Graph displays combined data from four independent experiments, for which at least 50 cells per experiment were counted. All error bars are standard deviations obtained from pooled data. Significant differences from wt DC infected with Ba WT are shown. *, $P< 0.05$; ***, $P<0.0001$. Absence of *P* value indicates that data are not statistically different.

Figure 4: The SLAMF1-Omp25 axis restricts *Brucella*-induced inflammation *in vivo* without affecting bacterial replication.

(a) C57BL/6J wild-type (wt) or *slamf1*$^{-/-}$ mice were intraperitoneally injected with PBSx1 (Mock, ○) or inoculated with $1x10^6$ CFU of wild-type *B. abortus* (Ba WT, ■), Ba Δ*omp25* (▲) or Ba Δ*omp25* complemented (Ba Δ*omp25c*, □) mutants (the latter only for wt mice). Eight days later, spleens were removed, weighted (upper graph), lysed in 0.1% Triton-X100 and CFU were enumerated (lower graph). Genotype of mice is indicated in the x axis. Each symbol represents one animal. Data obtained from 8 independent experiments, each with at least n=3 animals per condition, are shown and mean ± SD is represented by horizontal bar. Significant differences from wt mice infected with Ba WT are shown. *, $P< 0.05$; **, $P< 0.001$. Absence of *P* value or ns, non-significant.

(**b**) Survival curve of C57BL/6J wild-type (*ifng*$^{+/+}$, —) or *ifng*-deficient (*ifng*$^{-/-}$, —) mice were infected intraperitoneally with 1x10^6 CFU of *B. abortus* WT (plain lines) or Δomp25 (dashed lines) strains. Mice were weighted every two days; when reaching a weight loss of 30%, they were sacrificed. Five mice per group were used for this experiment, n=3. Significant differences from Ba WT are shown. *, $P< 0.05$.

(**c**) C57BL/6J wild-type (wt) or *slamf1*$^{-/-}$ mice were intraperitoneally injected with PBSx1 (Mock, ○) or inoculated with 1x10^6 CFU of wild-type *B. abortus* (Ba WT, ■), Ba Δ*omp25* (▲) or Ba Δ*omp25c* (□) mutants (the latter only for wt mice). Eight days later, mice were sacrificed, single-splenocyte suspensions were prepared and DC subsets were analysed by flow cytometry from total splenic CD11c$^+$ cells. Absolute number fold changes of cDC1(upper graph), cDC2 (middle graph) and pDC (lower graph) over mock-treated wild-type corresponding cells are shown. Genotype of mice is indicated in the x axis. Each symbol represents one animal. Data obtained from 3 independent experiments, each with at least n=3 animals per condition, are shown and mean ± SD is represented by horizontal bar. Significant differences from wt mice infected with Ba WT are shown. *, $P< 0.05$; ***, $P< 0.0001$. Absence of *P* value or ns, non-significant.

(**d**) Activated T cells subsets from single-splenocyte suspensions of C57BL/6J wild-type (wt) or *slamf1*$^{-/-}$ mice mock-treated or infected as described in (c) were analysed by flow cytometry eight days p.i.. Absolute number fold changes of CD4$^+$CD69$^+$ (upper graph) and CD8$^+$CD69$^+$ T cells (lower graph) over mock-treated wild-type corresponding cells are shown. Genotype of mice is indicated in the x axis. Each symbol represents one animal. Data obtained from 2 independent experiments, each with at least n=5 animals per condition, are shown and mean ± SD is represented by horizontal bar. Significant differences from wt mice infected with Ba WT are shown. *, $P< 0.05$; **, $P< 0.001$. Absence of *P* value or ns, non-significant.

(**e**) Thirty days p.i., spleens from C57BL/6J wild-type (wt) or *slamf1*$^{-/-}$ mice mock-treated or infected as described in (c) were removed, weighted (upper graph), lysed in 0.1% Triton-X100 and CFU were enumerated (lower graph). Genotype of mice is indicated in the x axis. Each symbol represents one animal. Data obtained from 5 independent experiments, each with at least n=3 animals per condition, are shown and mean ± SD is represented by horizontal bar. Significant differences from wt mice infected with Ba WT are shown. *, $P< 0.05$. Absence of *P* value or ns, non-significant.

Supplementary figures

Supplementary Figure S1: Flow cytometry characterisation of BMDCs *in vitro* upon *B. abortus* infection.

Representative FACS profiles of C57BL/6J BMDCs non-treated (Mock) or infected at a M.O.I. of 30 with wild-type *B. abortus* (Ba WT) 16 h p.i.. DCs were separated from macrophages ($F4/80^{+}$) and identified with the CD11c and MHCII cell surface markers. Expression levels of SLAMF1 and core DC activation markers (modal fluorescence) are shown.

Supplementary Figure S2: In infected BMDCs, SLAMF1 expression, *Brucella* intracellular localisation and replication are Omp25-independent.

(**a**) C57BL/6J BMDCs were non-treated (Mock), stimulated with 0.1 μg/ml of *E. coli* LPS or with 10 μg/ml of *B. abortus* CβG, or infected at a multiplicity of infection (M.O.I.) of 30 with wild-type *B. abortus* (Ba WT), Ba Δ*omp25* or Ba Δ*omp25* complemented (Δ*omp25c*) mutant strains for 16 h. SLAMF1 expression levels (MFI, Median of Fluorescence Intensity) in DCs were then measured by flow cytometry. Data obtained from at least 3 experiments, each with n=3 animals per condition, are shown. All error bars are standard deviations obtained from pooled data. Significant differences from Ba WT infected DC are presented; **, $P < 0.01$; ***, $P < 0.001$.

(**b**) Representative confocal microscopy images of C57BL/6J BMDCs infected with wild-type *B. abortus* (Ba WT, upper panel) or Ba Δ*omp25* mutant strain (lower panel) at a M.O.I. of 30. Cells were fixed at 24 h p.i and stained for calnexin (ER staining, red) and anti-*Brucella* LPS (green). Scale bar 10 μm. n= 4 independent experiments.

(**c**) C57BL/6J wild-type (*Slamf1*$^{+/+}$, plain line) or *Slamf1*-deficient (*Slamf1*$^{-/-}$, dotted line) BMDCs were infected with wild-type *B. abortus* (Ba WT), Ba Δ*omp25* or Ba Δ*omp25c* mutants at a M.O.I. of 30. At different time-points post-infection (p.i.), as indicated on the x axis, cells were harvested and lysed in 0.1% Triton-X100 to assess bacterial replication. CFU were counted and the mean ± SD of at least 4 independent experiments is shown. Absence of *P* value indicates that data are not statistically different.

Supplementary Figure S3: The Omp25-SLAMF1 axis does not impact MHCII expression in *B. abortus* infected BMDCs.

C57BL/6J BMDCs were non-treated (Mock), stimulated with 0.1 μg/ml *of E. coli* LPS or infected at a multiplicity of infection (M.O.I.) of 30 with wild-type *B. abortus* (Ba WT), or Ba Δ*omp25* or Δ *omp25c* mutant strains for 16 h. MHC expression levels (MFI, Median of Fluorescence Intensity) in DCs were then measured by flow cytometry. Data obtained from at least 3 experiments, each with n=3 animals per condition, are shown. All error bars are standard deviations obtained from pooled data. Significant differences from Ba WT-infected wt DCs are presented; *, $P< 0.05$. ns, non-significant.

Supplementary Figure S4: In presence of a SLAMF1 blocking peptide, wild-type *B. abortus* triggers NF-κB translocation in BMDCs similarly to the mutant strain devoid of Omp25.

C57BL/5J BMDCs were treated with 100 μg/ml of SLAMF1 blocking peptide or control scrambled peptide 3 h prior to infection. BMDCs were then non-treated (Mock) or infected with *B. abortus* (Ba) WT or Δomp25 at a M.O.I. of 30. Two hours p.i., cells were fixed, stained with TOPRO-3 for nucleus (yellow), anti-p65 (NF- κB, red) and anti-CD11c for DC (cyan) and analysed by confocal microscopy. Presence of NF-κB in the nucleus of infected cells was quantified and expressed as a percentage of DCs with nuclear NF-κB. Graph displays combined data from four independent experiments, for which at least 50 cells per experiment were counted. All error bars are standard deviations obtained from pooled data. Significant differences from DCs pre-treated with the control peptide and infected with Ba WT are shown. *, $P< 0.05$. Absence of *P* value indicates that data are not statistically different.

Supplementary Figure S5: *B. abortus* devoid of Omp25 upregulates pro-inflammatory gene expression in BMDCs.

BMDCs were infected at a M.O.I. of 30 with *B. abortus* (Ba) WT or Δomp25 mutant strains, or treated with PBS as negative control (mock). Six or twenty-four hours p.i., cells were harvested and total RNA extracted. mRNA expression levels of *tnfa*, *il12b*, *il6*, *il1b*, *ccl2*, *cxcl1*/*kc*, *nos2* and *ptgs2* genes were assessed by RT-QPCR. Data were normalised onto housekeeping gene (HPRT) and fold increase was calculated from mock-treated cells. The graphs show combined data from three independent experiments. All error bars are standard

deviations obtained from pooled data. Significant differences from BMDCs infected with Ba WT are shown. *, $P< 0.05$; **, $P < 0.01$; ***, $P<0.0001$. ns, non-significant.

Supplementary Figure S6: Effect of Omp25 and SLAMF1 loss on granuloma formation upon *in vivo* infection with *B. abortus*.

C57BL/6J wild-type (wt) or *Slamf1*$^{-/-}$ mice were intraperitoneally inoculated with 1x10^{6} CFU of wild-type *B. abortus* (Ba WT, ■) or Ba Δ*omp25* (▲) mutant. Eight days later, spleens were removed and fixed. Thin cryo-sections (7 μm) of spleen were stained with eosin-hematoxylin and granuloma counts performed. Each symbol represents one animal. Genotype of mice is indicated in the x axis. Pooled data from two independent experiments, each with at least n=3 animals per condition are shown and mean ± SD is represented by horizontal bar. Significant differences from wt mice infected with Ba WT are shown. One *P* value is indicated, although superior to 0.05 and therefore not significant but indicative of a trend. ns, non-significant.

Supplementary Figure S7: Flow cytometry characterisation of splenic DCs and T cells *in vivo* upon *B. abortus* infection.

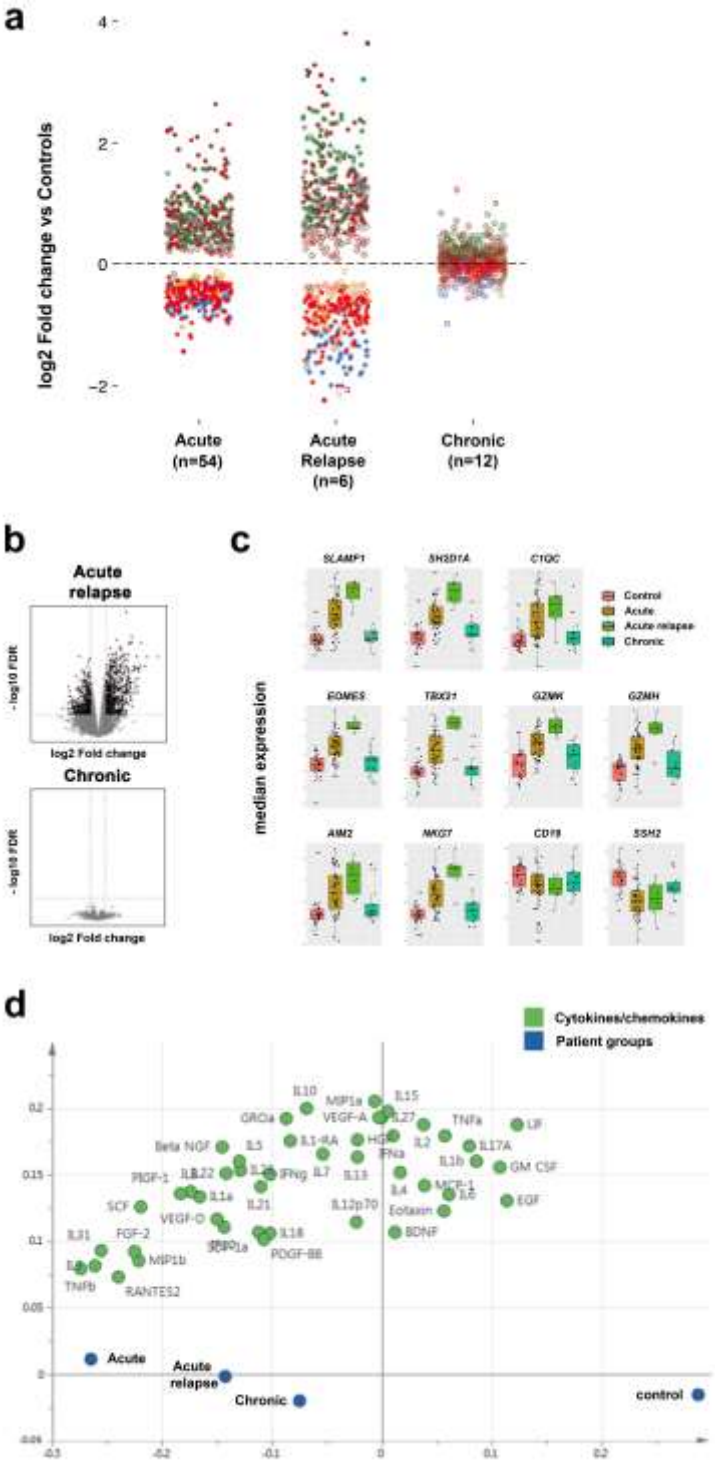
Single-cell suspensions from spleens were prepared and analysed by flow cytometry. Representative FACS profiles of one infected sample are shown. For DC analysis (upper panel), T cells, B cells, NK cells and neutrophils were first excluded and remaining cells were analysed for CD11c and CD11b expression. Amongst the CD11c^{int}CD11b^{-} cells, pDC were identified as Bst2^{+}SiglecH^{+} cells. The two conventional DC subsets were characterized among the CD11c^{hi} cells, as cDC1 cells for the XCR1^{+}SIRPα^{-} fraction and as cDC2 cells for the SIRPα^{+}XCR1^{-}CD24^{-} fraction. For T cell analysis (bottom panel), T cells were identified from splenic cells by CD3 positivity. CD4^{+} and CD8^{+} T cells were then assessed for their expression of the activation marker CD69.

Supplementary Figure S8: The Omp25-SLAMF1 interaction limits activation of CD4^{+} T cells in *B. abortus* infected BMDCs.

Activated T cells subsets from single-splenocyte suspensions of C57BL/6J wild-type (wt) or *slamf1*$^{-/-}$ mice mock-treated or infected as described in Figure 4 were analysed by flow cytometry eight days p.i.. Percentages of CD4^{+}CD69^{+} (left graph) and CD8^{+}CD69^{+} T cells (right graph) over % of splenic CD4^{+} or CD8^{+} cells respectively are shown. Genotype of mice is indicated in the x axis. Each symbol represents one animal. Data obtained from 5 independent

experiments, each with at least n=3 animals per condition, are shown and mean ± SD is represented by horizontal bar. Significant differences from wt mice infected with Ba WT are shown. *, $P < 0.01$; **, $P < 0.001$. ns, non-significant.

Figure 1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Acute Relapse (n=6)
Chronic (n=12)
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Chronic
log2 Fold change
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d
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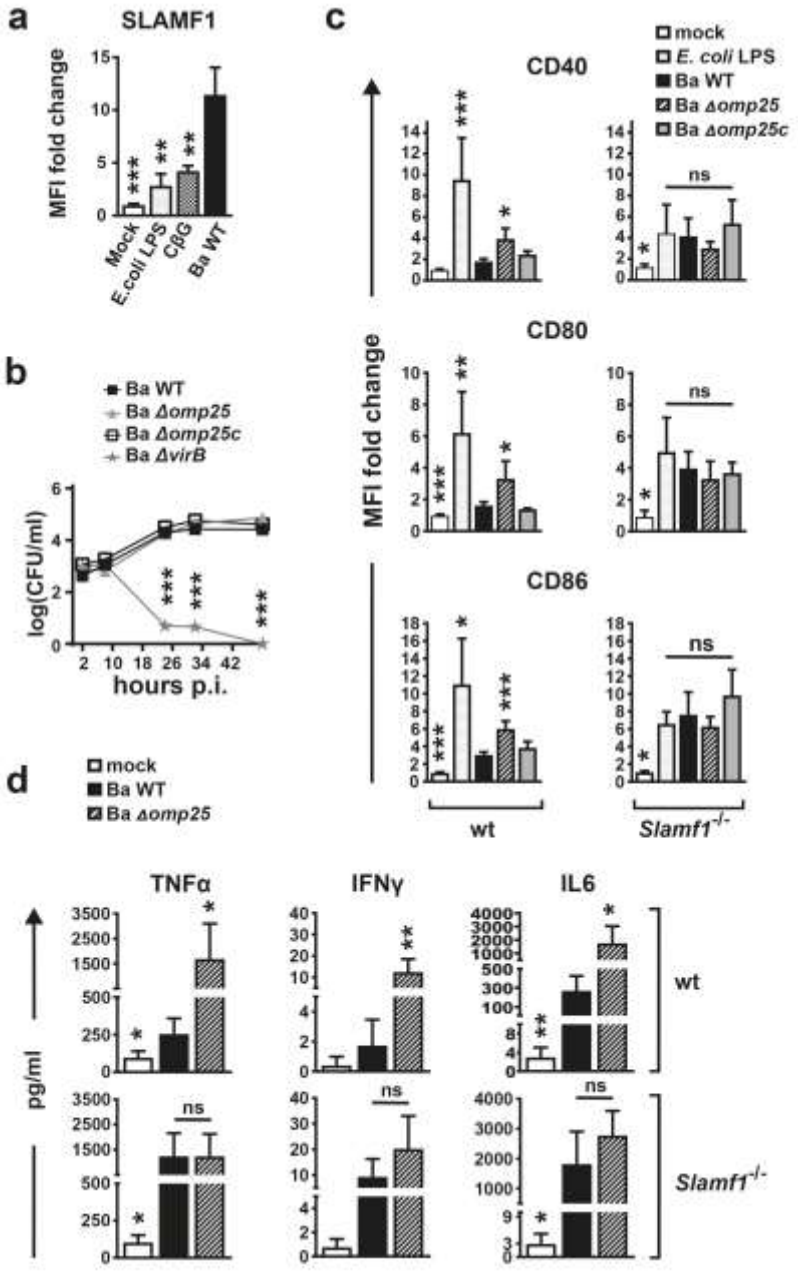
control

Figure 2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c
mock
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Ba Δomp25
Ba Δomp25c
CD40
CD80
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MFI fold change
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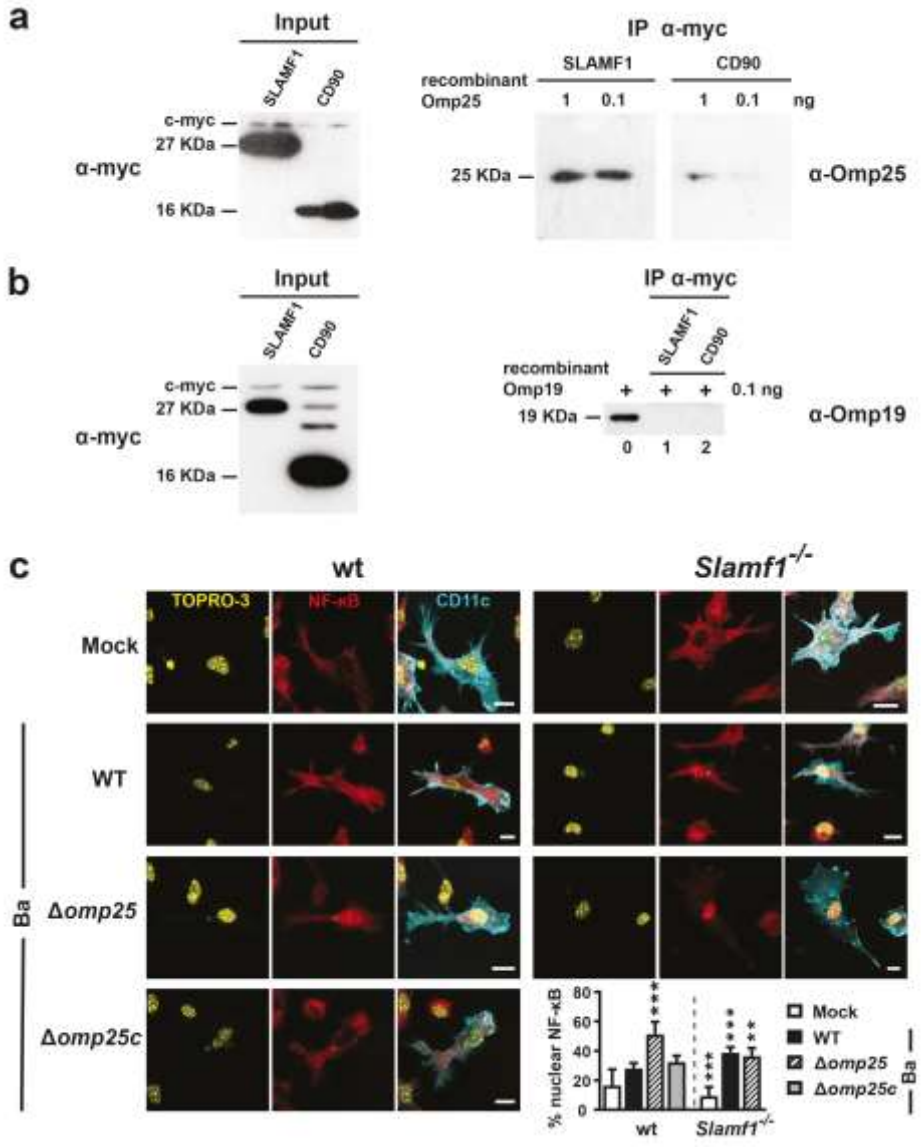
Figure 3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Δomp25c
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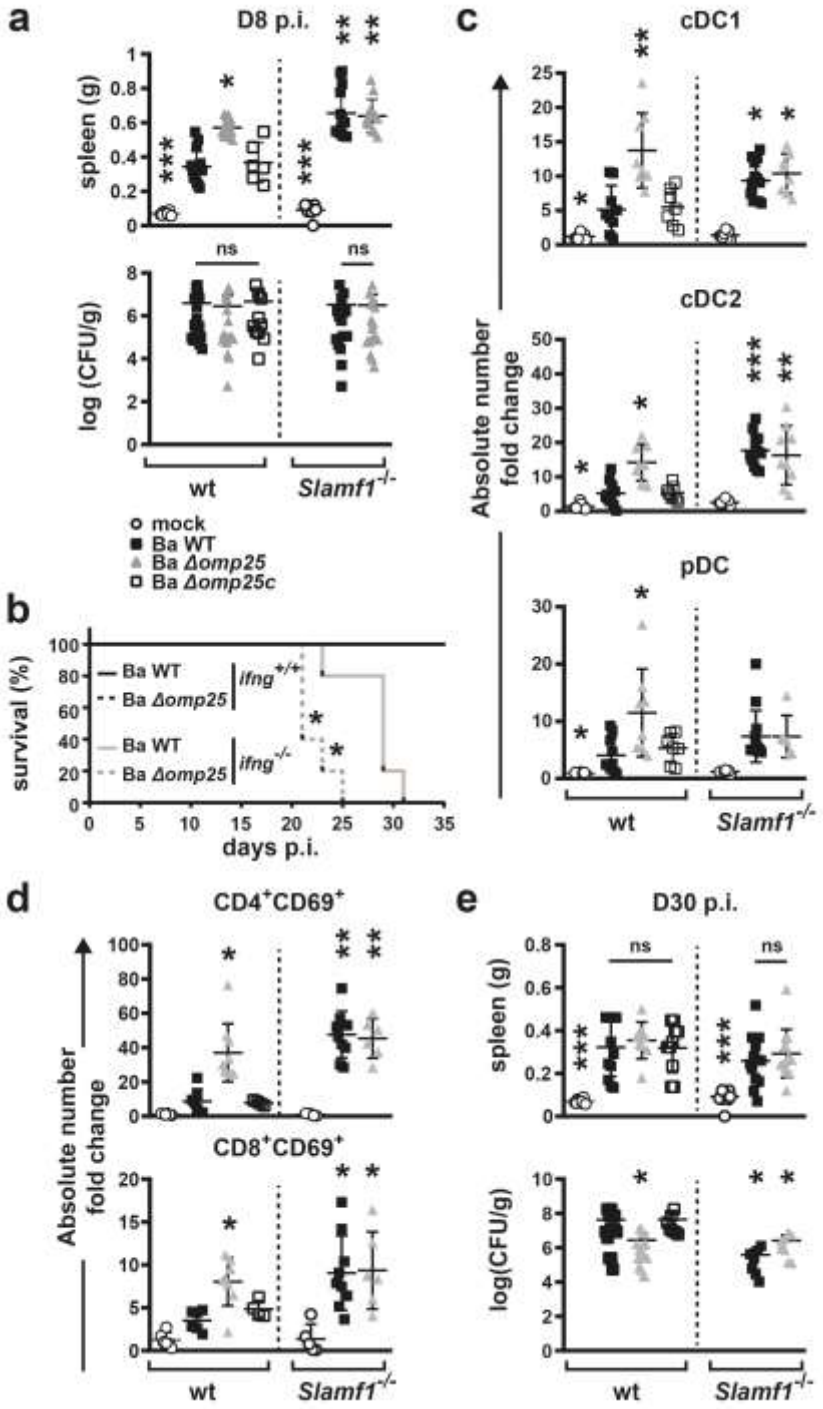
Figure 4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survival (%)
Ba WT
Ba Δomp25
ifng+/+
ifng-/-
days p.i.
c
cDC1
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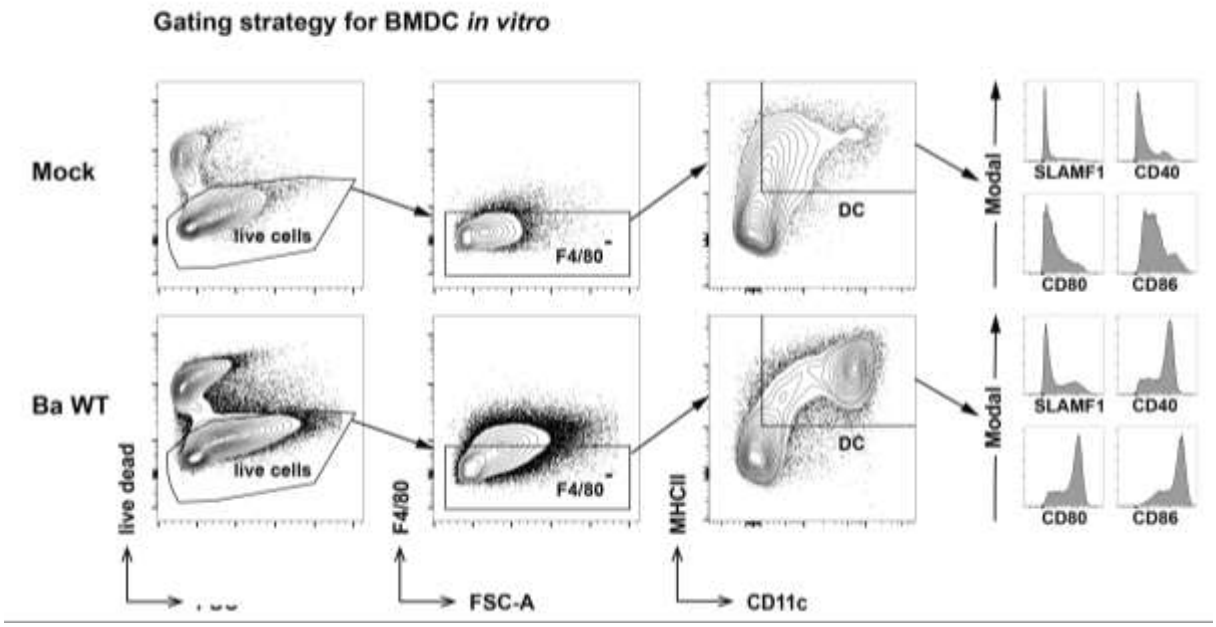
Supplementary 1



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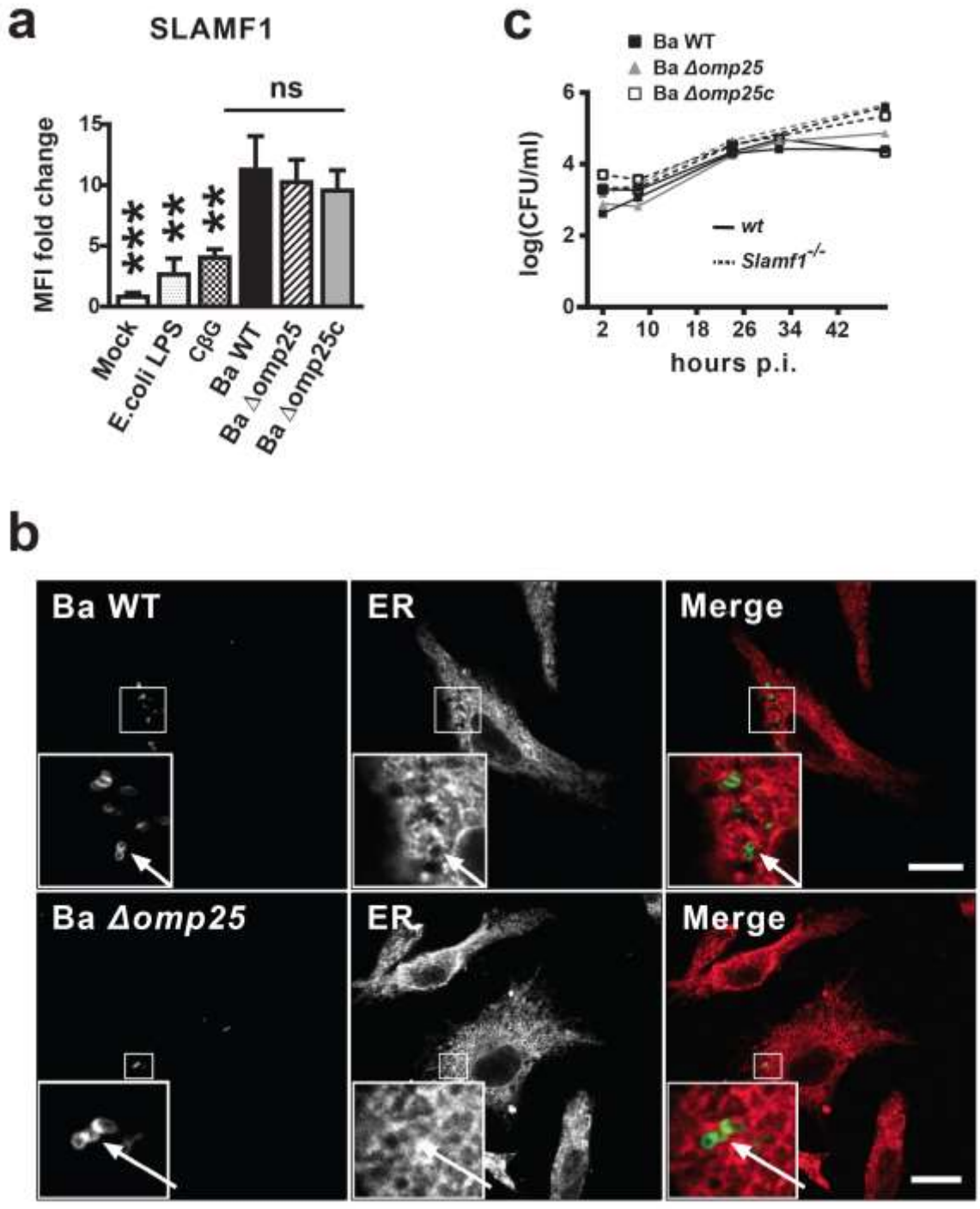

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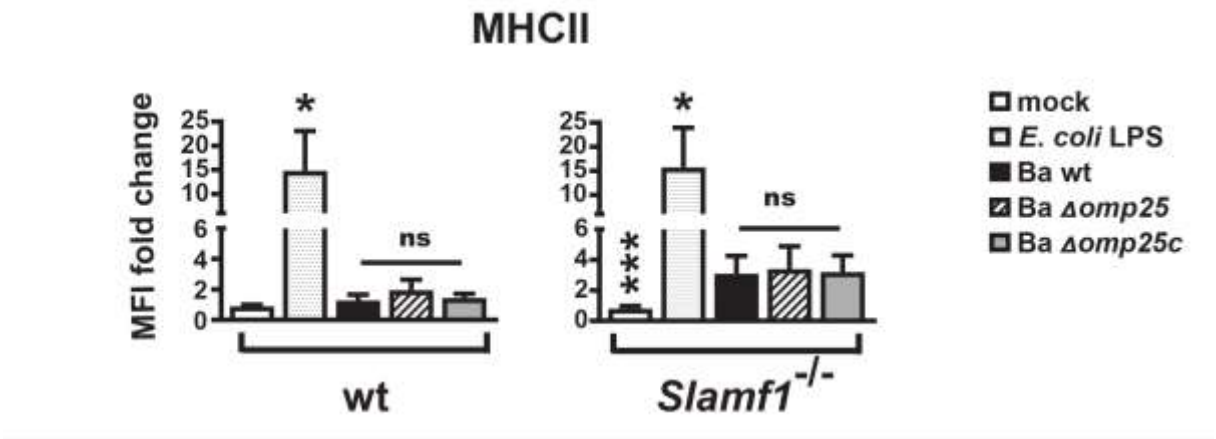

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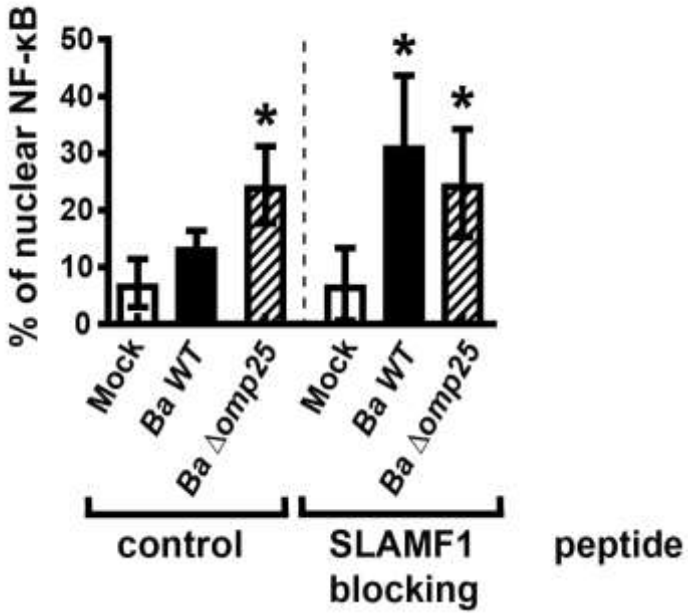

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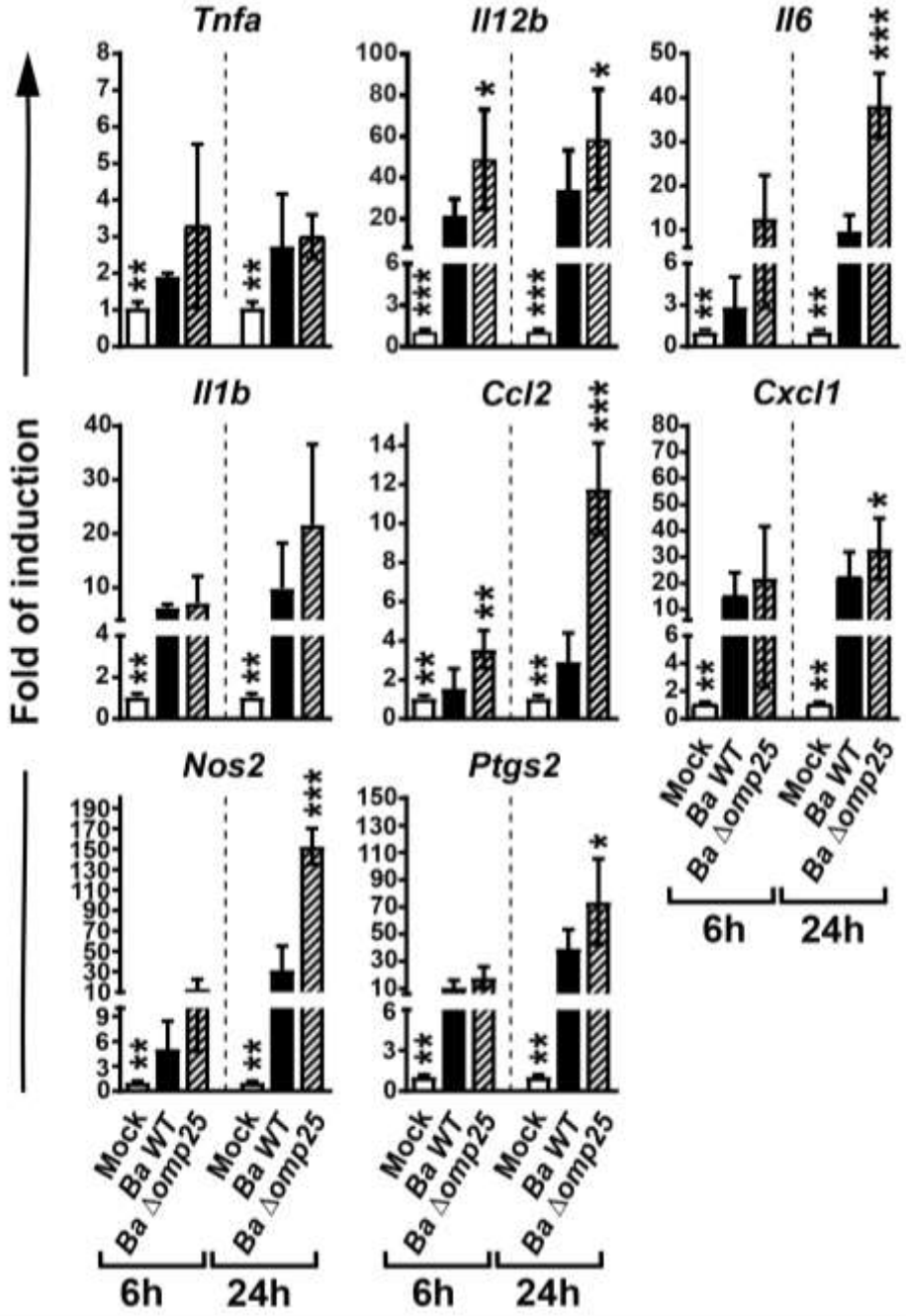

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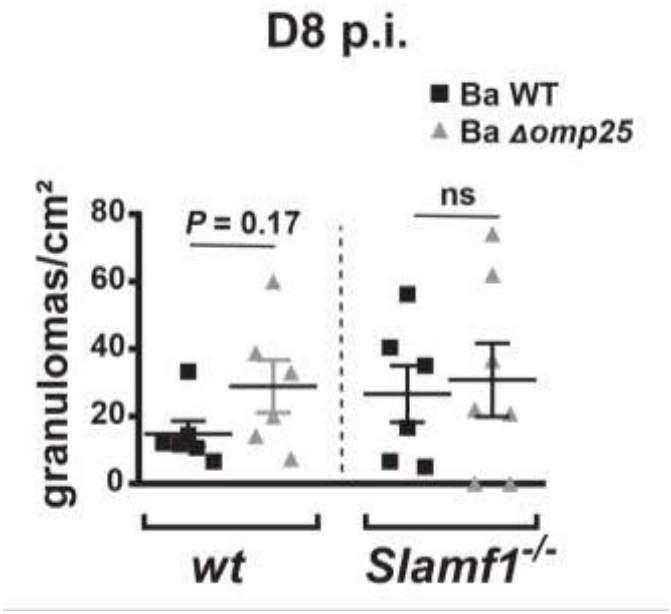
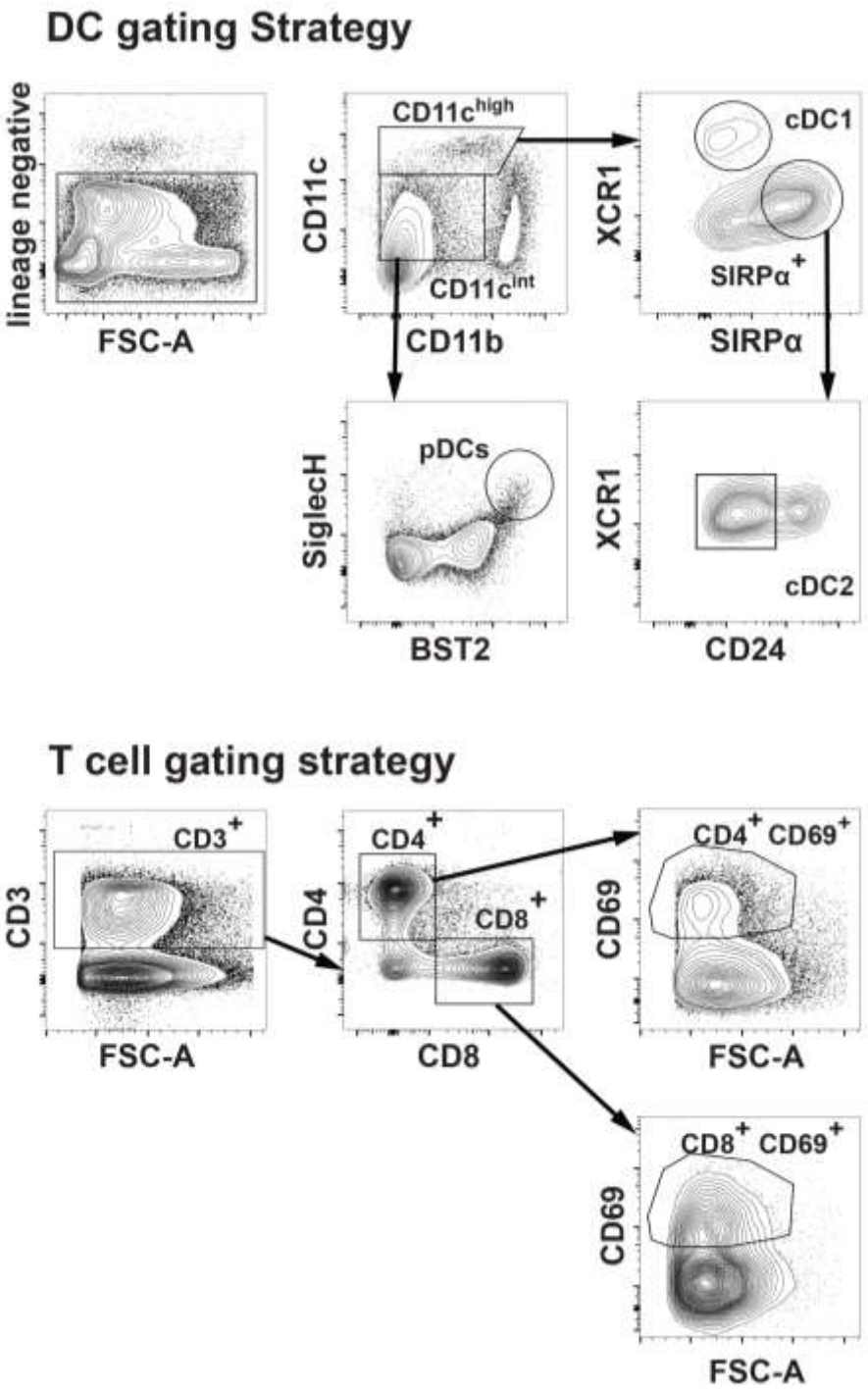


Supplementary 7



Supplementary 8

Manuscript 2: Persistence of *Brucella* abortus in the bone marrow of infected mice . (Gutterriez-Jimenez*, **Hysenaj*** et al., 2018, J. Immunol Res.)

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Research Article

Persistence of *Brucella abortus* in the Bone Marrow of Infected Mice

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Brucellosis is a zoonotic bacterial infection that may persist for long periods causing relapses in antibiotic-treated patients. The ability of *Brucella* to develop chronic infections is linked to their capacity to invade and replicate within the mononuclear phagocyte system, including the bone marrow (BM). Persistence of *Brucella* in the BM has been associated with hematological complications such as neutropenia, thrombocytopenia, anemia, and pancytopenia in human patients. In the mouse model, we observed that the number of *Brucella abortus* in the BM remained constant for up to 168 days of postinfection. This persistence was associated with histopathological changes, accompanied by augmented numbers of BM myeloid GMP progenitors, PMNs, and $CD4^+$ lymphocytes during the acute phase (eight days) of the infection in the BM. Monocytes, PMNs, and GMP cells were identified as the cells harboring *Brucella* in the BM. We propose that the BM is an essential niche for the bacterium to establish long-lasting infections and that infected PMNs may serve as vehicles for dispersion of *Brucella* organisms, following the Trojan horse hypothesis. Monocytes are solid candidates for *Brucella* reservoirs in the BM.

1. Introduction

Brucellosis is a zoonotic bacterial infection caused by members of the genus *Brucella* [1]. In humans, the disease is long-lasting, displaying a variety of clinical and pathological manifestations that may persist for months or years [2–5]. If the infection is not properly treated, it may cause death.

The ability of *Brucella* organisms to develop chronic infections is linked to their competence to invade the mononuclear phagocyte system, where they replicate within the endoplasmic reticulum [6]. In addition, the poor proinflammatory responses induced at the onset of the infection [7], together with the capacity of *Brucella* organisms to extend the life of infected cells, are factors that contribute to the pathogenicity of this microorganism [7, 8].

The persistence of *Brucella* organisms in humans occurs in the lymph nodes, spleen, liver, bone marrow (BM), reproductive organs, and joints [9, 10]. The bacterium is isolated from the BM in about half of the human patients with brucellosis [4]. However, in all brucellosis cases, the BM displays histopathological alterations, whether or not the bacterium is isolated from this tissue. Common hematological signs are neutropenia, thrombocytopenia, and anemia, and in severe cases, pancytopenia has also been

reported [4, 5, 11]. In most patients, the BM cellular alterations ameliorate or disappear after antibiotic treatment [4]. Moreover, brucellosis transmission by BM transplantation from seemingly healthy donors has been reported [12]. These data indicate that even in those cases in which the bacterium is not isolated from the BM, it still may be present, hidden within cells.

Following experimentation in mice, it has been proposed the BM may be the most relevant tissue for *Brucella* persistence [13]. In addition, *Brucella canis* has been shown to persevere in the BM at chronic stages of mouse infection [14]. Here, we describe the persistence of *Brucella abortus* in cells of the mice BM and propose that this tissue is essential for establishing long-lasting chronic infections.

2. Materials and Methods

2.1. Infection Protocols. *B. abortus* 2308W expressing red fluorescent protein from *Discosoma* coral (*B. abortus*-RFP), provided by Jean-Jacques Letesson (University Notre-Dame de la Paix, Namur, Belgium) was used in all experiments. BALB/c mice were supplied by the Escuela de Medicina Veterinaria, Universidad Nacional, Costa Rica, and Laboratorio de Ensayos Biológicos, Universidad de Costa Rica. C57BL/6 mice were purchased from Charles River Laboratories (Les Oncins, France), housed under specific pathogen-free conditions, and handled in accordance with French and European guidelines.

Mice were infected by the intraperitoneal route (i.p.) with 10^5 bacterial colony forming units (CFU) of *B. abortus*-RFP. At different phases of the infection, the spleen, liver, lymph nodes, and bone marrow (BM) were collected. Then, the organs subjected to bacterial counts, histopathological examination, and cells analyzed by flow cytometry, as described elsewhere [15, 16]. Experimentation in mice was conducted following the guidelines and consent of the "Comité Institucional para el Cuido y Uso de los Animales de la Universidad de Costa Rica" (CICUA-47-12) and in accordance with the corresponding Animal Welfare Law of Costa Rica (Law 9458). All animals were kept in cages with food and water *ad libitum* under biosafety containment conditions.

BM cells were also isolated and infected ex vivo in the presence of anti-*Brucella* antibodies, following previous protocols [16]. Briefly, BM cells were isolated from the tibia and femur of *B. abortus*-RFP-infected mice at 8 and 30 days of postinfection by flushing bones with HBSS (no calcium, no magnesium) or RPMI medium. BM cells were then incubated with *B. abortus*-RFP at MOI of 50 bacteria/cell at 37°C for 2 hours, washed with PBS, suspended in HBSS, and subjected to examination. The number of CFUs infecting enriched BM-derived PMNs was estimated by lysing the cells and counting bacteria in agar plates [17].

2.2. Immunofluorescence. BM cells (50 to 100 μl resuspended in DMEM at a concentration of 10^6 cells/ml) were loaded on alcian blue-coated coverslips (Sigma) and incubated for 20 min at 37°C to allow cell attachment. Twenty minutes Antigenfix (Diapath) was used for fixation. Once fixed onto coverslips, cells were washed with PBS and slides were mounted using ProLong Gold Antifade reagent containing DAPI (Thermo Fisher Scientific). Slides were observed with confocal microscope (Leica TCS SP8) as described before [18]. Image analyses were performed using the ZEN 2011 software.

2.3. Histopathology. For histopathological studies, the spleen, lymph nodes, and BM from infected and PBS-treated mice were fixed in 10% neutral buffered formalin, processed and stained with hematoxylin and eosin or Giemsa stain [19]. The histopathological score (from 0 (negative) to 4 (severe)) was determined by semiquantitative analysis as previously described [20–22].

2.4. Flow Cytometry. For flow cytometric analyses, cell surface markers were stained using the following antibodies: BV421 anti-CD11b (M170), BV711 anti-Ly6G (1A8), BV785 anti-F4/80 (BM8), and BV570 anti-CD4 (RM4–5) antibodies were purchased from BioLegend; AF647 anti-CD34 (RAM34), BV711 anti-CD8α (53-6.7), and BV650 anti-CD3 (245-2CII) from BD Biosciences; and Alexa Fluor 488 and APC both anti-CD115 (AFS98), PE anti- Ly6G (1A8), Ef450 anti-CD45R/B220 (RA3-6B2), PE Cy7 anti-CD19 (1D3), and AF700 anti-CD44 (1M7) antibodies from eBiosciences; and APC Cy7 anti-CD16/32 (2.4G2), BV510 anti-Sca-1 (D7), and BV605 CD117/c-kit (2B8) from BD Biosciences. An antibody staining scheme is provided in Table S1. Cells were identified according to the staining scheme and the percentage of each cell type determined in relation to all living cells of bone marrow at 8 and 30 days of postinfection. Cell viability was evaluated using Fixable Viability Dye UV (eBiosciences). Cells were fixed with Antigenfix for 20 min before the acquisition. Multiparameter flow cytometry was performed using a FACS LSRII UV (BD Biosciences) or Guava easyCyte (Millipore). Flow cytometry data were analyzed using the FlowJo software, version 10.0.7 (Tree Star Inc.).

2.5. Statistics. One-way analysis of variance (ANOVA) followed by Dunnett's test or multivariate analysis of variance (MANOVA) was used to determine statistical significance in the different assays. The JMP (https://www.jmp.com) and GraphPad Prism software (https://www.graphpad.com) were used for statistical analysis. Data were processed in Microsoft Office Excel 2015.

3. Results

According to bacterial loads, histopathological alterations, and immune response, murine brucellosis has been divided into four stages: onset of infection, acute phase, chronic phase, and chronic declining phase [23] (Figure 1(a)). After infection, *B. abortus* CFU counting was performed from the spleen, lymph nodes, and BM during the lapse of 168 days of postinfection (Figure 1(a)). Bacterial loads and kinetic profiles of the spleen and lymph nodes were similar. A significant bacterial increase was observed in the lymph nodes and spleen at the chronic steady phase III (28 days of postinfection), followed by a decrease in the bacterial numbers at the chronic declining phase IV and until the end of the experimentation. In the BM, *B. abortus* infection steadily

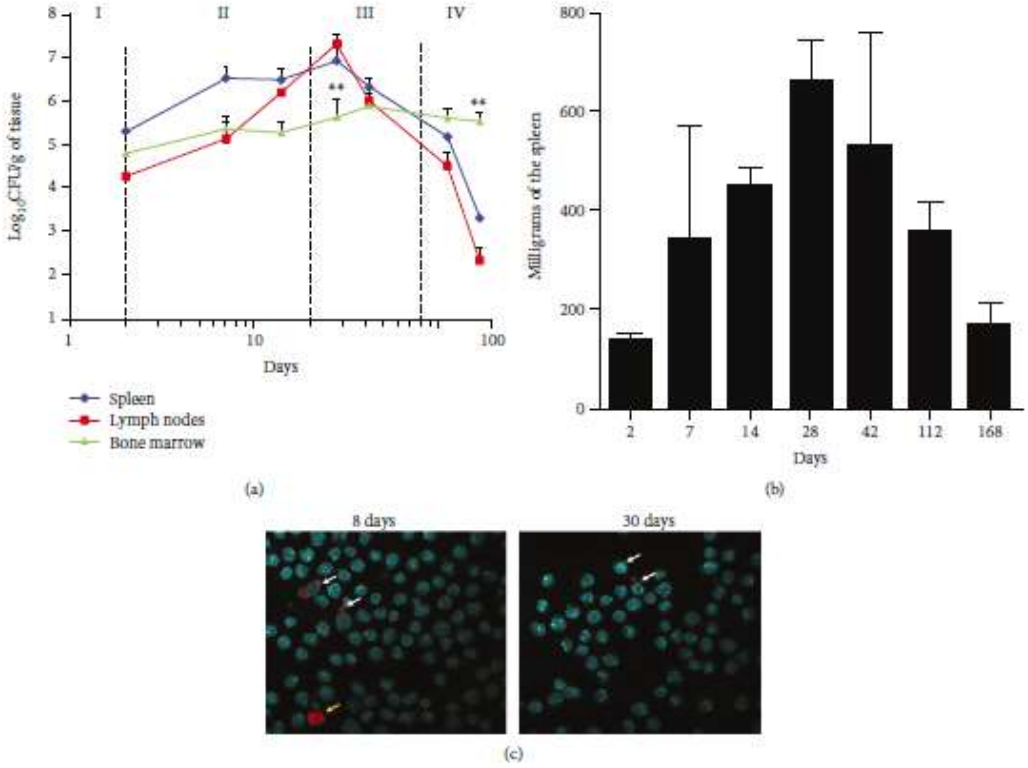

FIGURE 1: *B. abortus* persists in bone marrow during the course of infection. Mice were infected with *B. abortus*-RFP. (a) Spleen, lymph nodes, and BM were collected, and CFUs determined at different phases of infection [23]: the onset of infection (I), the acute phase (II), the chronic steady phase (III), and the chronic declining phase (IV). Each bar is the mean (±1 SD) of an experiment. Values of $^{**}p < 0.01$ are indicated in relation to spleen and lymph node bacterial loads. (b) Before CFU determination, the spleens were weighted at each time of examination. (c) BM cells were isolated from the tibia and femur of *B. abortus*-RFP- (red intracellular bacteria) infected mice at 8 and 30 days of postinfection mounted using ProLong Gold containing DAPI (blue nuclei). Microscope images are captured at 60x magnification confocal microscope.

persisted throughout all four phases, until day 168, when the CFU/g loads were significantly higher than those of lymph nodes and spleen. Similar results were obtained with C57BL/6 mice (not shown). The weight of the spleens increased until day 28 and then decreased until the end of the experiment, following a pattern similar to that of the kinetics of the CFU count (Figure 1(b)). Even though the number of CFU/g of BM was relatively high, the absolute numbers of *B. abortus* BM-infected cells were low at 8 (acute phase) and 30 (chronic phase) days of postinfection, suggesting that few infected cells harbored many bacteria (Figure 1(c)). However, a high number of bacteria was observed in some cells, a phenomenon that may account for the discrepancy between the CFU/g and the number of infected cells.

It has been demonstrated that most *Brucella*-infected human patients display histopathological alterations, whether or not the bacterium is isolated from the BM [4]. As shown in Figure 2(a), granulomatous inflammation was more severe and diffuse at acute stages than the multifocal chronic phase in the BM, spleen, and liver. At the acute phase, the inflammatory process was characterized by coalescing to diffuse inflammation with larger and cell-rich granulomas, while in the chronic phase, granulomatous inflammation was multifocal with smaller and fewer cellular lymphohistiocytic aggregates. Epithelioid macrophages predominate during the inflammatory process at early stages, reducing in number with chronicity. Classical granuloma formation was observed more clearly in the spleen and liver, while bone marrow developed an epithelioid macrophage-rich aggregate with scattered lymphocytes, which reduced its size and cellularity over time. Compared to the spleen, bone marrow granulomatous inflammation was more severe in the first two weeks of infection. After four weeks of infection, the spleen and bone marrow presented similar inflammation scores, though the granulomatous inflammation decreased in both tissues afterward (Figure 2(b)).

The cellular changes in the BM of infected mice were estimated by flow cytometry. At 8 days of postinfection, we observed changes in the hematopoietic cell population. At day 8 of postinfection, the percentage megakaryocyte-

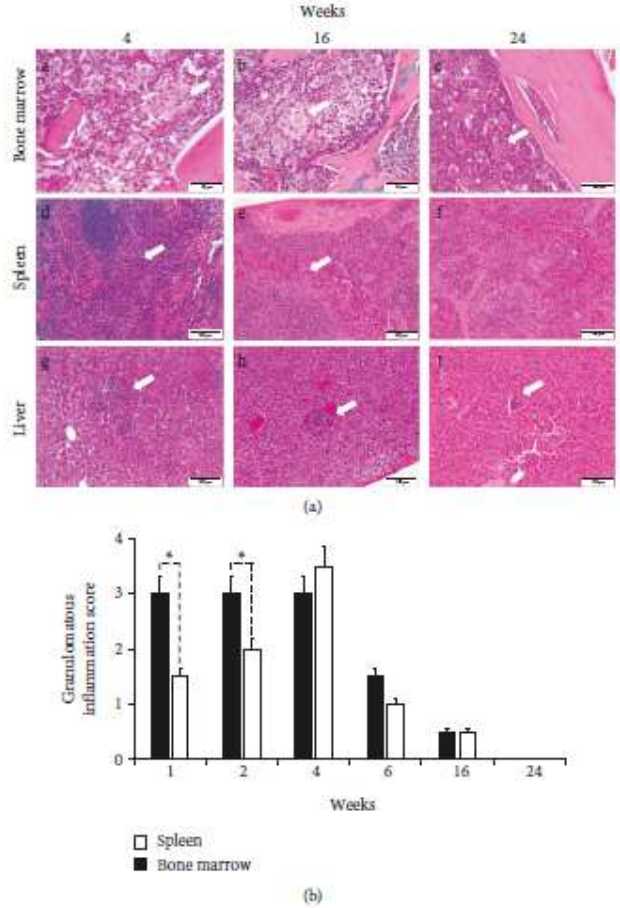

FIGURE 2: *Brucella abortus* induces a granulomatous inflammation in bone marrow. (a) Mice were infected with *B. abortus*-RFP. The spleen, liver, and BM were collected at different phases of infection and subjected to histopathological examination. (b) Granulomatous inflammation was scored from 0 (negative) to 4 (severe) [19] in BM over time. Each bar is the mean (±1 SD) of an experiment. Value of $^*p < 0.05$ is indicated in relation to BM and spleen granulomatous inflammation.

erythrocyte progenitor (MEP) decreased compared to the BM of noninfected mice (data not shown). Contrarily, the percentage of granulocyte-monocyte progenitors (GMP) significantly increased. Likewise, neutrophils (PMN) and $CD4^+$ lymphocyte populations significantly increased at 8 days of postinfection (Figure 3(a)). The increase of $CD8^+$ cells was evident, but not significant ($p < 0.05$).

In order to estimate the proficiency of BM cells to internalize *B. abortus*, we performed an ex vivo infection. For this, BM cells were infected with *B. abortus*-RFP in the presence of anti-*Brucella* antibodies. As shown in Figure 3(b), close to 32% of the BM cells were infected; of these, over 90% were identified as PMNs [16].

Flow cytometry analysis of BM from infected mice rendered three main cell types containing *B. abortus*: monocytes, PMNs, and GMPs (Figure 3(c)). At 8 days of postinfection, the proportion of PMN-containing bacteria was greater than other cells. Strikingly, the number of infected PMNs dramatically decreased after 30 days. The proportion of infected monocytes remained similar at 8 and 30 days of postinfection. Although at early stages of infection close to 3% of the GMP-contained bacteria, the number of infected cells practically disappeared at later times (Figure 3(c)).

4. Discussion

At initial stages of infection, *Brucella* invades target organs, before a strong activation of the innate immune system and stimulation of antimicrobial mechanisms [7, 24]. This immunological gap allows the bacterium to colonize, replicate, and hide within cells of the mononuclear phagocyte system. Linked to this is the observation that *B. abortus*

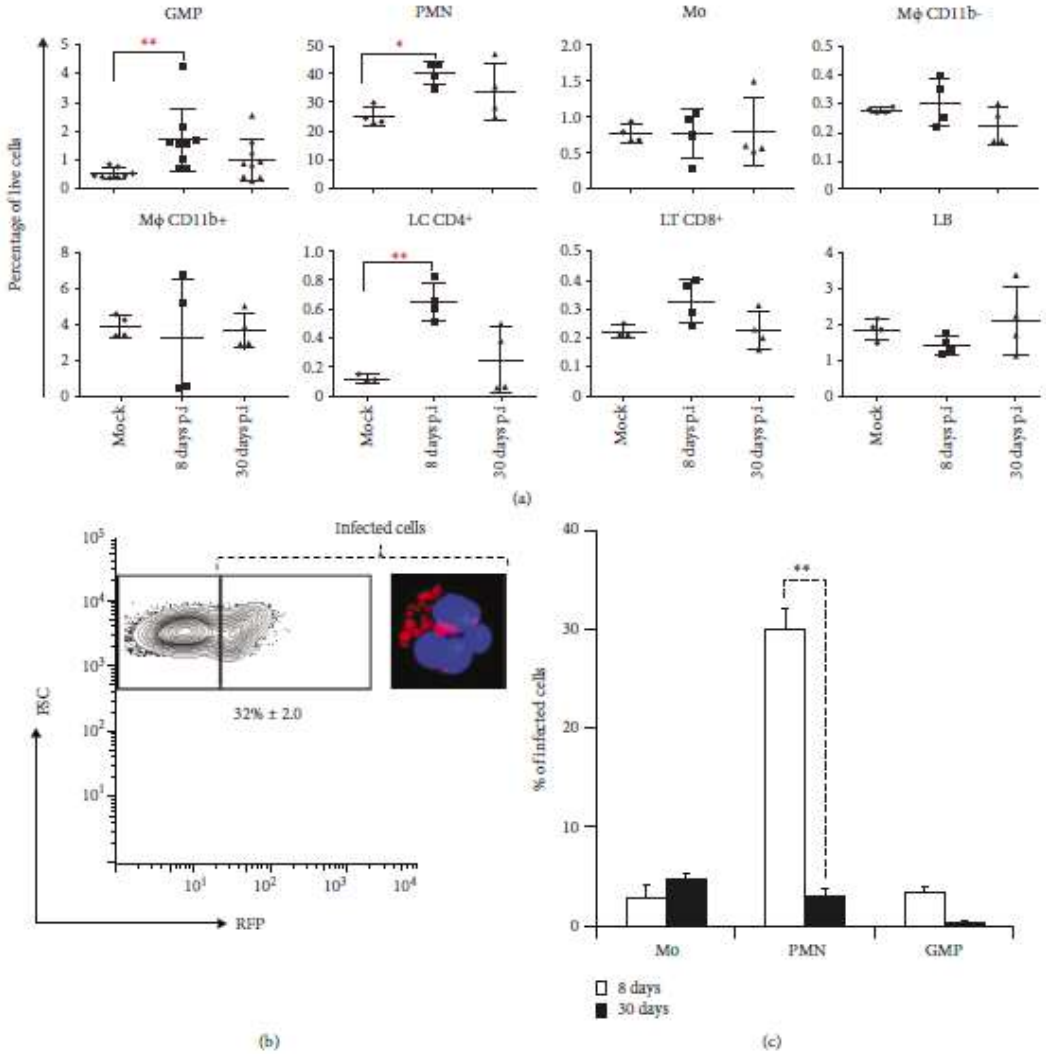

FIGURE 3: Bone marrow leukocyte variation at different stages of infection. (a) BM cells from *B. abortus*-infected mice were collected and subjected to multiparameter flow cytometry analysis. Cells were identified according to the staining scheme (Table S1) and the percentage of each cell type determined in relation to all living cells of bone marrow at 8 and 30 days of postinfection. Values of $^{*}p < 0.05$ or $^{**}p < 0.01$ are indicated in relation to control noninfected mice at 8 and 30 days of postinfection. (b) Whole BM cells were collected and infected ex vivo with *B. abortus*-RFP. Infected cells were gated based on the RFP (red) positivity, and the total percentages of infected cells were quantified. (c) BM cells from *B. abortus*-RFP-infected mice were collected and subjected to multiparameter flow cytometry analysis. Infected cells were gated based on the RFP (red) positivity, identified and quantified according to the staining scheme (Table S1) at 8 a 30 days of postinfection. Each bar is the mean (±1 SD) of an experiment. Values of $^{**}p < 0.01$ are indicated.

infection remains sequestered within BM cells for a protracted period, without significant changes in the bacterial loads. These phenomena propose a mechanism for *Brucella* persistence.

Granuloma formation, commonly observed in long-lasting infections, is an attempt to eliminate the microorganisms [25, 26]. In tuberculosis, it has been proposed that granulomas provide a bacterial safety shelter from the host immune response [27]. The higher number of granulomas in the BM and the permanence of these structures indicate the struggle of immune cells for eliminating *B. abortus*. This is also depicted by the significantly higher number of $CD4^{+}$ lymphocytes in the BM at early stages of infection, which in brucellosis correlates with Th1 polarization [28].

The most abundant infected BM cells at the acute phase of murine infection (once antibodies against *Brucella* have developed) were PMNs. This result is reminiscent of the ex vivo infection of BM cells. Indeed, we have demonstrated that a large proportion of *ex vivo B. abortus*-infected BM cells are PMNs and that these leukocytes are unable to kill the ingested bacteria [16]. Despite this, it is unlikely that PMNs are the main reservoirs for *Brucella* in the BM. Indeed, *B. abortus* does not replicate in these cells and these infected leukocytes died prematurely [16]. Rather, PMNs may serve as vehicles for dispersing the bacterium, functioning as Trojan horses, as previously proposed [16, 18].

A small proportion of GMP cells in the BM were also infected at the acute phase of infection. This is unexpected since uncommitted progenitors such us GMP cells are not yet considered phagocytic cells [29]. However, at later time points, the proportion of infected cells was negligible. Moreover, the total number of GMP cells increased at early times of infection, diminishing afterward. These cellular variations correlate with the pathological changes of the BM. A similar phenomenon has been observed in human brucellosis cases [4, 30].

To our knowledge, this is the first time that myeloid oligopotent progenitor stem cells, lacking a developed phagocytic machinery, have been shown to become infected with *Brucella* organisms. Even though it is common to observe extramedullary hematopoiesis in the spleen of *Brucella*-infected mice [23], here we demonstrate for the first time *Brucella*-infected hematopoietic oligopotent stem cells residing in the BM. During emergency myelopoiesis, self-renewing GMPs in patches (pGMPs) build GMP clusters and differentiate into clustering GMPs (cGMPs). These GMP clusters can differentiate into mature cells until complete disappearance of the GMP clusters [31, 32]. Moreover, it has been shown that the increasing number of myeloid progenitors can promote microbial persistence in the organism [33]. All these findings make us speculate that *B. abortus* infects myeloid oligopotent progenitor stem cells and may interfere to induce GMP differentiation into infected-differentiated cells. Whether the reduced number of infected nonphagocytic erythrocytes and B cells [34, 35] originates from BM-infected progenitor cells remains unknown.

Despite the histopathological changes of the *B. abortus*-infected BM, and the low numbers of infected monocytes, the proportion of these leukocytes remained constant and persistent. It is well known that during granuloma formation, monocytes differentiate into macrophages, epithelioid cells, and dendritic Langerhans-type giant cells [36]. Moreover, *Brucella* is able to survive in monocytes and inhibits their programmed cell death [8]. Join-Lambert et al. [37] showed that *Listeria monocytogene*-infected myeloid cells in the bone marrow play a crucial role in the pathophysiology of meningoencephalitis by releasing infected cells into the circulation. Therefore, BM monocytes are firm candidates for *Brucella* reservoirs in the BM. These cells may be the source of the frequent relapses observed in antibiotic-treated individuals, even several years after the primo infection [38, 39].

5. Conclusions

Bacterial persistence, chronicity, and relapses are major problems in brucellosis. Within this context, we concluded (i) that loads of *B. abortus* in the BM remain constant and are long lasting; (ii) that *B. abortus*-infected BM displays histopathological modifications associated with augmented numbers of multipotent progenitor and active hematopoietic stem cells, PMNs, and $CD4^+$ lymphocytes during the acute phase of the infection; and (iii) that the three types of infected cells in the BM are monocytes, PMNs, and GMP cells. In addition, we hypothesize that (iv) BM PMNs may serve as vehicles for dispersion of *Brucella*, following the Trojan horse hypothesis; (v) that *B. abortus*-infected myeloid oligopotent progenitor cells may differentiate into mature infected cells; and (vi) that monocytes are the most likely *Brucella* reservoirs in the BM and that these cells may be the source of the frequent relapses observed in antibiotic-treated individuals.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or nonfinancial interest in the subject matter or materials discussed in this manuscript.

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Supplementary Materials

Table S1: antibody staining scheme used for identifying each cell population. *(Supplementary Materials)*

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Manuscript 3: *Brucella* Omp25 elicits myeloid commitment of hematopoietic stem cells through SLAMF1 (Hysenaj et al., 2019, *in preparation*)

Brucella Omp25 elicits myeloid commitment of hematopoietic stem cells through SLAMF1

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Brucellosis is the world's most spread zoonosis with an incidence of 500,000 new cases per year. If not treated, human patients develop a chronic disease affecting several organs, including spleen, liver, bone marrow, and even die (Ertem, Kürekçi et al. 2000, Martirosyan, Moreno et al. 2011). We recently showed that *Brucella abortus* persists in murine bone marrow (BM) (Gutierrez-Jimenez, Hysenaj et al. 2018). BM specialized microenvironment provides with factors that regulate and support hematopoiesis (Li and Xie 2005). HSCs harbors self-renewal abilities and differentiate to replenish circulating blood cells (Fantuzzi and Faggioni 2000). HSCs express cytokine receptors and pathogen recognition receptors (PRRs) and as such can respond to infection (Boettcher and Manz 2017). HSCs as mature hematopoietic cells also express the SLAMF1 (CD150) receptor at their surface (Oguro, Ding et al. 2013). SLAMF1 contributes to immune modulation, B cell development, cell motility and bacterial sensing (Sidorenko and Clark 1993, Berger, Romero et al. 2010, Veillette 2010, Yurchenko, Shlapatska et al. 2011, van Driel, Liao et al. 2016). The role of SLAMF1 in HSC function along microbial challenge has not been explored yet. Here, we show for the first time that HSCs recognize the *B. abortus* outer membrane protein 25 (Omp25) through the SLAMF1 receptor and that HSCs can be considered as part of the innate immunity arsenal.

We inoculated intraperitoneally (i.p.) C57BL/6J *wild-type* (*wt*) mice with $1x10^6$ colony forming units (CFUs) of wild-type *B. abortus* . At 2, 8 and 30 days post-infection (p.i.) spleen, and bone marrow from tibias and femurs of each mouse were isolated and presence of bacteria was assessed by CFU assay. Figure 1a shows the presence of bacteria in spleen and bone marrow as long as 30 days post-infection. To evaluate if bacteria in the BM arestill virulent, we transplanted BM cells of infected mice to a new recipient mice (Figure 1b). At 8 weeks post-transplantation we could still count *B. abortus* CFUs in the BM and spleen of transplanted recipients. As such, *Brucella* keep being virulent in the BM. (Figure 1c).

Thereafter, we analysed by flow cytometry the distribution of BM hematopoietic progenitors and stem cell compartment (HSPC) upon infection. The absolute numbers of total BM cells or lineage negative progenitors (Lin-) were not affected by *B. abortus* infection (Supplementary 1a and 1b). In contrast, *Brucella* infection induced major changes in the HSPC compartment. The expansion of lin^-, Sca^+, $c\text{-}kit^+$ (LSK) progenitors noted at the onset of infection (Day 2 p.i.) (Figure 1d and 1e) was more important during acute infection (Day 8 p.i.). LSK expansion was

mainly due to the significant increase of multipotent progenitors (MPP) (KSL CD48^{+}) and to lesser extent to the increase of HSPCs (KSL, CD34^{-}, Flt3^{-}, CD48^{-}, SLAMF1^{+}). The long term hematopoietic stem cell (HSC^{LT}) (KSL, CD34-, Flt3-, CD48-, SLAMF1^{+}) slightly decreased. Overall, these data indicated that the presence of *Brucella* in the BM perturbs HSPC homeostasis. Indeed, other Gram negative bacteria such as *Escherichia Coli* or *Salmonella* Typhimurium also trigger HSPCs expansion that is associated with enhanced myeloid commitment. Myeloid commitment of HSCs is marked by the upregulation of PU.1 expression in HSCs (Pronk, Veiby et al. 2011, Takizawa, Boettcher et al. 2012, Mossadegh-Keller, Sarrazin et al. 2013, Pietras 2017). To test if *Brucella* infection induces myeloid skewing, we infected PU.1-KIGFP mice and analysed PU.1 expression in BM HSCs at Day 2 post infection. Thirty to forty percent of HSCs (KSL, CD34-, Flt3-, CD48-) upregulated PU.1. testifying that HSCs response to *Brucella* infection instructs PU.1 upregulation in BM HSCs (Figure 2a).
Brucella abortus persist in the BM (Gutierrez-Jimenez, Hysenaj et al. 2018). In addition, SLAMF1, the surface receptor of HSCs binds to the outer-membrane protein of *B. abortus* Omp25 (Degos *et al, submitted*) and restricts DC maturation. We therefore wondered if HSCs could also recognize *Brucella* outer membrane protein Omp25 via SLAMF1. For this, *wt* and *Slamf1*$^{-/-}$ mice were infected i.p. with *B. abortus* WT. *Slamf1*$^{-/-}$ infected mice displayed a similar infection rate as *wt* infected mice (Supplementary 2). Nevertheless, *Slamf1*$^{-/-}$ mice presented lower perturbations in the HSPC compartment compared to wild type infected mice (Supplementary 4b) suggesting that SLAMF1/Omp25 axis is responsible for HSPCs alterations but not for *Brucella* survival and replication in the host.

To test if SLAMF1/Omp25 axis is involved in PU.1 upregulation in HSCs during *Brucella* infection, we generated a new mouse line that expresses PU.1-GFP and lacks SLAMF1(*Slamf1*$^{-/-}$ PU.1 KIGFP). Strikingly, *B. abortus* infection of *Slamf1*$^{-/-}$ PU.1 KIGFP did not trigger upregulation of PU.1 in BM HSCs (Figure 2b) proving that SLAMF1/Omp25 axis elicits upregulation of PU.1 in HSCs. Thereafter, we analysed BM MPPs at D8 p.i. . An increase of myeloid biased MPPs (called MPP2-3 and defined as Lin-, Sca+, cKit+, CD48+, Flt3-) and GMPs was observed during infection. As expected, the increase in myeloid biased/committed progenitor cells was not observed in mice infected with *B. abortus Δomp25* or mice lacking SLAMF1. *wt* mice infected with *B. abortus* Δ*omp25c* (*B. abortus* Δ*omp25* complemented with pOmp25) showed similar results with *wt* mice infected with *B. abortus* WT evincing once again a direct role of Omp25 in controlling the increase of myeloid commitment (Figure 3a-c).

Remarkably, the increase of the myeloid ratio in the blood was observed only during acute phase of infection. At chronic phase of infection (Day 30 p.i. , Day 360 p.i.) the myeloid ratio returned to the same levels as in control mice (Figure 3d) arguing that SLAMF1/Omp25-dependent myeloid commitment is transient.

Direct pathogen sensing by HSCs has been reported to cause HSC dysfunction (Boettcher, Ziegler et al. 2012, Kobayashi, Kobayashi et al. 2015). To test HSPC function in response to *B. abortus* infection, we performed a competitive transplantation experiment. BM Lin- cells from *wt* or *Slamf1*$^{-/-}$ mice at D8 p.i. were mixed with CD45-2 BM lin^{-} cells from non infected mice and were transplanted into lethally irradiated CD45.2 recipients (Figure 3e). Lineage output and engraftment capacity were analysed at 4, 6, 8 weeks after transplantation. Engraftment capacity of cells arising from infected mice was akin to the control (Supplementary 3b) suggesting that *B. abortus* acute infection did not affect HSC self-renewal capacity. Conversely, the ratio between myeloid and lymphoid cells in the blood generated by HSPCs from *B. abortus* WT infected mice was higher than that of HSPCs from control mice (Figure 3f and supplementary 3d-f). Conversely, the ratio between myeloid and lymphoid lineage of blood hematopoietic cells arised from HSCs originating from *B. abortus* Δomp25 *wt* infected mice or from *Slamf1*$^{-/-}$ mice was comparable with blood myeloid ratio of control mice (Figure 3f). These findings attest that *Brucella* provokes myeloid skewing during acute infection without modifying HSC fitness.

To notice, *Slamf1*$^{-/-}$ mice present a different immune response compared to *wt* mice (Davidson, Shi et al. 2004). As such, to determine whether the phenotype observed in HSCs from infected *Slamf1*$^{-/-}$ mice was caused by the absence of Omp25 recognition and not to the *Slamf1*$^{-/-}$ environment, we generated hematopoietic chimeras (1:1) from BM cells of WT CD45-2 or *Slamf1*$^{-/-}$ CD45-1 mice (Figure 3i). The (wt: *Slamf1*$^{-/-}$) BM chimeras were then infected with *B. abortus* i.p. At day 8 p.i. the myeloid ratio of hematopoietic blood cells was higher in the *wt* compartment compared to the *Slamf1*$^{-/-}$ compartment in mice infected with *B. abortus* WT. Moreover, the generation of myeloid and lymphoid lineages in the blood was similar for *wt* and *Slamf1*$^{-/-}$ compartment of non-infected or *B. abortus Δomp25* infected BM chimeric mice (Figure 3j). In line with blood analyses, the percentage of GMP and MPP2-3 was higher in BM WT compartment compared to the *Slamf1*$^{-/-}$ compartment. The percentage of GMP and MPP2-3 was comparable in WT and *Slamf1*$^{-/-}$ compartments from non-infected or *B. abortus Δomp25* infected mice. To conclude, these data demonstrate that HSC myeloid commitment instructed *via* SLAMF1/Omp25 direct interaction is hematopoietic cell autonomous (Figure 3d).

To further asses if HSCs directly sense *B. abortus* Omp25, we sorted HSCs from PU.1 KIGFP mice and stimulated them for 16hwith *Brucella* outer membrane vesicles (OMV) (Figure 2b). OMVs are small vesicles generated from OM of the bacterium during stress conditions (Volgers, Savelkoul et al. 2018). One of the major proteins composing OMVs is Omp25.(Boigegrain, Salhi et al. 2004). HSCs stimulation with *B. abortus* WT OMVs induced PU.1 upregulation in HSCs (Figure 2c, 2e). Treatment with SLAMF1 blocking antibody abrogated the effect of *B. abortus* OMVs stimulation(Figure 2d).. Similar results were obtained when *Slamf1*$^{-/-}$ HSCs were stimulated with *B. abortus* WT or when *wt* HSCs were stimulated with *B. abortus ⊿omp25* OMVs demonstrating that the increase of PU.1^{+} HSC during *B. abortus* infection is due to downstream signalling triggered by Omp25 binding to HSCs.

To ensure, HSCs that direct stimulation of HSCs by *Brucella* provoke HSCs myeloid commitment *in vivo*, HSCs (KSL, CD48-, CD34-, Flt3-) from WT CD45-1 and *Slamf1*$^{-/-}$ CD45-1 mice were stimulated with *Brucella* for 30 min. Cells were then washed with gentamycin to kill extracellular bacteria and transplanted into *wt* lethally irradiated recipients (Figure 3g). Blood analysis at 4 weeks post-transplantation showed that HSCs stimulated with *B. abortus* WT generated more myeloid than lymphoid cells in the blood, compared to PBS-treated HSCs. (Figure 3h). Also, the ratio between myeloid and lymphoid blood cells generated by HSCs stimulated with *B. abortus ⊿omp25* and *Slamf1*$^{-/-}$ HSC stimulated with *B. abortus* WT was akine to the myeloid ratio generated by non-infected HSCs. Surprisingly, the increase of myeloid ratio in the blood generated by *B. abortus* WT mice was transient and was not observed at 6- and 8-weeks post-transplantation. (Figure 3h). Collectively, these results suggest that the SLAMF1/Omp25 heterophilic binding directly triggers HSC PU.1 upregulation in HSCs and they myeloid commitment. PU.1 is a pioneer transcription factor known as a myeloid master regulator. PU.1 is upregulated by several cytokines such as IL-1β, MCSF, TNFα and also by Toll like receptor activation (Mossadegh-Keller, Sarrazin et al. 2013, Pietras, Mirantes-Barbeito et al. 2016, Tyrkalska, Perez-Oliva et al. 2019, Yamashita and Passegue 2019). Therefore, it might be possible that SLAMF1/Omp25 downstream signalling shares the same intracellular partners TLR or inflammasome pathways. Further studies are required to clarify intracellular Omp25 induced SLAMF1 downstream signalling that triggers myeloid skewing.

Enhanced myeloid commitment is often associated with reduced red blood cell generation (Bi, Li et al. 2016). To investigate, if this is the case for murine brucellosis we infected *wt* mice i.p. with *B. abortus* WT (Figure 4c) and measured blood hematocrit. The level of hematocrit in the blood of infected mice was reduced compared to non-infected mice (Figure 4d). Moreover, the

decrease of hematocrit levels was present only along acute and not chronic infection (Figure 4d) suggesting that SLAMF1/Omp25 enhanced myeloid commitment is responsible of anemia. To determine if human brucellosis resembles to features we analysed data from 302 human patients. First both human (70 to 75 %) (Figure 4a) and mice (100%) (Figure 4e) suffered presented splenomegaly. Moreover, blood analyses showed that 31.8% of males and 25% of females were diagnosed like mice with anemia associated with a decrease of red blood cells, hematocrit and haemoglobin (Figure 4a and 4b). Hematological abnormalities observed in mice suggest that *Brucella* alters also HSC function in humans.

SLAMF1 does not seem to be expressed in human progenitor and stem hematopoietic cells. (Sintes, Cuenca et al. 2013). Indeed, human-induced pluripotent stem cells (hiPSCs) express SLAMF1(Naaman, Rabinski et al. 2018). Hence, it is not excluded that a minority of human HSCs express SLAMF1. Moreover, other SLAM family receptors that are expressed in human HSPCs can bind to microbial compounds. For instance, SLAMF2 binds FimH, a lectin from *E. coli* (Baorto et al., 1997; Cannons et al., 2011; Malaviya et al., 1999). Since SLAM Family receptors share the same intracellular partners (Veillette 2004), it is not excluded that Omp25 binds to SLAMF2 and induces MPP expansion and enhance myeloid commitment. More studies will be needed to determine whether SLAM members exhibit alterations in human brucellosis. However, brucellosis patients excepting those who develop endocardiditis do not die signifying that even in humans anemia is transient.

In most infectious cases, the enhanced myeloid commitment seems to promote pathogen clearance (Takizawa, Boettcher et al. 2012). Nevertheless for some pathogens HSPCs expansion is detrimental for the host and benefits the pathogen (Abidin, Hammami et al. 2017). For instance, *Leishmania donovani* parasite burden is decreased in Fzd6-/- mice compared to *wt* mice. Frizzled 6 (Fzd6) is a receptor of Wnt pathways. Absence of Fzd6 in mice results on defective HSC self-renewal capacity, and also dampens HSPC expansion upon LPS stimulation (Abidin et al 2015). In the case of murine brucellosis, 4 weeks post infection bacterial load in the spleen of *B. abortus* Δomp25 or *Slamf1*$^{-/-}$ infected mice decreases compared to WT infected mice (Supplementary 5) arguing that the enhanced transient myeloid commitment induced by SLAMF1/Omp25 benefits to the bacterium. Indeed, *Brucella* infects and replicates in myeloid cells (Salcedo, Marchesini et al. 2013, Gutierrez-Jimenez, Hysenaj et al. 2018).

In conclusion we show for the first time that *Brucella* manipulates HSCs in the BM to produce more myeloid cells through SLAMF1/Omp25 binding, to establish chronic infection. This mechanism is responsible of haematological abnormalities reported in humans.

MATERIALS AND METHODS

Mice

WT C57BL/6 mice were purchased from Janvier (France); C57BL/6 *Slamf1*$^{-/-}$ mice were provided by Yusuke Yanagi (Davidson, Shi et al. 2004). Animal experimentation was conducted in strict accordance with French and European guidelines relative (Authorization CE14 n°2017110214133256).

Bacterial strains

Brucella abortus 2308, *Brucella abortus Δomp25* (kan^R), *Brucella abortus Δomp22*(kan^R) or *Brucella abortus Δomp25c* (kan^R, Amp^R) were used for infection. *B.* abortus Δomp25 and *B. abortus Δomp22* were a gift from Ignacio Moriyón lab and have been described previously (Manterola, Guzman-Verri et al. 2007).

*B. abortus Δomp25:*pomp25 (Ba Δomp25c) was constructed by amplifying omp25 gene from a heat shock killed *B. abortus* by PCR using primers (5'-3'CCCGAATGCGCACTCTTAAGTCTCTCG) and (5'-3' CCCGGATCCTTAGAACTTGTAGCCG) (Invitrogene). The PCR product was inserted by the flanking EcoR1/BMHII sites (underlined) in the corresponding sites of pBBR1 MCS-4. (Kovach and al,1995). The ligation product was transformed into *Escherichia coli* DH5α. The integrity of the construct was confirmed by sequence analysis. The pBBR-MCS-4 *omp25* plasmid was then transformed into *E. coli* S17ʎpir. *E.coli* S17ʎpir. pBMR-MCS4 *omp25* and *B. abortus Δomp25* were culture in Trypic Soy Broth (TSB) for 4h at 37°C and 16h at room temperature (RT) for biparental mating.

B. abortus Δomp25c were selected after culturing in Trypic Soy Agar (TSA) plates supplemented with kanamycin (50µg/ml), ampicillin (50µg/ml) and nalidixic acid (50µg/ml). All experiments with *Brucella* were carried out in BSL-3 facility.

Brucella infection

6-8 weeks old female mice were inoculated intraperitoneally (I.P) with 10^6 CFU of *Brucella abortus* 2308, *Brucella abortus Δomp25* (kan^R), *B. abortus Δomp22* (kanamycin resistant) or with *B. abortus Δomp25c* (kan^R and Amp^R).

Strains were grown in Tryptic Soy Agar for 5 days then, overnight at 37°C for 16h under shaking in Tryptic Soy Broth (Sigma Aldrich) with kanamycin for Δomp25 until the OD (OD at 600nm) reached 1.8

For Colonie Forming Units (CFU) enumaration analysis, at different phases of infection, spleen and bone marrow were collected (Guitierrez-Jimenez et al,2018). Femur and tibia were flushed with 500ml of ice-cold PBS 0.1%Triton and then plated in appropriate dilution on TSA plates. Spleens were collected and splenocyte were isolated by mechanical disruption. Serial dilutions of infected tissues were plated on trypticase soy agar, incubated at 37°C for 72 hours in presence of 5% CO_2. CFU were enumerated 3-4 days after.

Transplantation assay

All donor cells are from WT and *Slamf1*$^{-/-}$ CD45.1 mice and transplanted into lethally irradiated (5,9 Gy) CD45.2 recipient mice.

For competitive assay, mice were transplanted with equal numbers of $1x10^6$ total BM cells or $1x10^6$ lineage negative cells.

For infected HSC transplantation, 1000 Sorted LT-HSC (KSL, CD48-, CD135-, CD34-) were infected with *B. abortus* WT or B.abortus *B. abortus Δomp25* (MOI) of 30:1. Bacteria were centrifuged onto cells at 400 g for 10 min at 15 ºC and then incubated for 45 min at 37 ºC with 5% CO_2. Cells were washed twice with medium and then incubated for 1 h in medium containing 100 µg/ml gentamicin (Sigma Aldrich) to kill extracellular bacteria. Cells were than washed 3 times with PBS. Infected cells were mixed in a ratio (2:1) with non-infected HSCs before transplantation.

Hematopoietic reconstitution and lineage determination were monitored over time in the peripheral blood. At terminal work-up, BM from chimeric mice was harvested and analyzed.

Flow Cytometry

For FACS sorting and analysis we used respectively FACSAriaIII and LSR-X20(BD) equipment and FlowJo software v10 (Treestar). For HSCs and progenitor analysis, total bone marrow cells were depleted of mature cells using direct lineage depletion kit (Miltenyi Biotec) and stain with antibodies anti- CD34-APC or -BV421 (cloneRAM34), anti-CD135-PE-CF594 or -PE (clone A2F10.1), anti-SLAMF1-PE-Cy7 or –BV711 (clone TC15-12F12.2), anti-CD117-BV605 (clone 2B8), anti-Sca-1-PrcpCy5.5 OR -PE (clone D7), anti-CD48-BV510 or –PE-Cy7 (clone HM48-1), anti-ESAM-APC (clone1G8/esam) and anti-CD16/32-PE or –APC-

Cy7 (clone 2.4G2). When needed, anti-CD45.1-APC or –BV421 and anti-CD45.2-FITC or PrcpCy5.5 were added. LIVE/DEAD was used as viability marker.

Blood cells were stained with anti-CD11b FITC (clone M1/70), anti-CD19-PE-Cy7 (clone 1D3), anti-CD45.2-PrcpCy5.5 (clone 104), anti-CD45.1-BV421 (clone A20), anti CD3e-APC (Clone 145-2C11) and anti Ly6G-PE (clone 1A8). Red blood cells were lysed using BD FACS lysing solution (BD) for 10 minutes then fixed for 20 minutes with Antigen Fix, prior to the acquisition.

Hematopietic Stem Cells *ex vivo* challenge with *Brucella abortus* membrane extracts

All cultures were performed at 37°C in a 5% CO2. Sorted hematopoietic stem cells (HSC) from wt or *Slamf1*$^{-/-}$ mice were cultivated in StemSpan SFEMII complemented with 50ng/µL TPO (Peprotech) and 20ng/µL SCF (Peprotech). Cells were stimulated with *Brucella* membrane extracts (blebs) from *B. abortus* WT or with *B. abortus Δomp25* (10µg/ml). Brucella membrane extracts were a gift from Moriyon lab and were previously described (Guzman-Verri, et al. ,1999).

Sorted HSC were also cultured with blocking peptide SLAMF1 (FCKQLKLYEQVSPPE, Auspep, 100µg/ml) or control peptide (DLSKGSYPDHLEDGY, Auspep,100µg/ml) provided by Sigma Aldrich. Blocking and control peptides were synthetized by Thermo Scientific and the sequences were described by Jordan et al , 2007.

Kaplan Meiersurvival following 5-FU administration

WT and *Slamf1*$^{-/-}$ mice were inoculated intraperitoneally (I.P) with 106 CFU of *Brucella abortus 2308*, *Brucella abortus Δomp25*. Thirty days p.i. 5-FU (150mg/kg) was injected intraperitoneally in a weekly interval. The survival of animals was followed over one month.

Statistics

Results were evaluated by GraphPad Prism v7 software (GraphPad Software, San Diego, CA, USA) using. Statistical test used is indicated in the figure legend. The value of $*P < 0.05$ was determined as significant.

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FIGURE LEGENDS

Figure 1: *Brucella abortus* persist in the BM and affect HSPC homeostasis.

a. C57BL/6J wild-type (wt) mice were intraperitoneally inoculated with $1x10^6$ CFU of *wild-type B. abortus*. Two, eight and thirty days later, spleens and bone marrow from tibia and femur of each mice were isolated, weighted, lysed in 0.1% Triton-X100 and CFU per gram of organ were enumerated. Each symbol represents one animal. Data obtained from 8 independent experiments, each with at least n=3 animals per condition, are shown and mean ± SEM is represented by horizontal bar.

b. Experimental scheme: Mice were intraperitoneally innoculated with $1x10^6$ CFU of wild-type *B. abortus*. BM cell were isolated from femur and tibia of the infected mice, resuspended in PBS and transplanted into previously lethally irradiated mice.8 weeks after transplantation CFU were performed as described in Fig a.

c. Enumeration of CFUs per gram of organ spleen and bone marrow at 8 weeks post transplantation as described in Fig. b.

d-e, C57BL/6J wild-type (wt) mice were intraperitoneally inoculated with $1x10^6$ CFU of wild-type *B. abortus* . Two, eight and thirty days later, FACS analyses were performed for BM cells. Representative FACS profiles (d) and frequency of LSK (lin^-,Sca^+,$cKit^+$) , LSK $CD48^+$ (lin^-,Sca^+,$cKit^+$ $CD48^+$), LSK $CD48^-$ (lin^-,Sca^+,$cKit^+$ $CD48^-$), MPP like (lin^-,Sca^+,$cKit^+$ $CD48^-$, $CD34^+$, $CD135^-$), HSC^{ST} (lin^-,Sca^+,$cKit^+$ $CD48^-$, $CD135^-$ $CD34^-$,$SLAMF^-$), HSC^{LT} (lin^-,Sca^+,$cKit^+$ $CD48^-$, $CD135^-$ $CD34^-$,$SLAMF1^+$) in lineage negative fraction of BM for PBS treated Mock (○) and infected mice (■). Data were obtained from 5 independent experiments, each with at least n=3 animals per condition, are shown and mean ± SEM is represented by horizontal bar. Significant differences from mock are shown. *** $P< 0.001$, ** $P< 0.01$, * $P< 0.05$. Absence of *P* value or ns, non-significant. Since data followed normal distribution, P-Value were generated using Brown-Forsyth followed by ANOVA Welch test.

Figure 2: *Brucella* induces PU.1 upregulation in a SLAMF1/Omp25 dependent manner

a) Experimental scheme PU.1 KIGFP and *Slamf1*$^{-/-}$ PU.1 KIGFP mice were intraperitoneally injected with PBSx1 (Mock, ○) or inoculated with 1x10^{6} CFU of wild-type *B. abortus* (Ba WT, ■), Ba Δ*omp25* (▲). b) Two days after, the percentage of HSCs (lin^{-},Sca^{+},cKit^{+} CD48^{-}, CD135^{-} CD34^{-}) was assessed by Flow Cytometry. Data obtained from 3 independent experiments, each with at least n=3 animals per condition, are shown and mean ± SD is represented by horizontal bar. Significant differences from mock are shown. *** $P< 0.001$, ** $P< 0.01$, * $P< 0.05$. Absence of *P* value or ns, non-significant. Since data did not follow normal distribution, P-Value were generated using Kruskal-Wallis followed by Dunn's test. c)Experimental scheme (left panel) HSC (lin^{-},Sca^{+},cKit^{+} CD48^{-}, CD135^{-} CD34^{-}) from PU.1 KIGFP mice were sorted, then stimulated *ex vivo* with PBSx1 (Mock, ○) or OMVs of *B. abortus* WT (Ba WT, ■), *B. abortus* Δ*omp25* (▲). After 24h, the level of GFP in cells was assessed by Flow Cytometry (right panel). In b, blocking peptide of SLAMF1 (100µg/ml) were used. In c, *Slamf1*$^{-/-}$ PU.1 KIGFP mice were added.

Figure 3. *B. abortus* induces HSC differentiation towards myeloid lineage.

a-d) C57BL/6J wild-type (wt) mice were intraperitoneally inoculated with 1x10^{6} CFU of *B. abortus*. Eight days later, FACS analyses were performed for bone marrow cells. Frequency of (a) MPP2-3 (lin^{-},Sca^{+},cKit^{+} CD48$^{+,}$ CD135^{-}), (b) MPP4 (lin^{-},Sca^{+},cKit^{+} CD48$^{+,}$ CD135^{+}), (c) GMP (lin^{-},Sca^{-},cKit^{+} CD34$^{+,}$ CD16/32^{+}), in Lin- BM cells is shown for (Mock, ○) or inoculated with 1x10^{6} CFU of wild-type *B. abortus* (Ba WT, ■), *B. abortus* Δ*omp25* (▲) or *B. abortus* Δ*omp25c* (□) mutants (the latter only for wt mice). d) Eight, thirty and three hundred sixty days post infection, ratio of myeloid cells (CD45^{+} ,CD11b^{+}) and lymphoid cells (CD3e^{+}CD19^{+}) in blood is shown for (Mock, ○) or inoculated with 1x10^{6} CFU of wild-type *B. abortus* (Ba WT, ■),*B. abortus* Δ*omp25* (▲) . e) Experimental scheme: WT CD45.1 and *Slamf1*$^{-/-}$ CD45.1 mice were intraperitoneally innoculated with 1x10^{6} CFU of *B. abortus.* BM cells were isolated from femur and tibia of the infected mice, lineage negative cells were sorted, resuspended in PBS and transplanted with Lin- cells from WT CD45.2 non infected mice into previously lethally irradiated WT recipient mice. Blood Flow Cytometry analyses were performed at 4, 6 and 8 weeks post transplantation. f) Ratio of myeloid cells (CD45^{+} ,CD11b^{+}) and lymphoid

cells ($CD3e^+CD19^+$) in CD45-1+ blood cells is shown for hematopoietic cells provided by CD45-1 WT or *Slamf1*$^{-/-}$ mice non infected (Mock, ○) or inoculated with $1x10^6$ CFU of wild-type *B. abortus* (Ba WT, ■), *B. abortus* Δ*omp25* (▲) as described in e. g) Experimental scheme: HSC from WT CD45-1 and *Slamf1*$^{-/-}$ CD45-1 mice were sorted and then incubated *ex vivo* with *B. abortus WT* and *B. abortus* Δ*omp25* for 30 minutes. After 30 minutes, cells were washed and treated for 1 hour with Gentamycin to kill extra-cellular bacteria. HSC were then transplanted into into lethally irradiated CD45-2 recipients. FACS analyses of blood samples were performed at 4, 6 and 8 weeks post transplantation. h) Ratio of myeloid cells ($CD45^+$,$CD11b^+$) and lymphoid cells ($CD3e^+CD19^+$) in CD45-1+ blood cells is shown for hematopoietic cells provided by CD45-1 WT or *Slamf1*$^{-/-}$ mice non infected (Mock, ○) or inoculated with $1x10^6$ CFU of wild-type *B. abortus* (Ba WT, ■), *B. abortus* Δ*omp25* (▲) as described in g. i) Experimental scheme: BM cells from *Slamf1*$^{-/-}$ CD45-1 mice and CD45-2 WT mice were isolated from tibia and femur of mice and transplanted into lethally irradiated recipient mice. Twelve weeks after transplantation mice were intraperitoneally injected with PBSx1 (Mock, ○) or inoculated with $1x10^6$ CFU of wild-type *B. abortus* (Ba WT, ■), Ba Δ*omp25* (▲) Blood and BM were analyzed 8 days after.

k), Ratio of myeloid cells ($CD45^+$,$CD11b^+$) and lymphoid cells ($CD3e^+CD19^+$) in blood in WT (○) and SLAMF1 (□)compartment is shown for BM chimeric mice intraperitoneal injected with PBS (Mock, non-filled symbols) or inoculated with $1x10^6$ CFU of wild-type *B. abortus* (symbol filled in black) *B. abortus* Δ*omp25* (symbol filled in grey) as described in i

l-m, FACS analyses were performed for bone marrow cells of chimeric mice as described in i . Frequency of (a) MPP2-3 ($lin^-,Sca^+,cKit^+$ $CD48^+$, $CD135^-$), (b) MPP4 ($lin^-,Sca^+,cKit^+$ $CD48^+$, $CD135^+$), (c) GMP ($lin^-,Sca^-,cKit^+$ $CD34^+$, $CD16/32^+$), in WT (○) and SLAMF1 (□) compartment is shown for BM chimeric mice intraperitoneal injected with PBS (Mock, non filled symbols) or inoculated with $1x10^6$ CFU of wild-type *B. abortus* (symbol filled in black) *B. abortus* Δ*omp25* (symbol filled in grey) as described in i.

Data were obtained from 4 (a-d) or 2 (e-m) independent experiments, each with at least n=3 animals per condition, are shown and mean ± SEM is represented by horizontal bar. Significant differences from mock are shown. *** $P< 0.001$, ** $P< 0.01$, * $P< 0.05$. Absence of P value or ns, non-significant. Since data did not follow normal distribution, P-Value were generated using Kruskal-Wallis followed by Dunn's test.

Figure 4: Finding about *Brucella* infection in mice can be extrapolated to human brucellosis.

a, Percentage of brucellosis patients that present anemia (a) and (b) splenomegaly before antibiotic treatment. Males upper panel and females lower panel. Anemia was characterized by a decrease of hematocrit, hemoglobin and erythrocytes (hematocrit <40% for males and <35% for females; hemoglobin <14g/dL for males and <12 g/dL for females, erythrocyte count <4 million for males and <3.8 million/mm3 for females). c) Experimental scheme: mice were intraperitoneally injected with PBSx1 (Mock, ○) or inoculated with $1x10^6$ CFU of wild-type *B. abortus* WT. 8 and 30 days post infection blood and spleen analyses are performed. d) Percentage of haematocrit in the d) blood and e) the spleen weight of non-infected (Mock ○) and *B. abortus* infected mice at at day 8 and 30 post infection (■) is showm.

Supplementary 1: *Brucella* infection does not affect the number of lineage negative progenitors and BM cells.

a-b, WT and *Slamf1*$^{-/-}$ mice were intraperitoneally injected with PBS or inoculated with $1x10^6$ CFU of *B. abortus*. Eight days later, bone marrow cells were isolated, cells were counted and than depleted for mature hematopoietic cells as shown in Methods. Lin- cells were also counted for (Mock, ○) or infected *B. abortus* (Ba WT, ■), *B. abortus* Δ*omp25* (▲) or *B. abortus* Δ*omp25c* (□) mutants (the latter only for wt mice). f) Experimental scheme:
Data obtained from 3 independent experiments, each with at least n=3 animals per condition, animals per condition, are shown and mean ± SEM is represented by horizontal bar. Significant differences from mock are shown. *** P< 0.001, ** P< 0.01, * P< 0.05. Absence of P value or ns, non-significant. Since data did not follow normal distribution, P-Value were generated using Kruskal-Wallis followed by Dunn's test.

Supplementary 2: Infection burden in PU.1 KIGFP mice

a-b, CFU count per gram of organ at Day 2 p.i. as described in Fig 2.a. for (a) spleen and (b) BM for PBSX1 injected (Mock, ○) or infected with *B. abortus* (Ba WT, ■), Ba Δ*omp25* (▲) PU.1 KIGFP and *Slamf1*$^{-/-}$ PU.1 KIGFP mice.

Data obtained from 3 independent experiments, each with at least n=3 animals per condition, animals per condition, are shown and mean ± SEM is represented by horizontal bar. Significant differences from mock are shown. *** P< 0.001, ** P< 0.01, * P< 0.05. Absence of P value or ns, non-significant. Since data did not follow normal distribution, P-Value were generated using Kruskal-Wallis followed by Dunn's test.

Supplementary 3 Acute *Brucella* infection does not affect HSC function

WT CD45.1 and *Slamf1*$^{-/-}$ CD45.1 mice were intraperitoneally innoculated with 1x10^6 CFU of *B. abortus.* BM cells were isolated from femur and tibia of the infected mice, Lin- cells were sorted, resuspended in PBS and transplanted with lineage negative cells from WT CD45.2 non infected mice into previously lethally irradiated WT recipient mice. Blood Flow Cytometry analyses were performed at 4, 6 and 8 weeks post transplantation as shown in fig 3i

d-f Percentage of engrafted (d)%CD11b+ cells (e) %CD19+ (f) %CD3+ in CD45-1 WT (left panel) and CD45-1 *Slamf1*$^{-/-}$ (right panel) cells from PBS injected (Mock, ○) or infected with *B. abortus* (Ba WT, ■), Ba Δ*omp25* (▲) mice at 4,6 and 8 weeks post engraftement. Percentage of engrafted CD45-1 WT (-) cells and *Slamf1*$^{-/-}$ CD45.1 (- -) cells from PBS injected mice in the blood of chimeric at 4, 6, 8 weeks

Data obtained from 2 independent experiments, each with at least n=3 animals per condition, animals per condition, are shown and mean ± SEM is represented by horizontal bar. Significant differences from mock are shown. *** P< 0.001, ** P< 0.01, * P< 0.05. Absence of P value or ns, non-significant. Since data did not follow normal distribution, P-Value were generated using Kruskal-Wallis followed by Dunn's test.

Supplementary 4. Acute *Brucella* infection induces changes in *Slamf1*$^{-/-}$ HSC compartment.

a. Experimental scheme: *Slamf1*$^{-/-}$ mice were intraperitoneally innoculated with 1x10^6 CFU of wild-type *B. abortus*. BM cell were isolated from femur and tibia of the infected mice, resuspended in PBS and transplanted into previously lethally irradiated mice.8 weeks after transplantation CFU were performed as described in Fig 1a.

b. Enumeration of CFUs per gram of organ spleen and bone marrow at 8 weeks post transplantation as described in Fig. b.

c-f, C57BL/6J wild-type (wt) mice were intraperitoneally inoculated with 1x106 CFU of wild-type B. abortus . Two, eight and thirty days later, FACS analyses were performed for BM cells. Representative FACS profiles (c) and frequency of LSK (lin-,Sca+,cKit+) , (d) LSK CD48+ (lin-,Sca+,cKit+ CD48+), (e) LSK CD48- (lin-,Sca+,cKit+ CD48-), HSC (lin-,Sca+,cKit+ CD48-, CD135- CD34-,),

Data were obtained from 4 independent experiments, each with at least n=3 animals per condition, are shown and mean ± SEM is represented by horizontal bar. Significant differences from mock are shown. *** P< 0.001, ** P< 0.01, * P< 0.05. Absence of P value or ns, non-significant. Since data followed normal distribution, P-Value were generated using Brown-Forsyth followed by ANOVA Welch test.

Supplementary 5 The SLAMF1/Omp25 promotes bacterial proliferation.

a-b, CFU count per gram of organ at Day 8 (a, b) and Day 30 p.i. (c,d) as described in Fig 2.a. for (a,c) spleen and (b,d) BM for PBSX1 injected (Mock, ○) or infected with *B. abortus* (Ba WT, ■), Ba Δ*omp25* (▲) PU.1 KIGFP and *Slamf1*$^{-/-}$ PU.1 KIGFP mice.

Data obtained from 4 independent experiments, each with at least n=3 animals per condition, animals per condition, are shown and mean ± SEM is represented by horizontal bar. Significant differences from mock are shown. *** P< 0.001, ** P< 0.01, * P< 0.05. Absence of P value or ns, non-significant. Since data did not follow normal distribution, P-Value were generated using Kruskal-Wallis followed by Dunn's test.

Figure 1

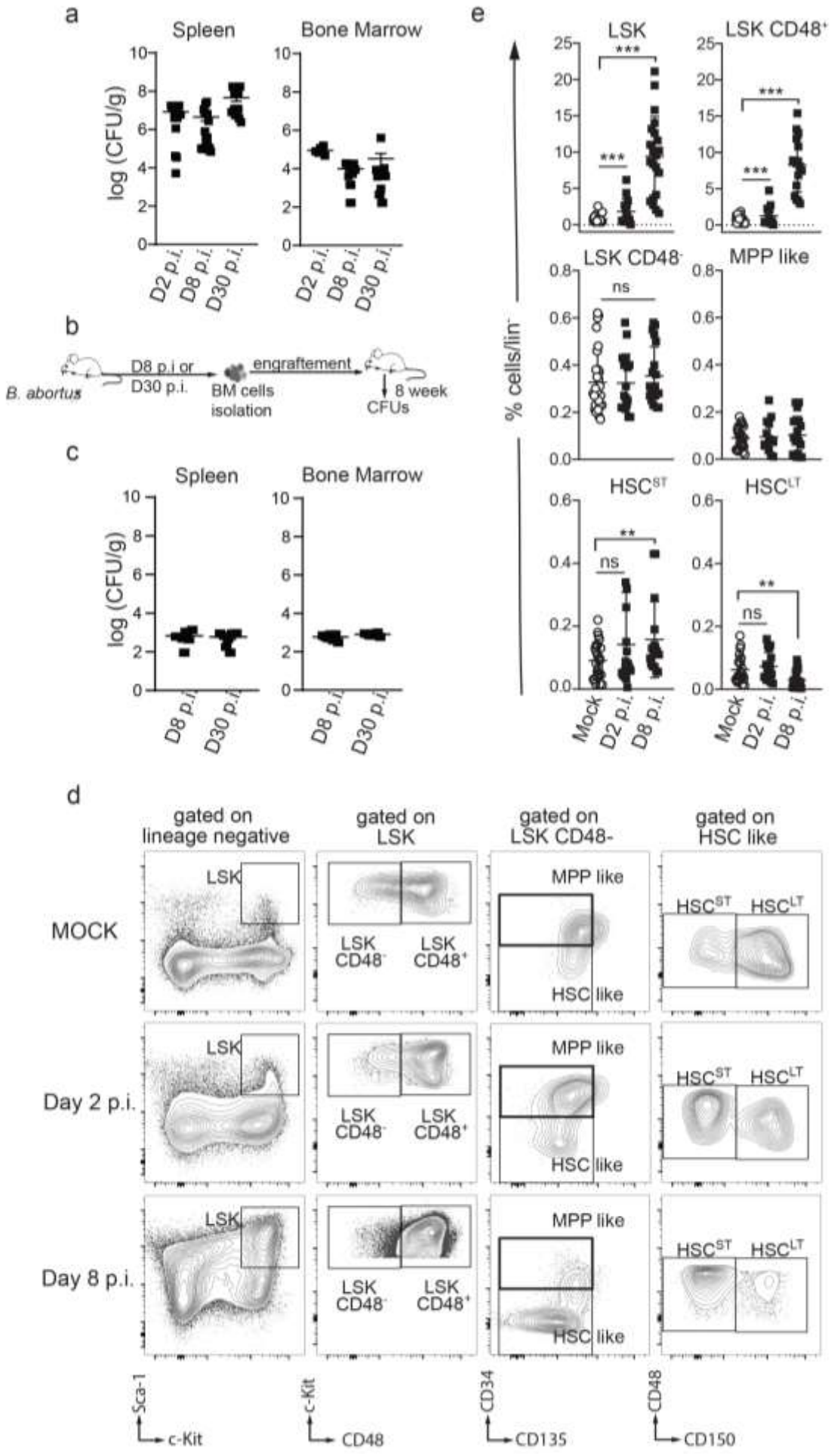

a
Spleen
Bone Marrow
log (CFU/g)
D2 p.i.
D8 p.i.
D30 p.i.
b
B. abortus
D8 p.i or
D30 p.i.
BM cells
isolation
engraftement
8 week
CFUs
c
Spleen
Bone Marrow
log (CFU/g)
D8 p.i.
D30 p.i.
e
LSK
LSK CD48+
LSK CD48-
MPP like
HSCST
HSCLT
% cells/lin-
Mock
D2 p.i.
D8 p.i.
***
**
ns
d
gated on lineage negative
gated on LSK
gated on LSK CD48-
gated on HSC like
MOCK
Day 2 p.i.
Day 8 p.i.
LSK
LSK CD48-
LSK CD48+
MPP like
HSC like
HSCST
HSCLT
Sca-1
c-Kit
CD48
CD34
CD135
CD150

Figure 2

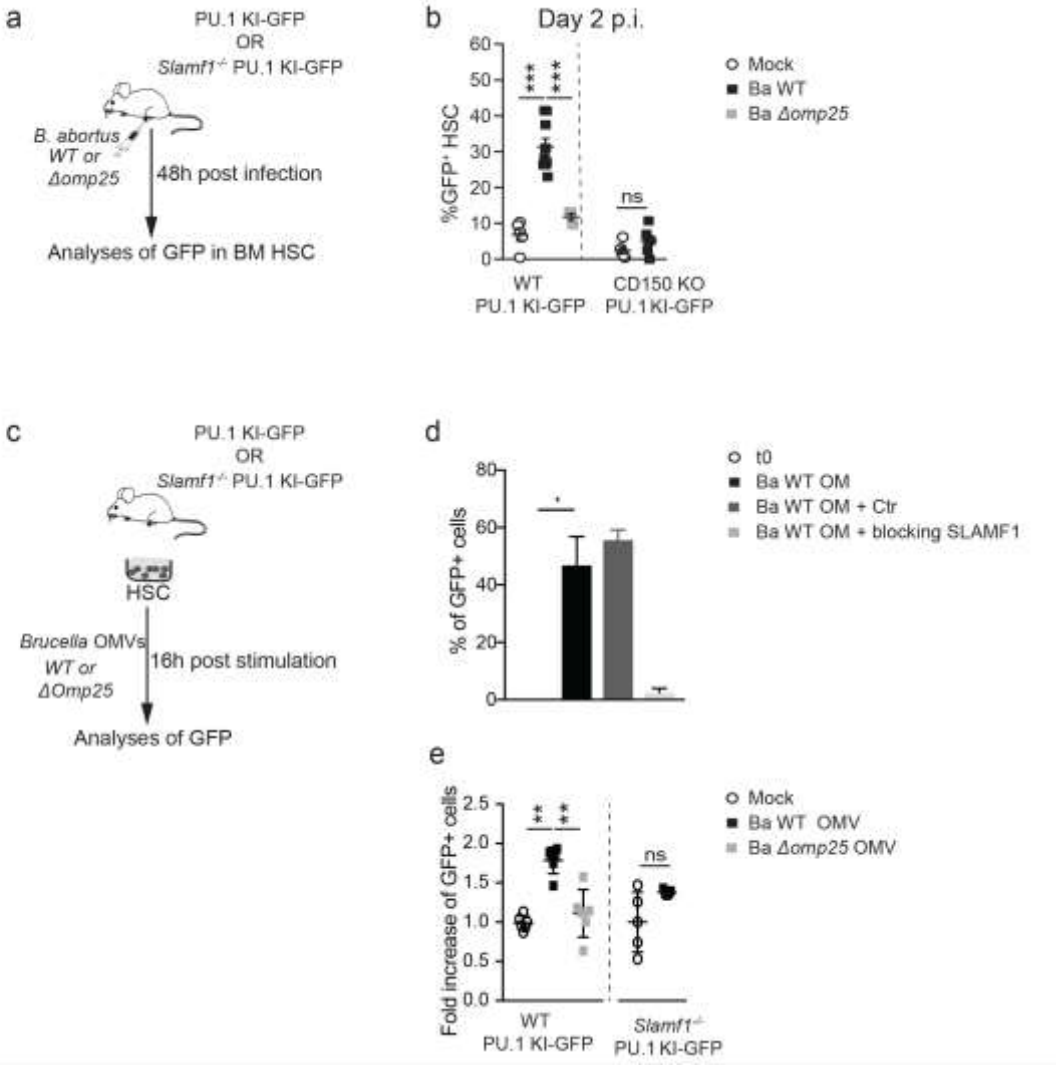

a
PU.1 KI-GFP
OR
Slamf1-/- PU.1 KI-GFP
B. abortus
WT or
Δomp25
48h post infection
Analyses of GFP in BM HSC
b
Day 2 p.i.
%GFP+ HSC
***
***
ns
Mock
Ba WT
Ba Δomp25
WT
PU.1 KI-GFP
CD150 KO
PU.1 KI-GFP
c
PU.1 KI-GFP
OR
Slamf1-/- PU.1 KI-GFP
HSC
Brucella OMVs
WT or
ΔOmp25
16h post stimulation
Analyses of GFP
d
% of GFP+ cells
t0
Ba WT OM
Ba WT OM + Ctr
Ba WT OM + blocking SLAMF1
e
Fold increase of GFP+ cells
**
**
ns
Mock
Ba WT OMV
Ba Δomp25 OMV
WT
PU.1 KI-GFP
Slamf1-/-
PU.1 KI-GFP

Figure 3

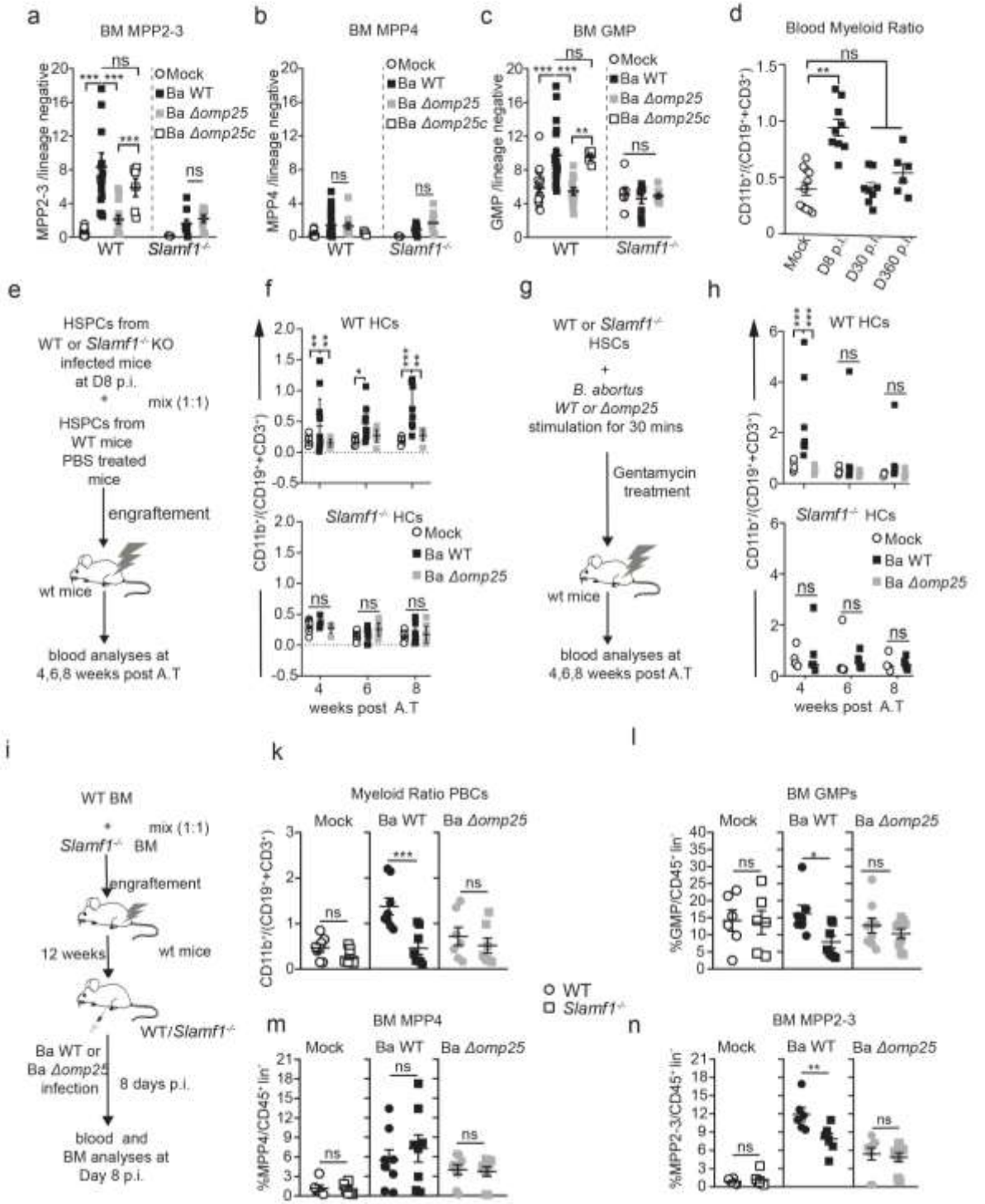
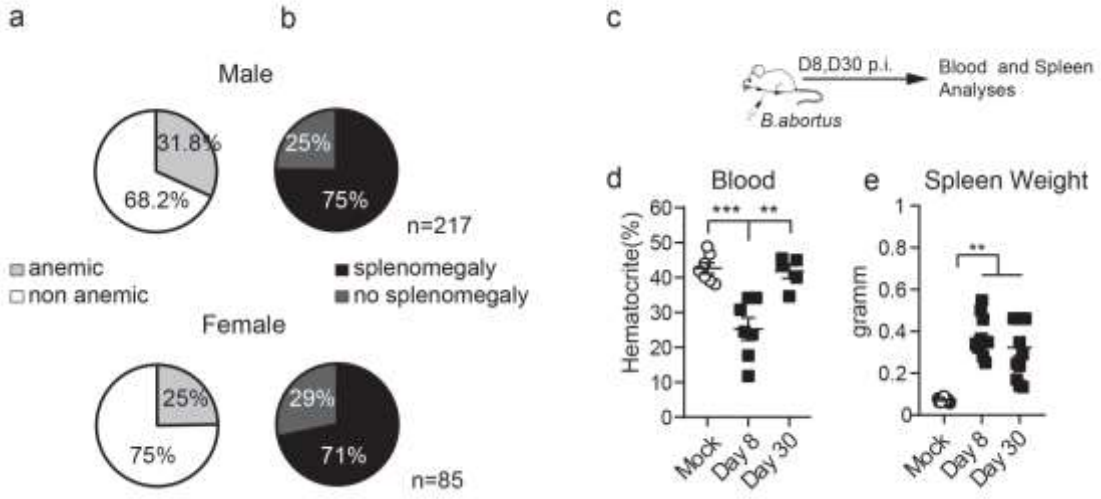
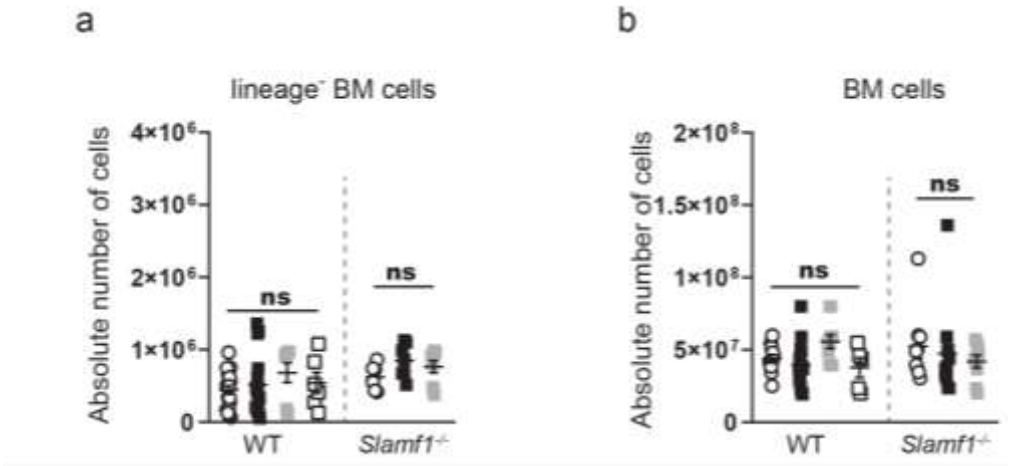

a
BM MPP2-3
MPP2-3 /lineage negative
Mock
Ba WT
Ba Δomp25
Ba Δomp25c
WT
Slamf1-/-
b
BM MPP4
MPP4 /lineage negative
c
BM GMP
GMP /lineage negative
d
Blood Myeloid Ratio
CD11b+/(CD19+CD3+)
Mock
D8 p.i
D30 p.i
D360 p.i
e
HSPCs from WT or Slamf1-/- KO infected mice at D8 p.i.
mix (1:1)
HSPCs from WT mice PBS treated mice
engraftement
wt mice
blood analyses at 4,6,8 weeks post A.T
f
WT HCs
Slamf1-/- HCs
weeks post A.T
g
WT or Slamf1-/- HSCs
B. abortus WT or Δomp25 stimulation for 30 mins
Gentamycin treatment
wt mice
blood analyses at 4,6,8 weeks post A.T
h
WT HCs
Slamf1-/- HCs
weeks post A.T
i
WT BM
mix (1:1)
Slamf1-/- BM
engraftement
12 weeks
wt mice
WT/Slamf1-/-
Ba WT or Ba Δomp25 infection
8 days p.i.
blood and BM analyses at Day 8 p.i.
k
Myeloid Ratio PBCs
CD11b+/(CD19+CD3+)
l
BM GMPs
%GMP/CD45+ lin-
WT
Slamf1-/-
m
BM MPP4
%MPP4/CD45+ lin-
n
BM MPP2-3
%MPP2-3/CD45+ lin-

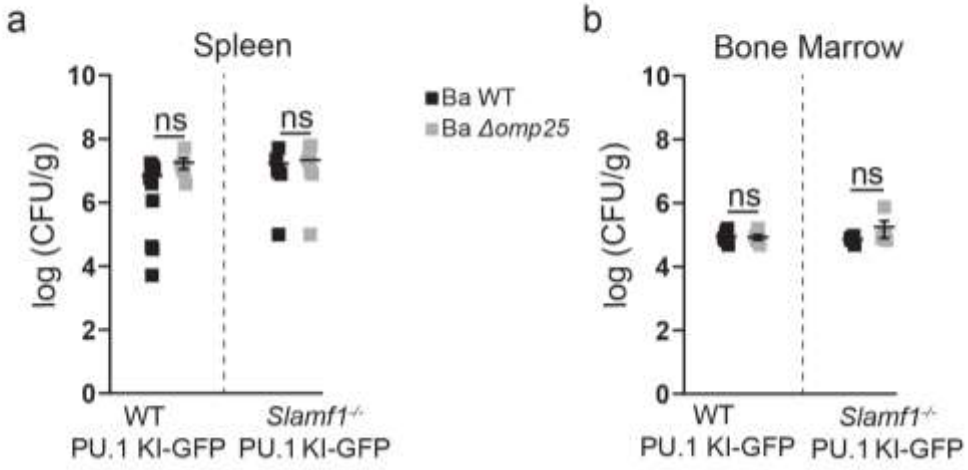
Figure 4


a
b
c
Male
31.8%
68.2%
25%
75%
n=217
anemic
non anemic
splenomegaly
no splenomegaly
Female
25%
75%
29%
71%
n=85
D8,D30 p.i.
Blood and Spleen Analyses
B.abortus
d
Blood
Hematocrite(%)
***
**
Mock
Day 8
Day 30
e
Spleen Weight
gramm
**
Mock
Day 8
Day 30

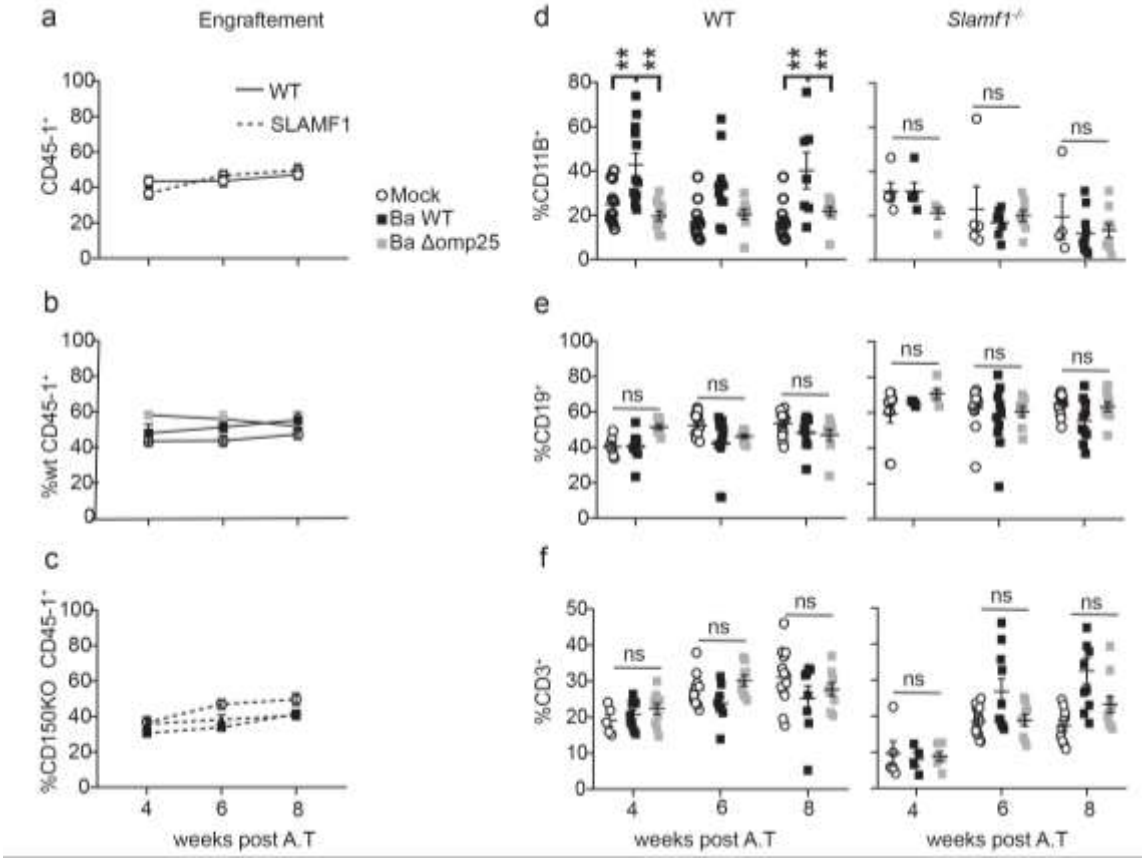
Supplementary 1



Supplementary 2



Supplementary 3



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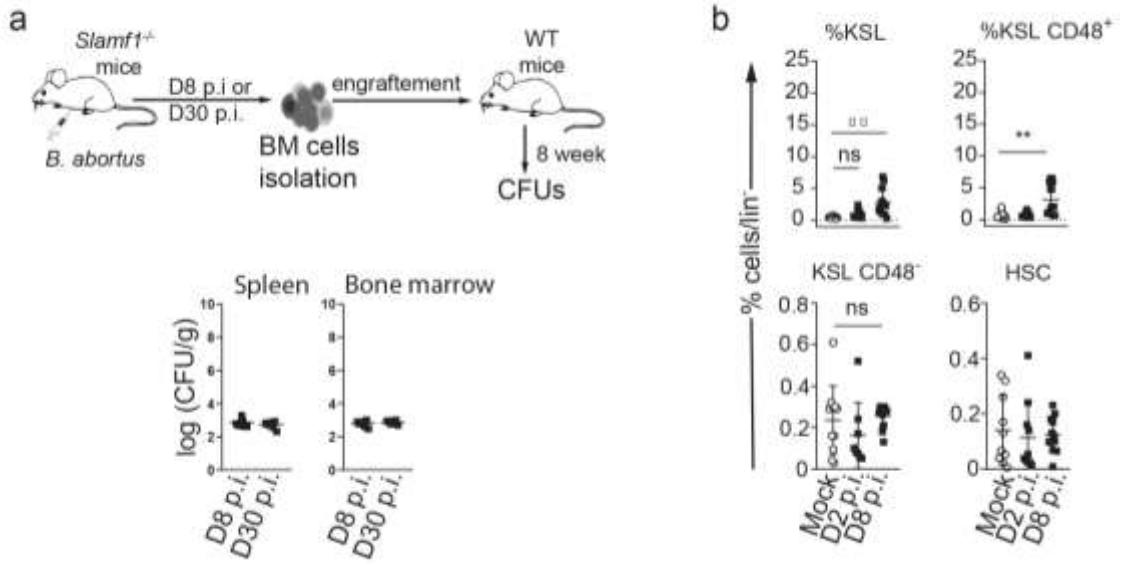
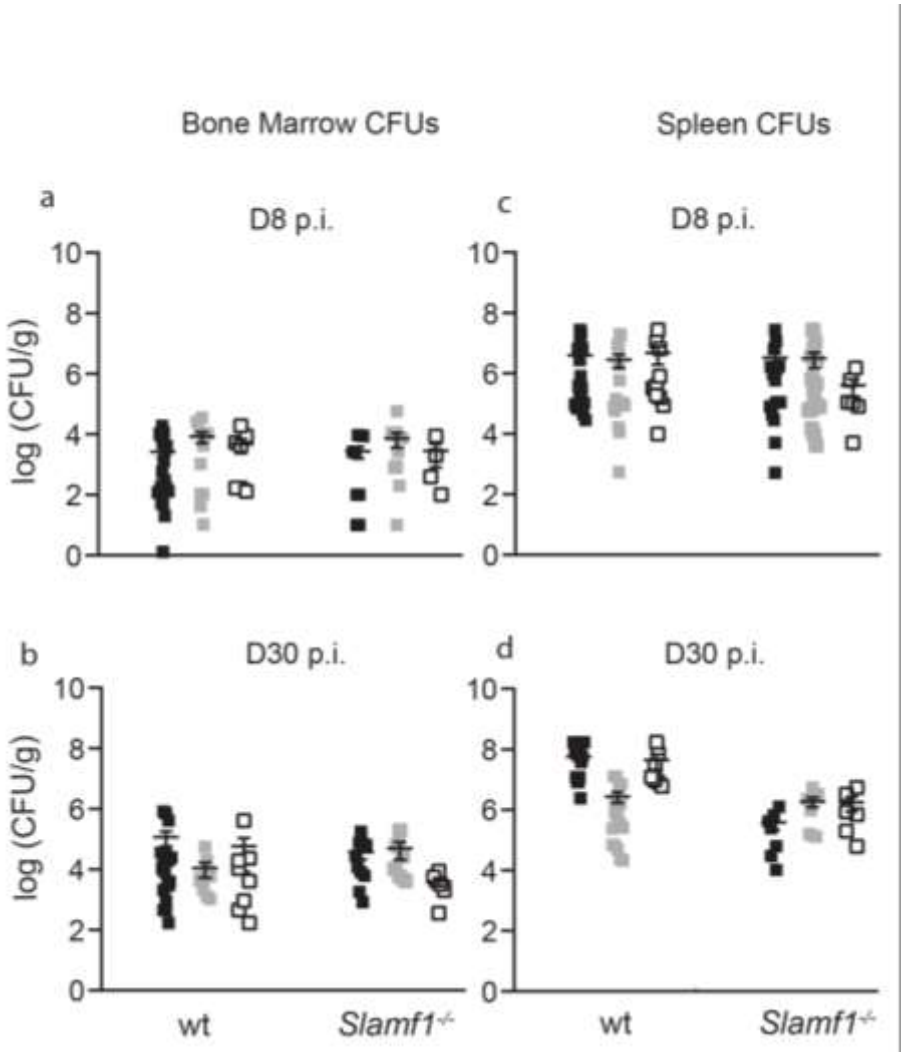


Supplementary 5



Discussion

Science is the acceptance of what works and the rejection of what does not. That needs more courage that what we think.

Jacob Bronowski

New markers for acute and chronic human brucellosis

Here, we have recapitulated the first blood transcriptional signature of human brucellosis using RNA seq that discriminates acute brucellosis from chronic brucellosis and healthy individuals. We show that this unbiased sequencing approach is robust in distinguishing patients with acute brucellosis, treated or relapse, from patients with chronic brucellosis. The latter presents an overall gene expression very close to that of uninfected controls. However, thanks to the comparative analysis of blood transcriptome and cytokine profiling in our clinical cohort, we have identified a number of molecules specific to each disease stage. As such, SLAMF1, a molecule overexpressed in several inflammation-related diseases (including infection, arthritis, systemic lupus erythematous and pulmonary allergy), has been identified as a marker of the acute phase of brucellosis in humans together with other classical markers of infection. This is in accordance with previous reports indicating a SLAMF1 upregulation at the phagocyte cell surface during the initial phase of infectious inflammation, followed by that of SLAMF8 (Kingsbury, Feeney et al. 2001, Wang, Abadia-Molina et al. 2012, Wang, van Driel et al. 2015). The *SH2D1A* gene, which encodes the SLAMF1 adaptor protein (SAP), varies accordingly to SLAMF1 suggesting that SLAMF1-SAP induced signaling is activated during acute brucellosis. A series of genes encoding other molecules shown to be involved in brucellosis were also overexpressed in *Brucella*-infected human blood such as C1QC, the first component of serum complement (Gonzalez-Espinoza, Barquero-Calvo et al. 2018), or genes encoding proteins involved in T_H1 response (Dornand, Gross et al. 2002, Billard, Cazevieille et al. 2005) (such as those of the transcription factors EOMES, TBX21/T-BET), innate response like Granzymes, GZMK and GZMH (Martirosyan, Ohne et al. 2013) or an inflammasome component AIM2 (Costa Franco, Marim et al. 2018, Costa Franco, Marim et al. 2019). As expected, acute patients secreted in their serum significantly more pro-inflammatory cytokines, in particular TNF-α, IL-9 and IL-31, while acute with relapse patients presented an exacerbated phenotype with more serum IL-1β together with IL-22, VEGF-D, CXCL10/IP-10 and βNGF. IL-22, a T-cell derived cytokine structurally related to IL-10, which protects against tissue destruction caused by exacerbated immune response, has been reported to be induced in murine T cells by IL-9, which itself is involved in the induction of T_H2 cell type immune response (Dumoutier, Louahed et al. 2000). The upregulation of this cytokine in acute blood patients might explain the rise in IL-22 when relapse occurs. Moreover, upregulation of such cytokines indicates an important contribution of the T_H2 response in human acute brucellosis, as confirmed by that of VEGF-D, shown to elicit T_H2 responses in DC through binding to its

receptor VEGFR3 (Lee, Link et al. 2004), and of NGF, which inhibits TLR-mediated inflammation in human monocytes (Prencipe, Minnone et al. 2014). CXCL10/IP-10, a pan-marker of viral or bacterial infection (Oved, Cohen et al. 2015) and an IFN-stimulated gene, like AIM2, is induced in bone marrow-derived murine macrophages via the STING pathway upon *Brucella* infection (Khan, Harms et al. 2016, Costa Franco, Marim et al. 2018), suggesting an important role of the IFN-type I in human brucellosis. Chronic brucellosis, although silent at the transcriptional level, is marked by the presence in patient serum of classical anti-inflammatory cytokines like IL-10 and IL1RA, as well as of two molecules known as pro-angiogenic chemokine for CXCL1/KC, and as a key pro-B and T cell differentiating cytokine for IL-7. Since *Brucella* elicits premature cell death of human neutrophils without inducing pro-inflammatory phenotypic changes (Barquero-Calvo, Martirosyan et al. 2013, Barquero-Calvo, Mora-Cartin et al. 2015), elevated blood CXCL1 levels might account for the neutropenia described in chronically-infected brucellosis patients (Crosby, Llosa et al. 1984). High levels of both IL-10 and IL1RA explain the low activation transcriptional signature of chronic patients and are thought to trigger an anergic state supporting persistence of bacteria (Mege, Meghari et al. 2006). As regards *Brucella*, IL-10 plays an essential role in its maintenance *in vivo* notably, by favoring *Brucella* replication in macrophages (Corsetti, de Almeida et al. 2013, Xavier, Winter et al. 2013). Interestingly, genetic polymorphism in the IL1RA gene has been linked to susceptibility to brucellosis (Hajilooi, Rafiei et al. 2006). High serum levels of IL-7 in the chronic patient group reflects an impaired T cell sensitivity to IL-7 as recently reported for tuberculosis patients (Lundtoft, Afum-Adjei Awuah et al. 2017) . These data highlight specific features of the chronic phase of human brucellosis and infer that combined detection of IL-10, IL1RA, CXCL1 and IL-7 should form a unique and promising biomarker serum signature for identifying *Brucella* chronicity. Collectively, these results disclose distinctive hallmarks of human brucellosis that are crucial for improving its diagnosis.

SLAMF1-Omp25 in DCs

We used the murine model to explore the function of SLAMF1 in brucellosis. We first looked at SLAMF1 expression in murine DCs and could confirm that similarly to human periphicaral blood cells, *Brucella* drove SLAMF1 upregulation in BMDCs in an Omp25-independent manner. Indeed, other factors such as *Brucella* CβG and *E. coli* LPS, flagellin or peptidoglycan (Farina, Theil et al. 2004) also upregulate SLAMF1 in human mo-DCs *in vitro*. Moreover, SLAMF1 has been recognized as the Measles virus entry receptor and has been reported to

interact with several *E. coli* outer membrane porins such as OmpC and OmpF (Berger, Romero et al. 2010). In the case of brucellosis, murine extracellular domain of SLAMF1 binds *B. abortus* Omp25 and not the related *B. abortus* outer membrane protein Omp19. The binding of OmpC and OmpF to SLAMF1 leads to macrophage activation and efficient killing of *E. coli* during infection, through phago-lysosome maturation (Berger, Romero et al. 2010, Yurchenko, Skjesol et al. 2018). In BMDCs, we show that neither SLAMF1 deletion nor lack of Omp25 affected *Brucella* intracellular trafficking and replication. This is in sharp contrast to what happens upon *E. coli* or *S. typhimurium* infection of macrophages where intracellular trafficking requires SLAMF1.

Interestingly, our *in vitro* data indicate that the SLAMF1-Omp25 axis limits nuclear translocation of NF-κB, subsequently restricting pro-inflammatory gene transcription, cytokine and chemokine secretion and co-stimulatory molecule expression. The signaling cascade involved in inhibition of NF-κB in the nucleus has to be explored. In the case of *Brucella* it might be possible that once inside the cell, the bacterium produce outer membrane vesicles (OMVs) containing Omp25. Omp25 is present in the extracellular media during Brucella macrophage infection and its blocking inhibits TNF-α production (Boigegrain, Salhi et al. 2004). This suggest that Omp25 binds to extracellular SLAMF1, triggering NFκB translocation in the nucleus resulting in inhibition of cytokine production. Other members of the SLAM receptor family have been shown to sense various microbial components (van Driel, Liao et al. 2016). For instance, SLAMF6 interacts with *E. coli* and *Citrobacter rodentium(van Driel, Wang et al. 2015)* while SLAMF2 binds FimH, a lectin from *E. coli* (Baorto, Gao et al. 1997, Malaviya, Gao et al. 1999, Cannons, Tangye et al. 2011). SLAMF1 is also expressed in other cells such as monocytes, macrophages, T, B lymphocytes and murine HSCs.

HSCs are the founder of the immune system and resides in the BM.

Bone marrow- the HSC niche- a furtive niche for *Brucella*

Osteo-articular complications are one of the most frequent symptoms of human brucellosis. In addition *Brucella* was isolated from a large number of human patients (Ertem, Kürekçi et al. 2000). However despite these clinical observations, the role of BM infection was not studied.

To explore this, we infected *BalbC* and C57BL/6 mice with *B. abortus* WT intraperitoneally and intravenously. In both cases *Brucella* was found in the BM 24hours post infection (p.i.) and persisted up to 1 year post-infection.

B. abortus DsRed was used to identify the infected cells by immunofluorescence techniques. Since BM cells show similar shapes and share common markers, confocal microscopy analyses were not appropriate to determine the phenotype of cells harboring *Brucella*. Flow Cytometry Analyses (FACS) were more adapted, and showed that monocytes, macrophages, neutrophils and granulo-myeloid progenitors (GMP) are the highly infected cells in the BM. Nevertheless, low infected cells could not be detected by FACS due to the fact that the DsRed signal of the intracellular bacterium is not very high in LSR561 laser of FACS. ImageStream (a technique coupling confocal microscopy and FACS) analyses could be performed to identify cells infected with less than five bacteria.

GMPs are multipotent stem cells with the ability to self-renewal and differentiate (Hérault, Binnewies et al. 2017). It can be possible that during GMP division, bacteria spread in both dividing daughter cells: the one that stays GMP and the one that differentiates in myeloid cell and egress from the BM spreading the infection in extra-medullary sites.

In addition, GMPs possess drug efflux pumps like the ABCG2 system that prevents antibiotic entry inside the cell (Scharenberg, Harkey et al. 2002). As such, GMPs might be the cause of relapse after antibiotic treatment. GMPs should be sorted and infected *in vitro* with *B. abortus* GFP or DsRed. Single cell video microscopy could trace infected GMPs during cell division and also investigate bacterial growth during antibiotic treatment.

Another important point of BM infection is granuloma formation. Granulomas show the efforts of the organism to clear bacteria. BM granulomas are enriched in macrophage aggregates with scattered lymphocytes, which reduced its size and cellularity over time. Compared to the spleen, bone marrow granulomatous inflammation was more severe in the first two weeks of infection. After four weeks of infection, granulomatous inflammation decreased in both tissues. The switch of metabolism in the macrophages is one of the explanations of the reduced inflammation. Indeed, in the spleen, during the acute phase of *B. abortus* infection, granulomas are predominantly constituted by classically activated macrophages (CAM) that produce IFN-γ. In contrast, during the chronic infection phase CAMs switch their metabolism and become alternatively activated macrophage (AAM). AAM produce IL-4 and IL-13 instead of IFN-γ (Byndloss and Tsolis 2016). Therefore, AAM macrophages can provide a safe shelter for *Brucella* to be protected from immune surveillance.

To sum up, *Brucella* infects and persist in the hematopoietic cells of BM *in vivo* and seems to provide a safe environment for the bacterium to be protected from immune surveillance and antibiotic treatement.

Brucella- journey to bone marrow

Brucellosis is accompanied by an increase of blood neutrophils in both humans and mice (Olt, Ergenç et al. 2015, Mora-Cartín, Gutiérrez-Jiménez et al. 2019). Neutrophils are very short lived cells that are generated from BM HSCs, migrate in the tissues through blood then return in the BM, where they are eliminated by $CD169^+$ macrophages by a process called neutrophil clearance (Christopher and Link 2007, Casanova-Acebes, Pitaval et al. 2013). In an *in vitro* system, Moreno *et al*. showed that murine neutrophils that have engulfed *Brucella* promote their phagocytosis by macrophages leading to macrophage infection (Gutierrez-Jimenez, Hysenaj et al. 2018) and consequently suggesting that neutrophils serve as a Trojan horse for *Brucella* dissemination in the BM. Once in the BM, *Brucella* can infect other cells such as osteoblasts, osteoclasts, plasma B cells and monocytes (Goenka, Guirnalda et al. 2012). In humans *Brucella* LPS induces pre mature death of infected neutrophils and thus it is very unlikely that infected human neutrophils egress in the BM. However, in humans and in mice, other cells are known to be infected by *Brucella* such as monocytes, long lasting plasma B cells and hematopoietic progenitors. All these cells have the capacity to return back in the BM (Shi and Pamer 2011) and thus transport the bacterium to this organ.

Hematological abnormalities along *Brucella* infection

In a cohort of brucellosis patients, we observed that thirty percent of patients suffer from anemia. Bacterial infections often provoke hematological changes due to BM HSC failure (Glatman Zaretsky, Engiles et al. 2014).

Some aspects of brucellosis in mice are not immediately transferable to humans (Grilló, Blasco et al. 2012). Therefore, to asses if the murine model is suitable for studying hematological abnormalities, I infected C57BL/6 mice with *B. abortus* i.p. and performed blood analyses. The level of hematocrit in the blood of *wt* mice infected with *B. abortus* was reduced compared with the non-infected condition demonstrating that like human mice suffer from anemia.

Moreover, BM analyses showed that mouse hematopoietic stem and progenitor cell (HSPCs) compartment was altered, indicating that hematological alterations are caused by HSPCs modification. Other bacterium such as *Plasmodium chabaudi, Pneumocystis carinii, Mycobacterium avium, Escherichia Cloli* are known to induce HSPCs expansion (Glatman Zaretsky, Engiles et al. 2014).

In the case of brucellosis, the level of systemic inflammation is very low (Barquero-Calvo, Chaves-Olarte et al. 2007). As well, the level of BM pro-inflammatory cytokines was lower compared to other infection such as MCMV, *Salmonella* Typhimurium, *Mycobacterium avium* (Matatall, Jeong et al. 2016, Hirche, Frenz et al. 2017, Takizawa, Fritsch et al. 2017). The slight increase of IL-12 and FN-γ resulted in a concomitant increase in IL-10. IL-12 and IFN-γ are known to induce HSC proliferation and cell cycle exit In contrast, IL-10 is known to suppress HSC expansion and emergency myelopoiesis (McCabe, Brendel et al. 2018). The low levels of inflammation in the BM sera and the simultaneous presence of pro-proliferative and anti-proliferative indicate that HSPC expansion is not caused by cytokines.

HSPCs can directly recognize pathogenic products via TLR receptors or STING pathway. For instance, recognition of LPS via TLR4 or recognition of bacterial DNA derivates via STING leads to HSPCs expansion. (Zhao, Ma et al. 2014, Kobayashi, Kobayashi et al. 2015).

Analyses of BM from infected mice demonstrated that *Brucella* also triggers expansion of HSPCs *in vivo* during acute phase of infection. The *Brucella* LPS have a peculiar structure that makes it a very weak TLR4 agonist (Martirosyan and Gorvel 2013, Zhao, Hanniffy et al. 2018), suggesting that HSPC expansion is not induced by *Brucella* LPS. Interestingly, as we saw previously, SLAMF1 binds to *Brucella* Omp25, indicating that this receptor place a role in HSPCs. Indeed, alterations in HSPCs of the BM compartment of *Brucella* infected *Slamf1*$^{-/-}$ were less important than in *wt* infected mice. In contrast, *Brucella* infected *Slamf1*$^{-/-}$ mice did not present any defect in hematocrit levels suggesting a potential role of SLAMF1 in hematopoiesis. In addition, at day 2 post-infection, the percentage of PU.1^{+} BM HSCs in *wt* mice infected with *B. abortus* WT was increased in a Omp25-SLAMF1-dependent fashion. PU.1 in combination with C/EBPβ enhances myeloid commitment (Heinz, Benner et al. 2010) and whose ectopic expression in B cells reprograms them into macrophages (Xie, Ye et al. 2004). In several studies PU.1 upregulation in HSCs is associated with myeloid commitment. (Mossadegh-Keller, Sarrazin et al. 2013, Pietras, Mirantes-Barbeito et al. 2016, Tyrkalska, Perez-Oliva et al. 2019, Yamashita and Passegue 2019). Analyses of *wt* and *Slamf1*$^{-/-}$ mice infected with *B. abortus WT* or *B. abortus Δomp25* demonstrated that *Brucella* transiently enhances myelopoiesis in a SLAMF1/Omp25-dependent fashion. Competitive hematopoietic chimeras demonstrated that the SLAMF1/Omp25 myeloid commitment is not due to a pollution of Sca^{+} cKit^{+} compartment with myeloid differentiated cells but to the HSC enhanced myeloid differentiation (Boettcher and Manz 2017).

SLAMF1-Omp25 in BM HSCs

SLAMF1 is also expressed in SLAMF1^{high} T_{reg} cells of the BM. These cells are important for HSCs maintenance and are in close vicinity with the HSCs (Hirata, Furuhashi et al. 2018). A hypothetical homophilic interaction between SLAMF1^{high} T_{reg} and SLAMF1^{+} HSCs is possible. This interaction can be altered during *Brucella* infection resulting in HSPC expansion. The absence of SLAMF1^{high} T_{reg} in the BM does not trigger myeloid skewing (Hirata, Furuhashi et al. 2018) suggesting a direct role of Omp25/SLAMF1 binding in HSCs. Analyses of blood and BM of *B. abortus WT* or *B. abortus Δomp25* BM chimeric mice with one half of hematopoietic cells expressing SLAMF1 and the other half not expressing it, clearly demonstrated that *B. abortus* WT induces HSC myeloid differentiation via SLAMF1 in a hematopoietic cell autonomous way.

We proved also that the observed phenotype is triggered by the binding of Omp25 to the SLAMF1 extracellular domain. Treatment of sorted HSCs with *Brucella* outer membranes (OMVs) causes the upregulation of PU.1 in these cells. PU.1 upregulation is abrogated when cells are treated with a SLAMF1 blocking peptide.

In addition, HSC pre-stimulated with *B. abortus* WT transplanted in irradiated recipients, generated more myeloid cells at 4 weeks post-transplantation. This increase in myeloid production was not observed anymore at 6 and 8 weeks post transplantation and was dependent on Omp25/SLAMF1 axis, implying that Omp25 binds directly to HSC to induce myeloid commitment.

It remains challenging to understand the downstream signaling of Omp25/SLAMF1 heterophilic interaction. SLAMF1 intracellular ITSM domain recruits the adaptor proteins SAP, EAT-2 or ERT for activatory signaling or SHP1/SHP2 for inhibitory signaling. Interestingly, based on transcriptomic analyses neither SAP neither EAT-2 nor ERT are expressed in HSCs (IMMGEN), indicating that ITSM might not exhibit its activatory function in HSCs. HSCs express the *Ptpn6* and *Ptpn11* gene coding respectively for SHP-1 and SHP-2. It will be interesting to block SHP1 and or SHP2 expression in sorted HSCs by using siRNA. But before it is crucial to test if the siRNA does not trigger changes in HSC biology. Then, if siRNA does not alter the HSC function, cells should be stimulated *ex vivo* with WT *Brucella* OMVs and OMVs lacking Omp25.

SLAMF1 has been shown to participate even in autophagosome generation (Bologna, Buonincontri et al. 2016). In HSCs, autophagy protect from metabolic stress during starvation

and thus participate in maintenance of quiescence. Decreased level of autophagy leads to HSCs expansion and enhanced myelopoiesis (Warr, Binnewies et al. 2013, Ho, Warr et al. 2017). It may be possible that *Brucella* Omp25 binding to SLAMF1 inhibits autophagosome generation triggering increased myeloid differentiation. GFP-LC3 transgenic mice infected with *Brucella* could be used to quantify autophagy in BM HSCs *in vivo*. LC3 (a mammalian homolog of Atg8) is a marker of the autophagosome membrane. In the GFP-LC3 mice, LC3 is fused with GFP enabling autophagosome quantification by fluorescence (Mizushima and Kuma 2008).

Transcriptomic and proteomic analyses of sorted HSCs from D8 p.i. of *Brucella* infected *wt* or *Slamf1*$^{-/-}$ mice would shed light on Omp25/SLAMF1 triggered signaling pathways.

To our knowledge, it is the first time that SLAMF1 is reported as bacterial sensor for HSCs. Therefore it is with great interest to find another pathogen that provides a distinct advantage to hematopoietic stem cells. As such, OmpC and OmpF of *Salmonella* Typhimurium bind to SLAMF1 (Berger, Romero et al. 2010). *Salmonella* LPS induces also HSCs expansion. Thus it will be challenging to decipher the downstream pathways triggered by concomitant stimulation of SLAMF1 and TLR4.

Brucella infection and hematopoietic stem cell exhaustion

At D30 p.i. the number of HSC^{LT} in the BM decreases in a Omp25/SLAMF1 showed for the first time that HSCs expand in the extra medullary sites upon recognition of microbial compounds (Massberg, Schaerli et al. 2007). Later, Takizawa *et al.* demonstrated that LPS administration *in vivo* induces an enhanced proliferation of HSCs resulting in the reduction of their competitive fitness, extramedullary hematopoiesis and increased myeloid cell production. Activation of STING pathway in HSCs also triggered HSCs loss of function and their mobilization in the spleen. (Takizawa, Fritsch et al. 2017). Both activation of STING pathway and also TLR4 signaling activation via LPS triggers a systemic infection. Due to this, these models do not allow observation of the specific effect of HSC pathogen recognition in hematopoiesis *in vivo.* Moreover, an increase of HSCs is observed in the spleen during chronic infection. It is not clear if the loss of HSCs in the BM is due to mobilization of HSCs in the spleen or to their exhaustion. In general, HSC expansion is followed by HSC exhaustion (Kobayashi, Kobayashi et al. 2015, Matatall, Jeong et al. 2016, Hirche, Frenz et al. 2017, Takizawa, Fritsch et al. 2017).

Intriguingly, *Brucella* infection did not alter the competitive engraftment capacity of HSC and did not induce extra medullary hematopoiesis in the spleen during acute infection (D8 p

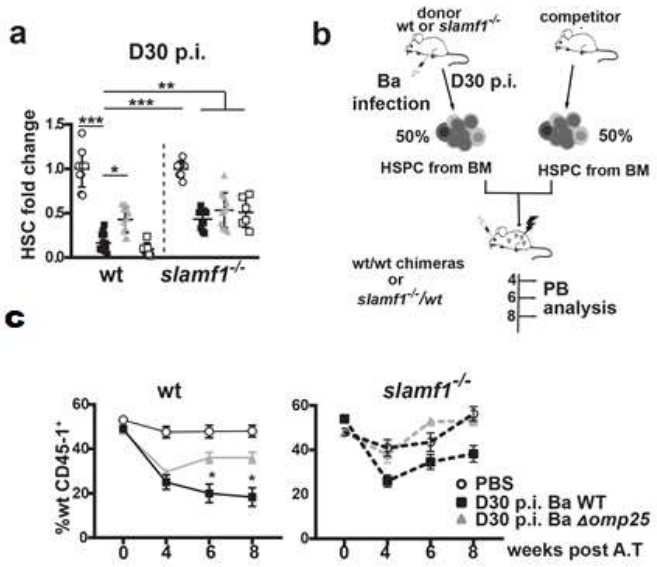


Figure 13. *B. abortus* induces HSC loss in the BM during chronic infection.

C57BL/6J wild-type (wt) mice were intraperitoneally inoculated with $1x10^6$ CFU of *B. abortus*. Thirty days later, FACS analyses were performed for bone marrow cells. HSC (lin^-,Sca^+,$cKit^+$ $CD48^-$, $CD135^-$) fold change of absolute number to Mock condition is shown for (Mock, ○) or inoculated with $1x10^6$ CFU of wild-type *B. abortus* (Ba WT, ■), *B. abortus* Δ*omp25* (▲) or *B. abortus* Δ*omp25c* (□) mutants (the latter only for wt mice). b) Experimental scheme: WT CD45.1 and *Slamf1*$^{-/-}$ CD45.1 mice were intraperitoneally innoculated with $1x10^6$ CFU of *B. abortus.* BM cells were isolated from femur and tibia of the infected mice at day 30 p.i., lineage negative cells were sorted, resuspended in PBS and transplanted with Lin^- cells from WT CD45.2 non infected mice into previously lethally irradiated WT recipient mice. Blood Flow Cytometry analyses were performed at 4, 6 and 8 weeks post transplantation. d) Percentage of engrafted CD45 cells is shown for cells from PBS injected (Mock, ○) or infected with *B. abortus* (Ba WT, ■), Ba Δ*omp25* (▲) mice. Data were obtained from 4 (a) or 2 (c-d) independent experiments, each with at least n=3 animals per condition, are shown and mean ± SEM is represented by horizontal bar. Significant differences from mock are shown. *** P< 0.001, ** P< 0.01, * P< 0.05. Absence of P value or ns, non-significant. Since data did not follow normal distribution, P-Value were generated using Kruskal-Wallis followed by Dunn's test.

In sharp contrast to acute infection, chronic infection triggered HSCs loss in the BM and extra medullary hematopoiesis in the spleen (Figure 13 and Figure 14). These results suggest that Omp25/SLAMF1 interaction is involved only during the onset of the infection in HSC function, and BM HSCs loss is a secondary effect of Omp25/SLAMF1 effect.

Further experiments should be done to determine if the decrease in HSC^{LT} number in the BM is due to HSC mobilization in the extra medullary sites or to extensive proliferation of HSC^{LT} in the BM. High levels of G-CSF in the blood could suggest HSC egress from the BM. Moreover, treatment of mice with BrdU or EdU (markers of proliferation) will bring evidence in the proliferation state of HSCs in the spleen and in the BM. It could also be possible that *Brucella* presence in the BM triggers HSC death that could explain the decrease of HSC numbers. Caspase-3 or AnnexinV staining may provide more arguments to answer this question.

SLAMF1-Omp25 axis promotes chronicity

Consistent with our *in vitro* data, *Brucella* replication is independent of Omp25 and SLAMF1 in mice during the acute phase of infection *in vivo*. However, when Omp25 or SLAMF1 are missing, the bacterial burden is significantly diminished during the chronic phase of infection. This indicates that by itself the Omp25-SLAMF1 axis does not directly control bacterial replication during early infection but alters the host response by tuning down inflammation, which as a secondary consequence influences bacterial load at the chronic stage. Removing the Omp25-SLAMF1 axis then allows the host to better control bacteria burden at chronic phase.

The transient increase of myeloid production triggered by Omp25/SLAMF1 interaction during acute infection seem to be a strategy for *Brucella* to promote infection since myeloid cells are the favorite niche of this bacterium.

This is the case for *Leishmania donovani*. The parasite burden is decreased in $Fzd6^{-/-}$ mice compared to *wt* mice due to incapacity of this mice to trigger enhanced myeloid commitment (Abidin et al 2015).To asses this , I propose to generate a SLAMF1-floxed mouse that will then be crossed with *Fgd5-CreERT2* mice. Fgd5 is only expressed in HSCs in the hematopoietic system(Gazit, Mandal et al. 2014). After crossing these 2 mice, the *Fgd5-Cre-fl-Slamf1*-fl mice

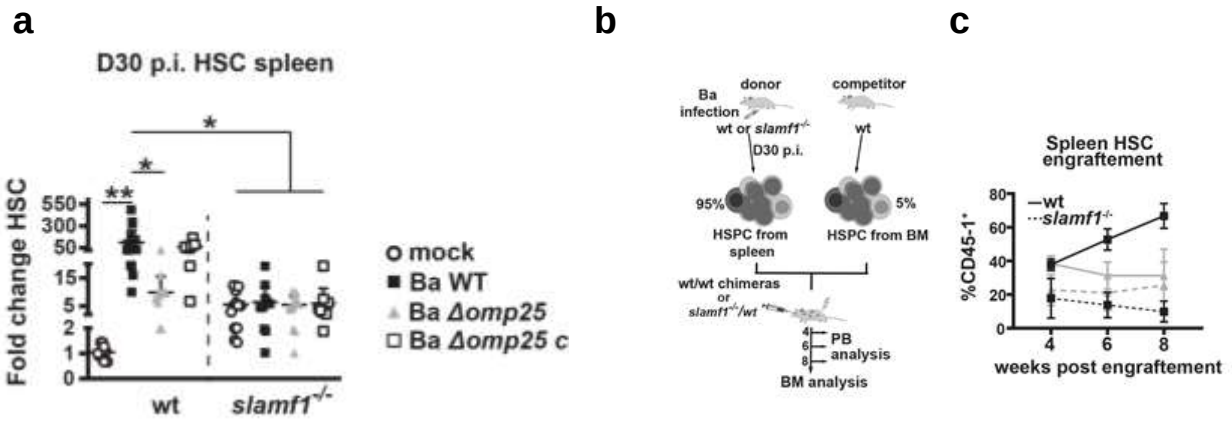


Figure 14. *B. abortus* induces HSC loss in the BM during chronic infection.

a) C57BL/6J wild-type (wt) mice were intraperitoneally inoculated with $1x10^6$ CFU of *B. abortus*. Thirty days later, FACS analyses were performed for spleen cells. HSC (lin^-,Sca^+,$cKit^+$ $CD48^-$, $CD135^-$) fold change of absolute number to Mock condition is shown for (Mock, ○) or inoculated with $1x10^6$ CFU of wild-type *B. abortus* (Ba WT, ■), *B. abortus* Δ*omp25* (▲) or *B. abortus* Δ*omp25c* (□) mutants (the latter only for wt mice). b) Experimental scheme: WT CD45.1 and *Slamf1*$^{-/-}$ CD45.1 mice were intraperitoneally innoculated with $1x10^6$ CFU of *B. abortus.* Spleen cells were isolated from femur and tibia of the infected mice at day 30 p.i., lineage negative cells were sorted, resuspended in PBS and transplanted with Lin^- cells from WT CD45.2 non infected mice into previously lethally irradiated WT recipient mice. Blood Flow Cytometry analyses were performed at 4, 6 and 8 weeks post transplantation. d) Percentage of engrafted CD45 cells is shown for cells from PBS injected (Mock, ○) or infected with *B. abortus* (Ba WT, ■), Ba Δ*omp25* (▲) mice. Data were obtained from 4 (a) or 2 (c-d) independent experiments, each with at least n=3 animals per condition, are shown and mean ± SEM is represented by horizontal bar. Significant differences from mock are shown. *** P< 0.001, ** P< 0.01, * P< 0.05. Absence of P value or ns, non-significant. Since data did not follow normal distribution, P-Value were generated using Kruskal-Wallis followed by Dunn's test.

can be generated. After tamoxifen injection, *Slamf1* will be specifically deleted in HSCs. If the transient myeloid commitment is beneficial to *Brucella*, at day 30 post-infection, the bacterial count in the spleen of tamoxifen-treated *Fgd5-Cre-fl-Slamf1*-fl mice should be lower than in infected control mice.

In addition, *in vivo* the Omp25-SLAMF1 interaction restricts the inflammatory response early on during *Brucella* infection. It promotes survival of *Ifng*-deficient mice upon *Brucella* infection, in contrast to mice infected with a mutant strain devoid of Omp25, thus revealing a Omp25/SLAMF1 down-regulated inflammation. Moreover, the SLAMF1-Omp25 axis prevents the increase of all types of splenic DCs (i.e. cDC1, cDC2 and pDCs), and inhibits downstream $CD4^+$ and $CD8^+$ T cell activation. The rise of activated $CD69^+$ T cells might augment tissue damage and explain the earlier death of Ba Δ*omp25*-infected mice compared to WT-infected ones in the lethal model of brucellosis. SLAMF1 was indeed shown to increase TCR-mediated cytotoxicity (Henning, Kraft et al. 2001). cDC1 cells are known to preferentially activate $CD8^+$ T cells and cDC2 $CD4^+$ T cells (Lewis, Williams et al. 2019). During MCMV infection *in vivo*, cDC2 cells first activate $CD4^+$ T cells, which then license $XCR1^+$ cDC1 cells to prime $CD8^+$ T cells in the spleen. cDC1 cells are thought to act as a platform to convey $CD4^+$ T cell help to $CD8^+$ cells (Chudnovskiy, Pasqual et al. 2019). Splenic pDCs are known as high producers of interferon upon activation and drivers of enhanced immunogenic response (Bauer, Dress et al. 2016). In this context, our results suggest that the binding of Omp25 to SLAMF1 *in vivo* might target these critical steps of DC-T cell interactions. Accordingly, SLAMF1 is recognized as a co-stimulatory molecule between DC and T cells (Cocks, Chang et al. 1995). Because of the absence of Omp25, *Brucella* is more potent in inducing all DC subset expansion and T cell activation *in vivo*, a possible mechanism might be that binding of Omp25 to SLAMF1 impedes SLAMF1 homophilic interaction and subsequent transduction of danger signals to host cells. As such, Omp25 might be used by the bacterium to control SLAMF1 signaling and consequently T cell activation as an immune response escape mechanism. SLAMF1, as other SLAMF receptors, associates via its cytoplasmic tail, which contains immunoreceptor tyrosine-based switch motifs (ITSM), with the SLAM Adaptor Protein (SAP) in a phosphotyrosine-dependent mode. The ITSM are used by phosphatases, such as SHP-2, and kinases, like FynT, recruited through SAP to SLAMF1 cytoplasmic domain, to control T cell activation (Detre, Keszei et al. 2010, Cannons, Tangye et al. 2011). The recruitment of the SAP/Fyn cascade may be impaired by the SLAMF1-Omp25 interaction. As SLAMF1 has recently been shown to be essential for TLR4-mediated TRAM-TRIF-dependent signaling in human macrophages

(Yurchenko, Skjesol et al. 2018), another possibility is that this interaction affects the recruitment of other signaling receptor(s) or adapter protein(s). Further investigations are clearly needed to understand how the interaction between *Brucella* Omp25 and the SLAMF1 receptor controls NF-κB nuclear translocation and defines the transduction pathways involved and their interplay.

Our data support an anti-inflammatory role of the Omp25/SLAMF1 interaction during the infection process early on *in vivo*, which mirrors what was seen in BMDC *in vitro*. Confirming prior reports (Jubier-Maurin, Boigegrain et al. 2001, Billard, Dornand et al. 2007), Omp25 stands as an important antigen synthesized by *Brucella* to control immune responses, and adds to the long list of *Brucella* proteins devoted to restrain DC immune responses, including BtpA, BtpB, PrpA, WadC or WboA (McQuiston, Vemulapalli et al. 1999, Fernandez-Prada, Nikolich et al. 2001, Salcedo, Marchesini et al. 2008, Conde-Alvarez, Arce-Gorvel et al. 2012, Salcedo, Marchesini et al. 2013, Spera, Comerci et al. 2014). The enhanced inflammation triggered by the Δ*omp25* mutant strain *in vitro* in BMDC and *in vivo* in wild-type mice or by the *Slamf1*$^{-/-}$ cells/mice infected by all *Brucella* strains tested, suggests that Omp25 plays a role in virulence *in vivo* via its association with SLAMF1. Altogether, the subtle SLAMF1-Omp25-dependent downsizing of the inflammatory response early on during the acute phase of *Brucella* infection *in vivo* promotes *Brucella* settling to proceed to chronic phase.

Final Conclusion

In conclusion, in order to better understand human brucellosis, we performed whole blood RNA seq analyses and blood cytokine measurements from human patients with acute, acute with relapse and chronic infected patients. These analyses identified SLAMF1 as the first significant blood marker for human acute brucellosis and IL-10, CXCL1/GROα, IL1RA, IL-7 cytokines as 4 combined biomarkers for detection of chronic human brucellosis.

In parallel, we discovered that *Brucella* infects and persists in the principal niche of adult HSCs: the bone marrow. This persistence was associated with granuloma formation, increased numbers of granulo-myeloid progenitors (GMP), neutrophils and $CD4^+$ T cells and also HSCPs during the acute phase of infection. Recent studies brought strong evidences that HSCs can recognize pathogen products *in vivo* through TLR or STING pathway. Both activation of STING pathway and TLR4 signaling triggers a high level of systemic inflammation (Takizawa, Fritsch et al. 2017). Because of this, it is difficult to distinguish between the direct and indirect effect of pathogen recognition in HSCs. During my thesis, we demonstrated that the murine

model of brucellosis is a very suitable system to study the effect of direct pathogen recognition in BM HSCs *in vivo* since *Brucella* is a stealthy pathogen that persists in the BM for more than six months without systemic immune activation. In a mechanistic point of view, we discovered that HSCs recognize *Brucella* in the BM via SLAMF1. On the one hand, this interaction triggers myeloid differentiation of HSCs suggested as responsible of anemia presentation during brucellosis. On the other hand, SLAMF1 finely tune inflammation down in mature hematopoietic cells during the acute phase of infection in order to allow *Brucella* to settle in its furtive replicative niche, the endoplasmic reticulum, and for progression to the chronic phase. The Omp25-SLAMF1 interaction therefore illustrates a novel discrete evasion strategy exploited by *Brucella* within infected DCs to mediate its dissemination in vivo by controlling innate immune responses in a timely manner thus fostering chronicity.

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A library is the delivery room for birth of ideas, a place where history comes to life.

Norman Cousins

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Résumé vulgarisé

La brucellose est une maladie qui se transmet de l'animal à l'homme. Elle est causée par la bactérie *Brucella*. Lors de ma thèse, j'ai montré que *Brucella* persiste dans les cellules de la moelle osseuse des animaux infectés. Ces observations sont très importantes car la moelle est un organe responsable de la génération des cellules du système immunitaires et c'est la principale niche des cellules souches hématopoïétiques. Au cours de ma thèse, j'ai montré que la protéine de la membrane externe 25 de *Brucella* (Omp25) est capable de lier au récepteur SLAMF1, une molécule exprimée par les cellules souches hématopoïétiques. Cette interaction conduit à la génération d'un plus grand nombre de cellules myéloïdes par les cellules souches hématopoïétiques. Les cellules myéloïdes sont la niche préférée de *Brucella*. Ainsi, cette stratégie permet à la bactérie d'envahir l'hôte et d'établir une infection chronique de longue durée. SLAMF 1 apparaît comme une nouvelle cible thérapeutique pour le contrôle des maladies infectieuses chroniques, ce qui représenterait une avancée importante dans la génération de nouveaux médicaments.

Outreach abstract

Brucellosis is a disease that is transmitted from animals to humans. It is caused by the pathogenic bacterium *Brucella*. During my thesis, I showed that *Brucella* persists in the bone marrow cells of infected animals. These observations are very important because the bone marrow is an organ of the immune system responsible for the generation of the immune cells, as it is the principal niche of hematopoietic stem cells. During my thesis, I showed that *Brucella outer membrane 25 (Omp25)* is able to bind SLAMF1, a hematopoietic stem cell molecule. This interaction leads to the production of more myeloid cells by the hematopoietic stem cell. Myeloid cells are the favorite niche of *Brucella*. Thus, this strategy allows the bacteria to invade the host and establish a long lasting chronic infection. SLAMF 1 appears as a new therapeutic target for controlling chronic infectious diseases, which would represent an important advance in the generation of new drugs.

Permbledhje per publikun

Bruceloza është një sëmudje infektive që transmetohet nga njeriu tek kafshet. Mikrobi pergjegjës për këtë sëmundje është bakteria e quajtur *Brucella*. Gjatë punimeve te doktoratures, une zbulova qe kjo bakteri qëndron ne qelizat e palcës se kockave. Këto vezhgime janë shumë të rëndësishme sepse në palcë gjenden qelizat steminale te sistemit imun, pergjegjëse për prodhimin e qelizave te gjakut. Për më tepër, une vura re që një molekulë e bakteries që quhet Omp25 lidhet me molekulën e siperfaqes te qelizave steminale SLAMF1. Bashkeveprimi midis këtyre dy molekulave detyron qelizat steminale te prodhojn ë më shumë rruaza te bardha te typit myeloik sesa rruaza te kuqe duke shkaktuar keshtu anemi tek te semuret. Zakonisht qelizat myeloike sherbejnë për të luftuar kundër mikrobeve por në rastin et *Brucelles* keto qeliza jane fole te infeksionit. Si rezultat, rritja e qelizave myeloike shoqërohet me shtimin e bakterieve ne trup që shkaktojne me vonë nje infeksion kronik. Zbulimi i nje ilaci që bllokon bashkeveprimin midis molekules SLAMF1 dhe Omp25 do te ishte nje avancim kolosal për trajtimin e sëmudjeve infektive.