MASTER IN BIOCHEMISTRY AND MOLECULAR AND CELLULAR BIOLOGY

Impact of Trypanosoma brucei infection on the contrai of Brucela melitensis infection in mice

Van Vyve, Margaux

Award date:
2017

Awarding institution:
University of Namur

UNIVERSITÉ DE NAMUR

Faculté des Sciences

IMPACT OF *TRYPANOSOMA BRUCEI* INFECTION ON THE CONTROL OF *BRUCELLA MELITENSIS* INFECTION IN MICE

Mémoire présenté pour l'obtention

du grade académique de master 120 en biochimie et biologie moléculaire et cellulaire

Margaux VAN VYVE

Janvier 2017

Table of contents

ABSTRACT

Université de Namur
FACULTE DES SCIENCES
Secrétariat du Département de Biologie
Rue de Bruxelles 61 - 5000 NAMUR
Téléphone: + 32(0)81.72.44.18 - Téléfax: + 32(0)81.72.44.20
E-mail: joelle.jonet@unamur.be - http://www.unamur.be

Impact of *Trypanosoma brucei* infection on the control of *Brucella melitensis* infection in mice

VAN VYVE Margaux

Summary

Brucellosis is one of the most common worldwide zoonosis caused by a facultative intracellular gram negative coccobacilli of the genus *Brucella.* It affects a wide range of mammals and induces abortion and sterility causing huge economic issue. *Brucella* is a furtive bacteria generating a chronic disease if not treated. At this time, no treatment nor safe or effective human vaccines exist.

In this master thesis, we demonstrated that *Brucella melitensis* persists in a $CD11c^+$ reservoir cells able to resist to protective memory response in wild type C57B/6 mice. Surprisingly, the co-infection by the parasite *Trypanosoma brucei* appears able to increase the immune control of chronic *Brucella* infection. Following co-infection, the mice become able to eliminate *Brucella* thanks to a $CD4^+$ T cells dependent Th1 immune response, as demonstrated by using IL-12 and IFNγ deficient mice. This mechanism is not antigen specific as *T. brucei* co-infection is also able to favor the elimination of *B. suis* and *B. abortus* and that *T. cruzi* co-infection display a similar effect on *B. melitensis*.

Another central point of this work was to characterized *Brucella*'s dissemination following several route of infection. We highlighted the fact that the route of dissemination affects the dissemination and could thus affects the pathology of brucellosis.

Mémoire de master 120 en biochimie et biologie moléculaire et cellulaire

Janvier 2017

Promoteurs: E. Muraille

J-J. Letesson

Université de Namur
FACULTE DES SCIENCES
Secrétariat du Département de Biologie
Rue de Bruxelles 61 - 5000 NAMUR
Téléphone: + 32(0)81.72.44.18 - Téléfax: + 32(0)81.72.44.20
E-mail: joelle.jonet@unamur.be - http://www.unamur.be

Impact de l'infection par *Trypanosoma brucei* sur le contrôle de l'infection par *Brucella melitensis* chez la souris

VAN VYVE Margaux

Résumé

La brucellose est une des zoonoses les plus courantes dans le monde. Cette pathologie est causée par une bactérie gram négative intracellulaire facultative du genre *Brucella.* Une large gamme de mammifères peuvent être touchés par cette maladie qui induit l'avortement ainsi que la stérilité chez les animaux causant de graves problèmes économiques. *Brucella* est une bactérie furtive qui, non traitée, induit une maladie chronique. A l'heure actuelle, il n'existe pas encore de traitement ni the vaccin sûr et efficace pour l'homme.

Durant ce mémoire, nous avons démontrés que *Brucella melitensis* persiste dans dans cellules réservoirs $CD11c^+$ capable de conférer une résistance face à la réponse mémoire protectrice dans des souris wild type C57BL/6. De façon surprenante, une co-infection avec le parasite *Trypanosoma brucei* permet d'améliorer le contrôle de l'infection chronique générée par *Brucella.* Suite à une co-infection, la souris devient capable d'éliminer *Brucella* grâce à une réponse immunitaire de type Th1 dépendante des lymphocytes T $CD4^+$, comme démontrer en utilisant des souris déficientes poour l'IL-12 et l'IFNγ. Ce mécanisme n'est pas antigène spécifique sachant qu'une co-infection avec *T. brucei* est également capable de favoriser l'élimination de *B. suis* et de *B. abortus* mais aussi qu'une co-infection avec *T. cruzi* présente un même effet sur *B. melitensis*.

Un autre point central de cette étude est la caractérisation de la dissémination de *Brucella* suivant différents modes d'infection. Nous avons pu observer que la voie d'infection impactait fortement la dissémination et pourrait donc jouer sur l'expression de la pathologie de la brucellose.

Mémoire de master 120 en biochimie et biologie moléculaire et cellulaire

Janvier 2017

Promoteurs: E. Muraille

J-J. Letesson

INTRODUCTION

Introduction

Individual variability

Individual variability is a very important element to assure the robustness of populations and species to natural selective pressures. Every single individual has a different response to infection and present his own capacity to adapt to. Identify the at-risk person for an epidemic could lower the mortality risk but to do so, the implicated factors in this susceptibility to face an infection must to be identify. On the other hand, some persons are resistant to infection. Different factors are already known to have an effect on the ability of the host to control an infection. The most obvious parameter is the genetic variability that determine the ability to develop an effective immune response. Other elements also are taken into account. The microbiota, depending on the diet, but also the immune history of the host can influence the ability of the person to face the infection.

The microbiota is composed by the micro-organisms colonizing the host from its birth. It is a community of fungus, bacteria and viruses modeling the immune response and thus, influencing the ability of the host to face an infection (Kitano & Oda 2006; Cerf-Bensussan & Gaboriau-Routhiau 2010). It has been shown that "germ-free" mice were more susceptible to an infection, indicating the role of the microbiota in the maturation of the immune system (Buffie & Pamer 2013). The microbiota also protects from the infections by competition with pathogenic. Microbiota competes with the pathogens on different fields: for the nutrients, by producing toxic compounds or by making less accessible the structure facilitating the adherence of the pathogen to the epithelium. But the microbiota can also trigger a cross-reactive immune response facilitating the elimination of the pathogen (Zeng et al. 2016).

The microbiota is not the only element to act on the individual ability to face an infection: the immunological background of the host also plays a great role in this variability. Many epidemiological and experimental studies have shown that the vaccination or the exposition to an infection modify the immunological status and allow the host to be more resistant to other infection even when the host is free from the infectious strain. It is called "heterologous immunity" (Welsh & Selin 2002). As an example, the Bacillus Calmette-Guérin (BCG) vaccin gives protection against tuberculosis but also against secondary infections like *Listeria monocytogenes* (Blanden et al. 1969), *Staphylococcus aureus* (Sher et al. 1975) and *Plasmodium yoelii* (Matsumoto et al. 2000).

With the immune history, the cross pathologies are another important point. When an infection occurs, the host is rarely naive. It is usual in nature to be co-infected by several pathogens at the same time. Almost everybody is concerned by latent infections like those caused by the herpes virus, Epstein–Barr virus, *Plasmodium falciparum* or *Mycobacterium tuberculosis*. Those cross-pathologies influence the immune status of the host on different ways like the alteration of the tissue architecture and the modification of the metabolism of the host cell but also the activation or inhibition of the protective immune response (Hakansson et al. 1994; Potian et al. 2011; Lokken et al. 2014; Furman et al. 2015).

While my master thesis we worked on the cross-pathologies aspect of the individual variability during an epidemic. This aspect is little studied but it is a very common condition of an immunological response. It is also important to be able to predict the behavior of a host when he is facing a co-infection and in a therapeutic interest, to determine the protection mechanisms and susceptibility markers. That could lead to identify individuals responsible for

a big part of the dissemination and thus, responsible for the spread of the pathogen. In this model, we study the impact of cross-pathologies on a disease affecting many individuals, humans and animals included, covering a large part of the globe and furthermore persisting in the host in absence of a treatment. Taking all this into account, brucellosis is a well-fitting model.

Brucella

Brucella: the causative agent of brucellosis

Brucella is a gram negative α-proteobacteria and the causative agent of a chronic infectious disease: brucellosis. This pathogen is a facultative intracellular coccobacillus. The gender *Brucella* is divided into 12 species and each are specific for a mammalian host but this specificity is not stringent (**Table 1**). 6 of them are able to infect accidentally humans. The main species infecting humans are *B. melitensis, B. abortus* and *B. suis*. Brucellosis, also known as Malta's fever, is a worldwide spread zoonosis with more than 500.000 new human cases diagnosed per year (Pappas et al. 2006) but this incidence is widely underestimated because of the difficulty of access to healthcare in some endemic countries but also because of the variability of symptoms. The most concerned regions are Central America, South America, Middle-East, Africa and West Asia but also in southern Europe (**Figure 1**)(Pappas et al. 2006; Medzhitov 2007; Corbel 1997).

Brucella species and their preferential hosts

Species	**Preferential host**
B. melitensis	Sheep, goat, camel
B. abortus	Cattle, buffalo, elk, yak, camel
B. suis	Pig, boar, deer, rodent
B. canis	Dog
B. ovis	Sheep
B. neotomae	Rodents
B. ceti	Porpoise, dolphin, wale
B. microti	Fox, vole
B. pinnipedialis	Orca
B. papionis	Monkey
B. vulpis	Fox
B. pyxicephali	Buffalo toad

Table 1:* Brucella *species and their preferential hosts

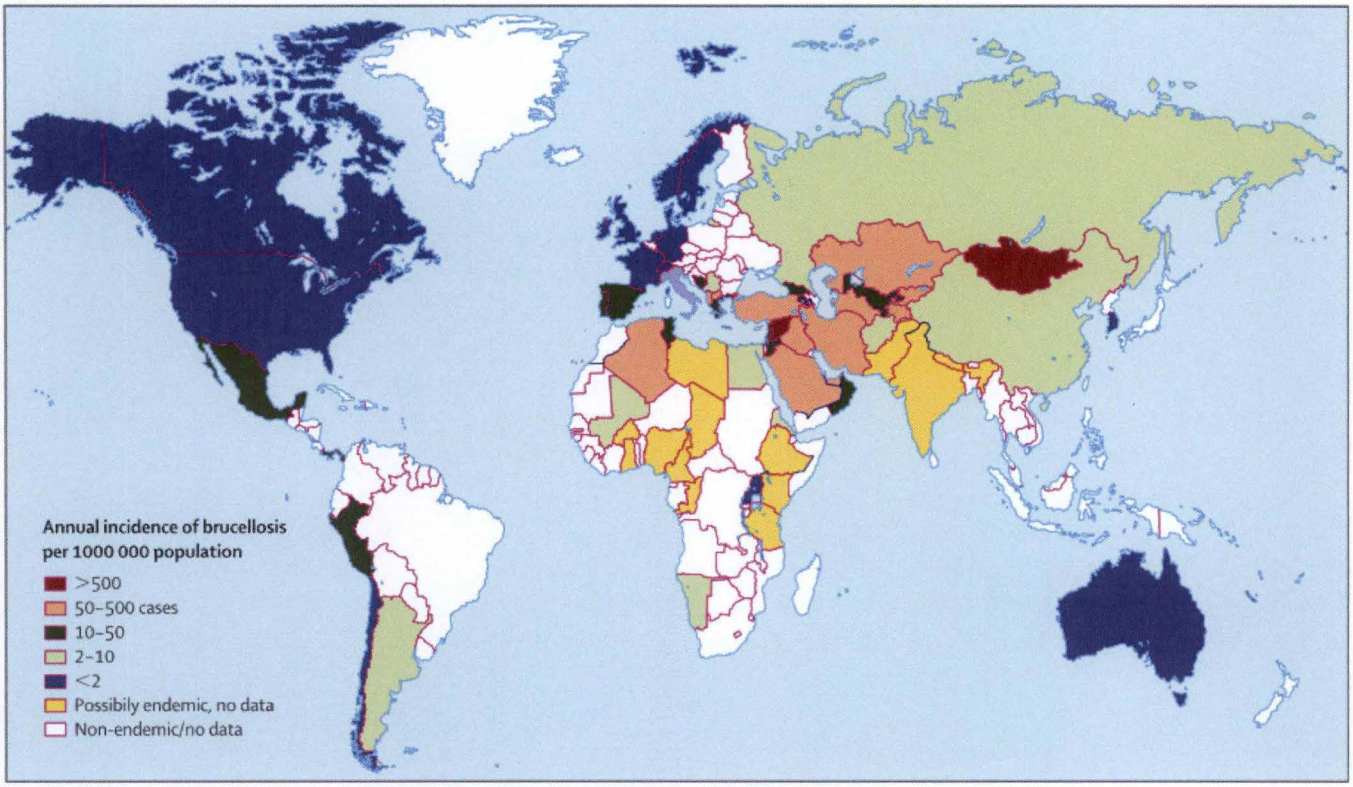


Figure 1: Incidence of human brucellosis worldwide (from (Pappas et al. 2006) *)*

Brucellosis: symptoms and transmission

The human form of brucellosis is characterized by an undulant fever specific of the acute phase. Without treatment, the disease enters into a chronic form with debilitating complications such as encephalitis, meningitides, endocarditis, arthritis, and in some cases it can conduct to abortion (Schneider et al. 2011). It is estimated that only 10 to 100 bacteria are enough to infect humans, combined with its ability to aerosolize makes *Brucella* a potential bioterrorism agent (De Figueiredo et al. 2015). Humans can be infected by different routes: ingestion of contaminated unpasteurized milk or cheese, skin contact and inhalation of aerosol (Atluri et al. 2011).

The animal form of brucellosis is characterized by abortion in females and sterility in males. *Brucella* is known to have a tropism for trophoblastic cells (Saenz et al. 2008) and for the placenta in pregnant mice causing the destabilization of the homeostasis and the death of the fetus. The animal contamination can be either horizontal: the most frequent and due to the aerosol produce during the abortion or the contamination of the food and the water by animals' secretion, either vertical by the ingestion of contaminated milk (Díaz Aparicio 2013). In endemic region, the economic cost due to the loss of milk production and the abortion can be significant (Seleem et al. 2010).

Diagnostic

In Belgium, 5 to 10 new human cases of brucellosis are diagnosed each year (Infectious diseases of Belgium, Stephan Berger, Ed. Guideon 2015, p. 63). The disease can be diagnosis by an isolation of the bacteria in corporal fluid such as the blood, cerebrospinal fluid and synovial fluid. This technic is not very effective and an alternative exists: the serological test. It consists on testing the presence of anti-*Brucella*'s LPS antibody in the serum. (details in the "Manual of diagnostic tests and vaccines for terrestrial animals", 2016, published by the

IAO). A combination of several tests is still recommended because of the low sensibility of those test but also because of the cross-reaction that can be observed following an infection with *Yersinia enterolitica* O9 (Ducrotoy et al. 2016).

Existing treatments and vaccines

In endemic regions, animals can be vaccinated to prevent risk of infection. There are two main vaccine strains that are both life-attenuated: S19 strain for bovine vaccination and Rev1 for ovine and caprine vaccination. Those vaccines can induce abortion and infect humans. Furthermore, Rev1 is streptomycin resistant which is an antibiotic used to cure virulent strains (Garcia-Yoldi et al. 2006). Those vaccines induce anti-LPS antibody that can interfere with the diagnostic tests (Makala et al. 2002).
Infected animals are euthanized to restrict the spread of the bacteria. Infected humans have to take a combination of two antibiotics (doxycycline and rifampicin) during 6 to 8 weeks to avoid complications. Those medicines control the division of the bacteria and limit the risk of relapse.

Discovery of brucellosis

David Bruce is the first man to characterized *Brucella* in 1850 during Crimean War. He isolated the bacteria from the spleen of soldiers who died from an unknown pathology characterized by an undulant fever. He first named the pathogenic agent as *Micrococcus melitensis*. Half a century later, in 1905, *Brucella melitensis* is isolated from the milk of a contaminated goat highlighting its zoonotic feature (Seleem et al. 2010).
The first reliable hint of brucellosis comes from bones from the 10^{th} to 13^{th} centuries with typical lesions of brucellosis or tuberculosis that were found in ancient Albanian cities. Genetics analysis revealed the presence of *Brucella*'s DNA showing that brucellosis was endemic in those regions at that time (Mutolo et al. 2012).

Intracellular trafficking of *Brucella*

The intracellular traffic of *Brucella* has been described in macrophages and dendritic cells *in vitro*: the bacteria is opsonized or phagocyted by the cells. Inside the cell, *Brucella* resides in a vacuole, called *Brucella* containing vacuole (BCV), carrying early endosome marks. Fusion with late endosome and lysosome acidifies the vacuole and this acidification induces the expression of *virB* operon which encodes for the type IV secretion system (T4SS). T4SS is a virulence factor allowing the liberation of effectors in the cytoplasm of the host cell that deflect the intracellular traffic of the cell (Billard et al. 2008; Marchesini et al. 2011; De Jong et al. 2008). After 4 to 6 hours, the bacteria then reach the endoplasmic reticulum before starting to replicate (Deghelt et al. 2014). 72h post infection, some bacteria reside in a double membrane compartment with autophagy markers that allowed *Brucella* to leave the cell to invade others (Starr et al. 2012) (**Figure 2**).

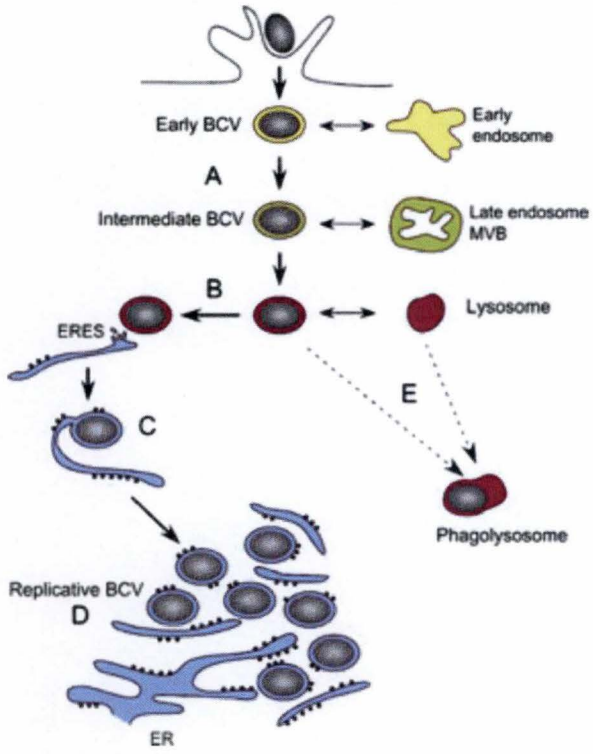


Figure 2: Intracellular trafficking of* Brucella *(from (Starr et al. 2008)) After its entry in the host cell, *Brucella* resides in a vacuole: *Brucella* Containing Vacuole (BCV) which merges with an early endosome to become an early BCV and then with a late endosome to turn into an intermediate BCV that fuses with a lysosome. This vacuole interacts with the exit site of the endoplasmic reticulum (ERES) in order to form a replicative BCV providing an environment conducive to *Brucella*'s replication.

Brucella's niche

Brucella is a furtive pathogen causing chronic disease due to its ability to persist in cells that we called "reservoir cells". Those cells seem give a protection against the immune system and to be favorable to the replication of the bacteria. Following an intraperitoneal infection, *B. melitensis* infects principally the dendritic cells and the macrophages in the spleen: the macrophages of the red pulp and the metallophilic macrophages of the marginal zone (MMMs). As the infection evolve into its chronic phase, the infected cells are mainly localized in the white pulp of the spleen (Copin et al. 2012). The reservoir cells have been described in the spleen of susceptible IL-12p40$^{-/-}$ mice, following an intranasal infection. They presented a specific phenotype such as the expression of CD11c and CD205 receptors whose are markers of the dendritic cell (DC) population. These cells are distinct from classical myeloid-related CD8α $^{-}$ CD11b^{+} DC present in the spleen of naive mice and from CD11b^{+} inflammatory DC present during the acute phase of infection by the absence of CD11b and F4/80 staining. These reservoir cells are also distinct from the CD8α^{+} DEC205^{+} lymphoid-related DC subset located in the T cell area of naive mice. The infected cells possibly derive from the marginal zone and differentiate from MOMA-1^{+} MMM (metallophilic marginal zone macrophages) and ER-TR9^{+} MZM (marginal zone macrophages) because of their expression of MOMA-1 or SIGN-R1/ER-TR9. Those cells display also other specific markers such as Bodipy (indicating a high lipid content) and arginase 1 (Hanot Mambres et al. 2015) **(Figure 4) (Box1).**

Box 1: Phenotypic markers of macrophages

Macrophages are part of a heterogeneous cell population polarizing in response to local stimuli. It exists two main phenotype of macrophages: M1 and M2, based on the receptors expressed, the cytokines and the effector molecules produced but also on their function.

Pro-inflammatory or M1 macrophages are induced by IFN-γ which can be combined with TNF, LPS but also with Granulocyte Macrophage Colony Stimulation Factor (GM-CSF). Those cells are inducer and effector cells in the Th1 inflammatory response. Activated M1 macrophages express a high level of IL-12 and IL-23 and metabolize arginine into NO which secreted along with inflammatory cytokines (Brown et al. 2009).

The activation of M2 macrophages is induced by diverse cytokines: IL-4, IL-13 and IL-10; by glucocorticoid and also by immune complex. When activated those cells express a high level of IL-10 and via the metabolization of arginase, produce ornithine which is involved in a Th2 response. M2 macrophages facilitates tissue repair (Verreck et al. 2006; Gordon & Taylor 2005).

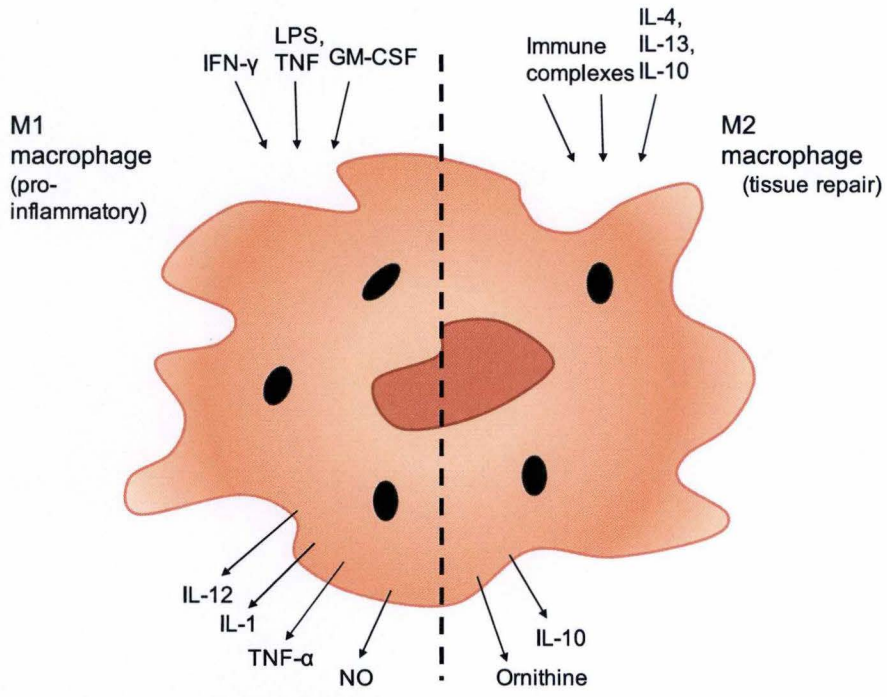


Figure 3: Macrophages M1 and M2 polarization IFNγ, LPS combined with TNF or GM-CSF induce the polarization into M1 macrophages. When activated, they express inflammatory mediators. The presence of immune compexes, IL-4, IL-13, IL-10 induce the polarization into M2 macrophages. In their activated, those repairing tissue macrophages secrete ornithine and IL-10.

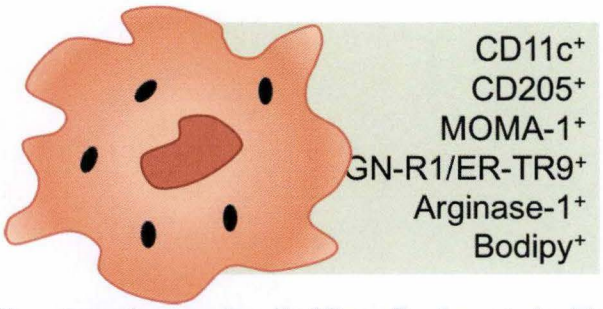


Figure 4: Phenotype of reservoir cell of Brucella characterized in susceptible mice

Particularities of *Brucella suis, abortus* and *melitensis*

B. melitensis preferentially infects sheeps and goats whereas *B. abortus* is a bovine pathogen and *B. suis* is a swine pathogen. *B. melitensis* is known to be the most pathogenic one for human, followed by *B. suis* (Young 2002). In the literature, little can be found about the pathogenic differences in mice between the different strains of *Brucella*. Following an intranasal mode of infection in mice, it has been observed that *B. abortus* and *B. suis* are able to persist longer than *B. melitensis* in the lungs.

Concerning the dissemination of *Brucella* in mice, it has been shown that following the route of infection the bacteria behavior is different. Splitter's team has shown that following an intraperitoneal injection, *B. melitensis* is found almost instantaneously in almost every organ (Rajashekara et al. 2005) and our team has shown that following more physiological route of infection like the intranasal infection, *B. melitensis* resides in the lungs and only 5 days after the infection the bacteria can be found in the spleen and in the liver (Hanot Mambres et al. 2015) but also that following an intradermal infection, the bacteria was found in the lesion in early times and only after 5 days, in the spleen and in the liver (A. Demars, 2015).

It could be interesting to investigate more the differences between the different species in a multi-organ way.

Immune response against *Brucella*

The innate immune system is able to detect the presence of pathogens by the recognition of conserved domains, called PAMPs (Pathogen Associated Molecular Patterns), by receptors specific of those domains: the PRRs (Pattern Recognition Receptors). Once the pathogen is identified, an inflammatory response coupled with an adaptive immune response is triggered.

Brucella has established different strategies to escape the immune system; this furtive bacterium is able to limit the expression of PAMPs but also to modify their structure to lower the recognition by the PRRs (Gorvel 2008). For example, the lipopolysaccharide (LPS) of *Brucella* contains a longer O-chain that causes a hindered limiting the recognition by the Toll-Like Receptor 4 (TLR4) (Cardoso et al. 2006). The bacteria express no pili nor fimbriae and capsule that are also elements detectable by the immune system (Martirosyan & Gorvel 2013) and the flagellin expressed by *Brucella* contains no recognition domain for the TLR5 (Terwagne et al. 2013). When in the host cell, *Brucella* is able to inhibit the development of the immune response. For example, *Brucella abortus* expresses two proteins: *Brucella* Tir-containing protein 1 and 2 (Btp1, Btp2), presenting homologies with TLR and thus, interfering with the pathways of TLR2 and TLR4. As a consequence of that, infected dendritic cells have a lower maturation and activation decreasing the production of pro-inflammatory cytokines such as Tumor Necrosis Factor α (TNFα) and interleukin-12 (IL-12) (Cirl et al. 2008; Salcedo et al. 2008).

Despite all the mechanisms designed by this furtive bacterium to escape the immune system, some receptors are still able to detect this pathogen and trigger an immune response. For

example, NLCR4, a cytosolic receptor, detects the flagellin of the bacterium (Terwagne et al. 2013).
The immune response against *Brucella* following an intranasal infection triggers an IL-17RA dependent immune response in the early stage of infection in the lungs and it represents the first line of defense against the pathogen. The production of interleukin-17 (IL-17) by Th17 lymphocytes is triggered by the secretion of IL-12, IL-23 and IL-27 by dendritic cells and macrophages (Ulrich E. Schaible and Albert Haas, Ed. Wiley Blackwell, pp. 220-221). IL-17 targets the neutrophils and induces their migration, maturation and activation starting a local inflammatory response. However, a deficiency of IL-17RA has only a minor impact on the first days of *Brucella* infection and is rapidly counterbalanced by the Th1 immune response (Pasquevich et al. 2011; Hanot Mambres et al. 2016).

The effective primary immune response against *Brucella* during the plateau stage of the infection in the spleen is mainly an IFNγ dependent Th1 immune response. This response is initiated by the presentation of the microbial antigens by the major histocompatibility complex II (MHCII) and the secretion of interleukin-12 (IL-12) by the infected cells (mainly macrophages and dendritic cells) to the naive $CD4^+$ T cells inducing their activation and differentiation. IFNγ production by the $CD4^+$ T cells leads to the secretion of antimicrobial effectors such as nitric oxide (NO) by myeloid cells (Vitry et al. 2014). These results have been obtained by using genetically deficient mice for IL-12 and MHCII ($CD4^+$ T cells activation). Those mouse were susceptible to *Brucella* infection when TAP1 ($CD8^+$ T cells activation) deficient mice were not (Copin et al. 2012), **(Table2)**.

Deficient mice	
$TAP\text{-}1^{-/-}$	Transporter Associated with the Antigen Processing 1. Subunit for a transporter involve in the delivery of peptide across the RE membrane to class I molecules. → Defective in the assembly and in the transport of class I molecules. Their cells expressed a severe reduced level of MHC I and thus, deficient in the activation of $CD8^+$ T cells.
$IL\text{-}12p35^{-/-}$	Gene encoding for a subunit of IL-12 (IL-12p35). IL-12p35 and is involve in the induction of IFNγ and in the Th1 immune response.
$MHCII^{-/-}$	Major Histocompatibility Complex II. Receptor involve in the presentation of peptides of extracellular proteins to $CD4^+$ T cells. → $CD4^+$ T cells in the thymus but they are not able to maturate.

Table 2: Deficient mice and impact of the deficiency

Protective immune response against secondary infection with *Brucella*

After a first contact with *Brucella*, the host is able to better control the pathogen thanks to a protective memory immune response. Following an intranasal inoculation, secondary infection is principally controlled in the lungs: the immunized mice show a bacterial load 60% lower in the spleen than primary infected. The $CD4^+$ T cells keep an important role in the control of a second infection in mice (Hanot Mambres et al. 2016). It is interesting to note that $CD8^+$ T cells also play a role upon a secondary intranasal infection in this model (Hanot Mambres et al. 2016; Clapp et al. 2016). Other studies have shown that in an intraperitoneal secondary infection model, circulating specific antibodies have a crucial role in the control of the dissemination of *Brucella* but that in this case, but there is also an important role of $CD4^+$

T cells and the MyD88/IL-12p35 signaling pathway (Vitry et al. 2014). The nature of the protective immune response seems thus to be depend on the mode of infection.

Brucellosis is a chronic disease which if untreated leads to diverse complications such as endocarditis, osteoarthritis and neurological disorders. Suggesting that external factors like the co-infection are able to influence the control of the pathology, we develop different model of cross-pathologies and in this case, we studied the impact of *Trypanosoma brucei* on the ability of mice to control *Brucella*'s infection. Trypanosomiasis induces a strong inflammatory response and we aim to destabilize the furtivity of *Brucella*, which is little inflammatory, by triggering a strong inflammatory response.

Trypanosoma brucei

Trypanosoma brucei: ID and disease

Sleeping sickness or Human African Trypanosomiasis (HAT) is a vector-borne disease due to a flagellate protozoan: *Trypanosoma brucei*. This parasite belongs to the *Trypanosomatidae* family and to the *Trypanosoma* genus. It has the structure of a eukaryotic cell with a tubular mitochondria and a kinetoplast containing the circular mitochondrial DNA. It exists a large panel of *Trypanosoma* species but *Trypanosoma brucei* is the only species that causes HAT. *Tyrpanosoma brucei* can be divided on 3 different subspecies: *Trypanosoma brucei brucei, Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*. *T. brucei* infects mammalians but only *T. brucei gambiense* and *T. brucei rhodesiense* infect humans.

The most concerned regions by this disease are rural regions of sub-Saharan Africa. Sleeping sickness, or Nagana as it is called for animals, is transmitted from an infected mammalian to another via the bite of a carrying Tsetse fly but the mother-to-child infection also occurs because of the capacity of the parasite to cross the placenta.

Millions of people in sub-Saharans countries are concerned by the disease and many of the affected population live in remote rural areas with limited access to health services. It complicated the diagnosis but also the treatment and the surveillance of the disease.

The human trypanosomiasis exists under two different forms depending on the subspecies causing the disease: *T. brucei gambiense* cracks down in 24 countries in west and central Africa and this subspecies represented 98% of the human cases of trypanosomiasis. It causes chronic infection with detectable symptoms that occur few months to years after being infected. It is mostly too late for the patient who is already in an advanced stage of the disease. *T. brucei rhodesiense* can be found in 13 countries in eastern and southern Africa and unlike *T. brucei gambiense*, it causes an acute disease and the first symptoms occur weeks to months post infection but this form of the disease knows a rapid development into the central nervous system stage. Animals can host the humans' pathogens and act as a reservoir, especially for *T. brucei rhodesiense* but it is also true for *T. brucei gambiense*.

The development of the disease is divided onto two stages: the haemo-lymphatic stage corresponding to the multiplication of the parasite in subcutaneous tissues, the blood and the lymph and with intermittent fever, and the neurological or meningo-encephalic stage when the parasite has cross the brain-blood barrier. This stage has more obvious symptoms such as a change of behavior, confusion, sensory disturbance, coordination problem but also disturbance of the sleep cycle giving its name to the pathology.

Life cycle of *T. brucei*

Trypanosoma brucei is an extracellular parasite that needs a part of its life cycle in a mammalian host but also a part in a Tsetse fly of the genus *Glossina*. By taken the bite by a

Tsetse fly of an infected mammalian as the first step of the cycle, the parasite enters into the midgut of the fly along with the blood meal into the bloodstream trypomastigote stage. It then differentiates into procyclic trypomastigote and starts a division. After a journey through the fly, the parasite reaches the salivary glands where it turns into an attached epimastigote which is able to complete its life cycle part into the *Glossina* by an asymmetric division generating a metacyclic trypomastigote: the mammalian's adapted form. The infection of a mammalian host bitten by a carrying Tsetse fly allowed the rest of the life cycle. Following the bite, metacyclic trypomastigotes that are in the bloodstream of the host turn into the so-called bloodstream trypomastigote which is able to multiply but also to cross the blood vessel endothelium in order to colonize extravascular tissues including the central nervous system (Langousis & Hill 2014; Vickerman 1985) **(Figure 5)**.

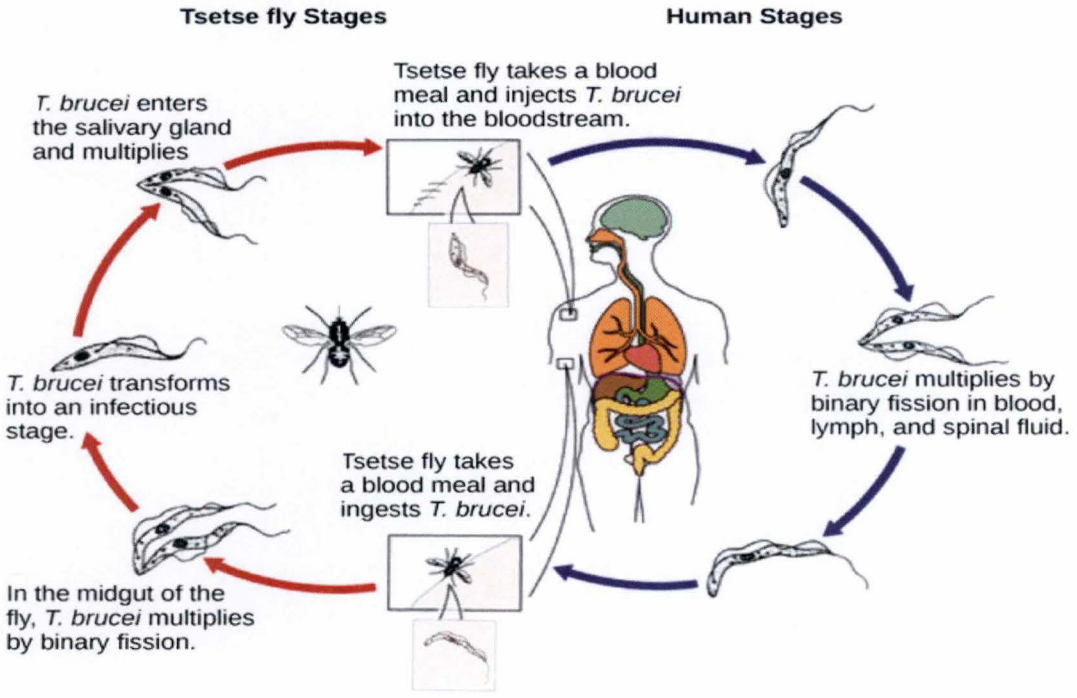


Figure 5: Life-cycle of* Trypanosoma brucei *inside human host and Tsetse fly (from A. de Silva and M. Moser, Centers for Disease Control and Prevention) The blood form of *Trypanosoma brucei* is ingested by the Tsetse fly along with the blood while taking a blood meal. In the midgut of the fly, *T. brucei* turns into its procyclic trypomastigote form and multiply in a binary fission way. It then transforms into an infectious stage and migrates to the salivary gland where it multiplies and turns into a metacyclic form that will be injected in the bloodstream of a mammalian host during the next blood meal of the Tsetse fly. When in the bloodstream, *T. brucei* turns into the bloodstream trypomastigote form and reach other body fluid (blood, lymph and cerebrospinal fluid) where it multiplies itself by binary fission.

Immune response against *T. brucei*

During *T. brucei* infection, the surface glycoproteins allowed the immune system to detect the presence of the parasite and to trigger an immune response against it. A strong Th1 immune response depending on the B cells lymphocytes is induced by the recognition of those Variant Surface Glycoprotein (VSG). Macrophages and dendritic cells exposed the VSG via MHCII to the T cells lymphocytes to trigger the Th1 immune response. This response is characterized by a strong production of IFNγ by the $CD4^+$ T cells. Activated macrophages produce

antimicrobial factors such as TNFα and oxygen-derived reagents (H_2O_2) and nitrogen (NO) (Pays et al. 2006; Namangala et al. 2001).
T. brucei is able to escape the immune system by switching its VSG every 5 days, translated by peak of parasitaemia (Horn 2014). About 2000 genes implicated in the VSG are encoded in *T. brucei*'s genome but only one form is expressed at once (Mugnier et al. 2015). The recognition of the surface glycoproteins induces antibodies production, IgMs and IgGs, specific to those proteins. The parasite is able to change its glycoproteins in response to the immune pressure making it invisible to the immune system until it produces new antibodies (Marcello & Barry 2007).

Immunosuppressive effect of *T. brucei*

Trypanosoma brucei is known to induce an immunosuppressive effect. The immunosuppressive effect revokes the protective responses induced by vaccines but also prevent the development of a B cells-mediated memory response which causes a public health issue in countries where African trypanosomiasis is endemic (Radwanska et al. 2008). It has been shown that mice infected by *T. brucei* present a declining antibody response as the infection progresses until a low rate of IgM anti-VSG (Sacks & Askonas 1980). Radwanska highlighted that *Trypanosoma*'s infection provokes a rapid destruction of the splenic architecture inducing a loss of the germinal center formation needed for the maturation of the B cells lymphocytes (Radwanska et al. 2008).

Diagnosis

A diagnosis of the disease during the early stage of infection is crucial knowing that the late stage of infection is characterized with a high rate mortality even treated. For *T. brucei gambiense* a massive screening of the population is allowed by the Card Agglutination Test for Trypanosomiasis (CATT). This technic tests the presence of *Trypanosoma* specific antibody in the blood, the serum and the plasma (Pansaerts et al. 1998). As a first step of the diagnosis, this test is very interesting because it is cheap and allow the screening of many patients at once. It also has a high sensitivity (87-98%) but a positive result needs to be confirm (Inojosa et al. 2006). The presence of the parasite has to be confirmed by microscopic observation of body fluids such as the blood, the lymph and the cerebrospinal fluid (Chappuis et al. 2005). This diagnosis is also true for *T. brucei rhodesiense*. The last and significant step of the diagnosis is the determination of the stage of the disease. To do so, a cerebrospinal fluid puncture needs to be performed to see if the parasite had already reach the central nervous system and enter into the neurological stage of the disease (Kennedy 2013).

Treatment

The treatment depends on the stage of the patient. For a patient in the first stage of the disease, a safer and easier to administer treatment is preferred but a follow-up every 6 months for 24 months needs to be perform. For patient in the second stage of the disease the treatment is highly toxic and needs to cross the blood-brain barrier (Kennedy 2013).
Pentamidine and suramin are the treatments use for patients in early stage of the disease. Suramin is used as a first line treatment in case of an infection with *T. brucei rhodesiense* and as a second line treatment when trypanosomiasis is due to *T. brucei gambiense*. Whereas pentamidine is a first line treatment for *T. brucei gambiense* and a second line treatment for *T. brucei rhodesiense*. Pentamidine is effective but has side effects such as cardiac and gastrointestinal disordes, hyperglycaemia or hypoglycaemia and hypotension (Kennedy 2013). Suramin is an effective treatment especially when taken very early but also has its side

effects like renal failure, skin lesions, neurological complications, anaphylactic shock, bone marrow toxicity. Those early stage treatments are more effective when combined.
The medicines use for treating the late stage of the disease are highly toxic. The most toxic is the melarsoprol, the only one effective for the neurological stage of *T. brucei rhodesiense*. This arsenic-based drug cause encephalopathy such as cerebral edema (Baker et al. 2013) and lead to death on 5% of cases. For *T. brucei gambiense*, a combination of eflornithine and nifurtimox is used. It has a lower death rate than melarsoprol of 0.7% and the side effect are also less severe. The use of eflornithine and nifurtimox causes bone marrow toxicity, alopecia, seizures and gastrointestinal symptoms (Kennedy 2013).
In a vector-borne disease, the best treatment is still the prevention by avoiding the vector.

Trypanosoma cruzi

Another important protozoan's pathology is Chagas' disease caused by *Trypanosma cruzi*. It could also a well-fitting model to study the cross-pathologies implication in the individual variability.
Trypanosoma cruzi is the causative agent of Chagas' disease that rages in the Americas. About 60.000 new cases of this parasitic disease occur each year. In contrast to *T. brucei*, *T. cruzi* is an intracellular parasite that is transmitted via the contaminate feces of a triatomine bugs which is the vector the disease, blood transfusion, organs transplant but also congenitally (Rassi et al. 2010). The life cycle of the parasite begins when a bug bites an infected mammalian host, the parasite is ingested under its trypomastigote form along with the blood. In the midgut of the bug, *T. cruzi* turns into an intermediate form called epimastigote. The epimastigote migrates in the bug and turns into the metacyclic trypomastigote form that will be excreted with feces (**Figure 6**). The metacyclic trypomastigote enters into the host through the bite site or mucous membranes. Once inside the mammalian host, it is able to invade several types of nucleated cells and differentiate into its intracellular form: the amastigote. It replicates every 12 hours for 5 days and then turns into a trypomastigote. The cell then breaks allowing the parasites to invade new cells and start new replicative cycle (Burleigh & Andrews 1995; Bern et al. 2011).

Chagas' disease: symptoms, diagnosis and treatment

Chagas' disease presents different clinical aspects such as an acute phase, a chronic phase and a congenital form. An incubation of 1 to 2 weeks occurs before the acute phase which lasts 8 to 12 weeks (Rassi et al. 2010). This phase is characterized by circulation hypomastigotes that are detectable by microscopy of the blood. Most patients are asymptomatic or have mild and non-specific symptoms like fever which makes the control of the parasite difficult. 20 to 30% of the patients have typical symptoms like inflammation and swelling at the site of infection called "Chagoma" and less than 1% of the infected population declare severe symptoms such as myocarditis, pericardial effusion and meningoencephalitis (Acquatella 2007; Bern et al. 2011).
The chronic phase of the disease occurs 8 to 12 weeks post infection when the parasite become invisible in the blood and thus undetectable by microscopy but patients remain a reservoir and still have the potential to transmit the disease. After years of infection, patients develop cardiac and digestive disorders (Rassi et al. 2010). During the chronic phase it is only possible to detect the presence of *T. cruzi* by a serological method by detecting the presence of IgG anti-*T. cruzi* via an enzyme-linked immunosorbent assay (ELISA) or indirect

fluorescent antibody (IFA) test (Bern et al. 2011). The treatment of the chronic phase only concerns the treatment of the symptoms (Castro et al. 2006).
New borne with congenital form are largely asymptomatic but in some cases they have severe life-threatening disease (Bern et al. 2009; Torrico et al. 2004). This form can be detected in early life by microscopy, just like in the acute phase via the visualization of the circulating tripomastigotes but also by molecular method via a PCR (Bern et al. 2011).
For an effective treatment of the disease, patients must be taken care of during the acute phase. The drugs used are benznidazole and nifurtimox and these two are effective against the extracellular form of *T. cruzi* which is present only during the acute phase. The side effects of the treatment are numerous and include among other things: anorexia, nausea, insomnia, nervous, convulsions, forgetfulness (Castro et al. 2006; Clayton 2010).

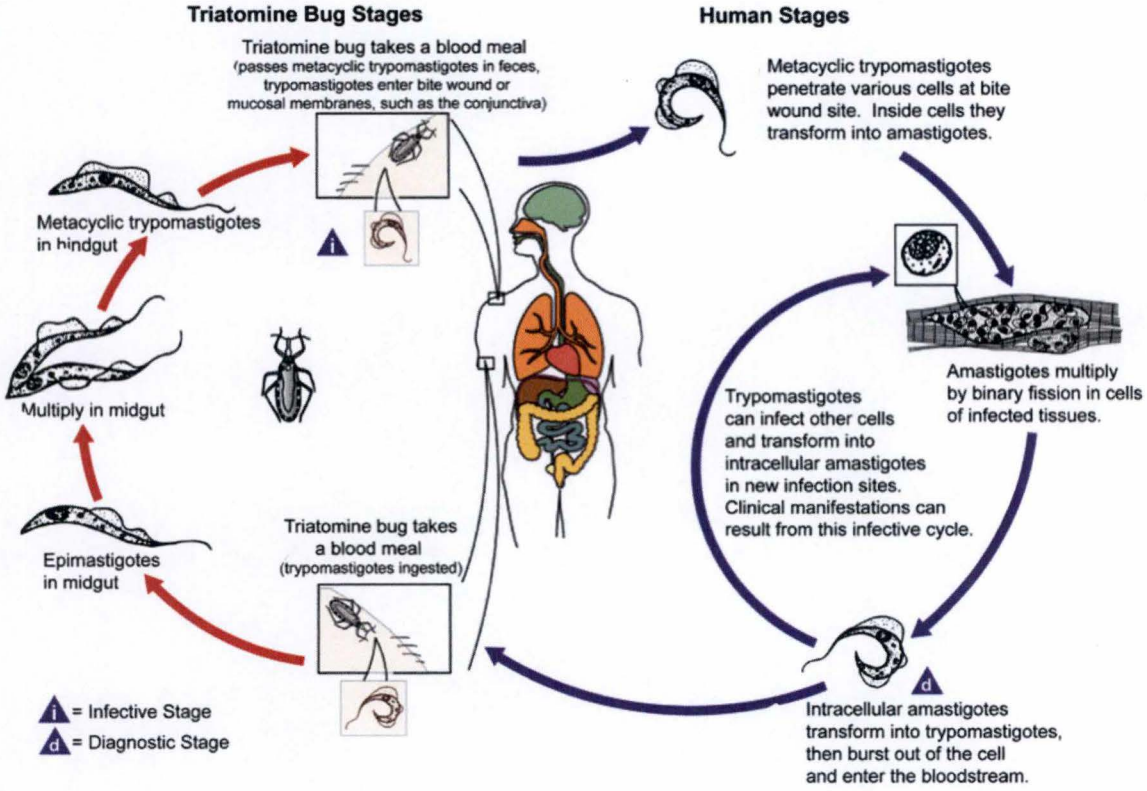


Figure 6: Life-cycle of* Trypanosoma cruzi *inside human host and Bugs (from Centers for Disease Control and Prevention) A tritomine ingested circulating blood parasites while taking a blood meal. The ingested trypomastigotes transform into the epimastigote form in the midgut of the bug where it replicates it then turns into the infective metacyclic trypomastigote in the hindgut. This form is able to go in the fece of the bug and enters into a mammalian host through a bite wound or mucosal membrane while the bug is taking a blood meal. The metacyclic trypomastigote is able to enter into various cells and inside turns into the amastigote form. It then multiply and turns into trypomastigotes that reach the bloodstream or infect other cells and transform into amastigote.

Interest of a cross-pathologies model

The epidemiological data and the similarities between the hosts suggest that it is likely to find mammals infected with *Brucella* and *Trypanosoma brucei*. Those two pathogens cause zoonosis covering a large part of the globe and both infect cattle. Furthermore, if we enlarge the thematic to the impact of parasitic infection on the ability of the host to control brucellosis, it is important to note that many parasites cause asymptomatic infection and increase the risk to find co-infected host.
T. brucei is also a good tool to modulate the immune environment of the mice infected with *Brucella*. The parasite is known to induce a strong Th1 immune response with a IFNγ-producing $CD4^+$ T cells which correspond to the immune response against *Brucella* (Liu et al. 2015). Knowing that *Brucella* is a furtive bacterium that induces a weak immune response, it could be interesting to show if a strong Th1 immune response could modulate the ability of *Brucella* to reach its niche and establish its chronicity.

Objectives

The aim of my master thesis was to generate an experimental model to study the impact of a cross-pathology on the ability of the mice to control the infection with *Brucella* and thus, to control the establishment of the bacteria to avoid the chronicity of brucellosis. Previous study in our laboratory have showed that *Trypanosoma brucei* infection reduce dramatically the number of *Brucella melitensis* in the spleen. We will identify the mechanisms implicated in this phenomenon.
First, we will compare the impact of *T. brucei* on different species of *Brucella* (*suis* and *abortus*) and of another *Trypanosoma* species (*T. cruzi*) on *B. melitensis* to determine is an antigenic cross-reaction can explain the impact of the immune response against *T. brucei* on *Brucella*.
Second, by using a battery of mice genetically deficient for key element of immune response, we will try to identify the immune mechanism induced by *T. brucei* implicated in the elimination of *Brucella* in spleen.

MATERIALS AND METHODS

Materials and methods

Ethics statement

The procedures used in this study and the handling of the mice complied with current European legislation (directive 86/609/EEC) and the corresponding Belgian law "Arrêté royal relatif à la protection des animaux d'expérience du 6 avril 2010 publié le 14 mai 2010". The Animal Welfare Committee of the Université de Namur (UNamur, Belgium) reviewed and approved the complete protocol (Permit Number: 12-188).

Mice

Wild-type C57BL/6 mice and wild-type BALB/c mice were acquired from Harlan (Bicester, UK). IL-12p35$^{-/-}$ C57BL/6 mice were acquired from Dr. B. Ryffel (University of Orleans, France). TAP1$^{-/-}$ and MHCII$^{-/-}$ C57BL/6 mice were acquired from Jörg Reimann (University of Ulm, Ulm, Germany). CD11c-DTR C57BL/6 mice were obtained from Dr. G. Holdenhove (Université Libre de Bruxelles, Belgium) and treated with diphtheria toxin (Sigma-Aldrich). All wild-type and deficient mice used were bred and housed in an institutional conventional animal facility of the Gosselies campus of the Université Libre de Bruxelles (ULB, Belgium) under the ethical regulation of the ULB. All mice used were 6 to 8 weeks old.

Mice infection with *Brucella*

Brucella melitensis 16M and *Brucella abortus* 2308 strains are Nal^R Kan^R and constitutively expressing a rapidly maturing variant of the red fluorescent protein DsRed, the mCherry protein (mCherry-Br), under the control of the strong *Brucella* spp. promoter, PsojA. Construction of the mCherry-Br strains has been described in detail in (Copin et al. 2012). *Brucella* strains were grown in biosafety level III laboratory facilities. Cultures were grown overnight in a 2YT liquid medium (luria-Bertani broth with double quantity of yeast extract) at 37°C with shaking. The following day, the bacteria are harvested. Cultures are washed twice in RPMI 1640 (Gibco Laboratories) after centrifugation at 3500g for 10 minutes. The measurement of the OD at 600 nm allows to prepare the appropriate infectious dose after dilution in RPMI. The infectious doses were validated by plating serial dilutions of the inoculums. For intranasal infection, mice were anesthetized with a cocktail of Xylasine (450 $\mu\ell$/kg) and Ketamine (720 $\mu\ell$/kg) in PBS before being inoculated intranasally with $2x10^4$ CFU of *B. suis, B. abortus* or mCherry-expressing *B. melitensis* in 30$\mu\ell$ of RPMI. For intraperitoneal infection, mice were inoculated with $2x10^4$ CFU of mCherry-expressing *B. melitensis* in 500$\mu\ell$ of RPMI. For intradermal infection, mice were inloculated with $4x10^4$ CFU of mCherry-expressing *B. melitensis* in 20$\mu\ell$ of RPMI in the paw of the mice. The infectious doses were validated by plating serial dilutions of the inocula.

Mice infection with *Trypanosoma*

The pleomorphic AnTat 1.1E *Trypanosoma brucei brucei* and a dominant-negative adenylate cyclases (DNc) mutant were stocked in infected mice blood at -80°C. *T. brucei* infection is characterized by a multi-waves parasitemia development and each wave corresponds to a switch of the antigenic population. DNc mutant parasitemia displays no peak of infection. Parasitemia is evaluated by using a counting chamber and a light microscope before dilution in PBS to prepare the infectious dose. Mice were infected by intraperitoneal injection with 5000 parasites/mouse in 200$\mu\ell$ of PBS.

Concerning the infection with *T. cruzi*, we used the Tuhalen strain (genotype TcVI). Mice were infected by an intraperitoneal injection of 1000 parasites under the blood trypomastigote form.

Injection of Diphtheria toxin

48 hours before the selected time after infection, mice were injected in an intraperitoneal way with 200ng/mice of Diphtheria Toxin from *Corynebacterium diphtheriae* (Sigma-Aldrich)

Harvesting of organs

At the selected time after infection, mice were anesthetized with 250$\mu\ell$ of Isoflurane under a bell in order to take blood by a retro-orbital sampling. Then mice were sacrificed by cervical dislocations. Immediately after sacrifice, spleen, a lobe of the liver, one lung, thymus, brain, 1 cm at the base of the tail, a piece of the muscle of the posterior leg, heart, bronchial draining lymphoid node, lesion from the paw, popliteal draining lymphoid node (for those two organs see A. Demars thesis) and one ovary) cells were collected for bacterial count.

Bacterial count

Organs were crushed and transferred to PBS/0.1% X-100 triton (Sigma-Aldrich). We performed successive serial dilutions in RPMI to get the most accurate bacterial count and plated them on 2YT medium Kan^+ for *B. melitensis* and *B. abortus* and on 2YT medium $polymyxine^+$ for *B. suis*. The CFU were counted after 5 days of culture at 37°C.

Cytofluorometric analysis

Spleens were harvested after sacrificed of the mice and cut into small pieces in a six-well plate with a drop of a mix of DNase I (Sigma-Aldrich - 100 mg/ml) and collagenase (Roche-400M and 1 U/ml). Spleens are then incubated for 30 min at 37°C, under 5% CO_2 with 1mL of collagenase/DNase. Cells are washed in RPMI 1640 and filtered with a bridal veil and a syringe into a Falcon 15 and are then incubated for 5 hours in 1mL of RPMI 1640, 10% FCS with 1μl/ml Golgi Stop (BD Pharmingen) at 37°C, under 5% CO_2. Cells are put overnight in 1mL of rich medium (RPMI 1640 + decomplemented Foetal Bovine Serum + L-Glutamine + Non-essential amino acids + pyruvate sodium + Gentamycin 50mg/mL) after centrifugation at 1400g for 7 minutes. The next day, cells are resuspended and 200$\mu\ell$ are put into a 96-well plate. Cells are washed twice with FACS buffer after centrifugation (2 minutes at 2000 RPM at 4°C). Cells are incubated with saturating doses of purified 2.4G2 (anti-mouse FcR; American Type Culture Collection) in 200 μl of PBS, 0.2% BSA, and 0.02% NaN3 (FACS buffer) for 20 min at 4°C to prevent Ab binding to the FcR. After being washed in FACS buffer, cells are incubated for 30 minutes in surface staining fluorescent mAb combinations in FACS buffer that were used to stain 3–5x10^6 cells. We acquired the following mAbs from BD Biosciences: Fluorescein (FITC)-coupled HL3 (anti-CD11c), FITC-coupled M1/70 (anti-CD11b), FITC-coupled 145- 2C11 (anti-CD3ε), phycoerythrine (PE)-coupled 1-A/1-E (anti-MHCII), PE-coupled RM4-5 (anti- CD4). After washing with FACS buffer and staining for cell surface markers, cells are fixed in PBS/1% PFA for 15–20 min at 4 °C. They are permeabilized for 30 min using a saponin-based buffer (103 Perm/Wash in FACS buffer ; BD Pharmingen) and stained with allophycocyanin-coupled XMG1.2 (anti– IFN-g; BD Biosciences) and with anti NOS2 (anti iNOS, Rabbit polyclonal IgG ; Santa-Cruz biotechnology). After final fixation in PBS/1% PFA, cells were analyzed on a FACSCalibur cytofluorometer. No signal was detectable with isotype controls. The cells were analyzed on a

FACSCalibur cytofluorometer. Dead cells and debris were eliminated from the analysis according to size and scatter.

Statistical analysis

We used a (Wilcoxon-) Mann-Whitney test provided by the GraphPad Prism software to statistically analyze our results. Each group of deficient mice was compared to wild-type mice. We also compared each group with each other and displayed the results when required. Values of $p < 0.05$ were considered to represent a significant difference. *, **, ***, **** denote $p<0.05$, $p<0.01$, $p<0.001$, $p<0.0001$, respectively.

RESULTS

Results

1. Characterization of the impact of *T. brucei* infection on *B. melitensis* infection in mice

1.1 *Brucella melitensis* persist in CD11c$^+$ reservoir cells in spleen providing a resistance to the immune response

The kinetic of *B. melitensis* in C57BL/6 mice have been established in the lungs, spleen and the liver following an intranasal infection with $2x10^4$ CFU of mCherry-*B. melitensis*. The bacteria persist until 12 days post infection in the lungs of the mice whereas the spleen and the liver are colonized from 5 days post infection. From 28 days post infection, the bacteria persist only in the spleen where it establishes a niche to persist for a long period (Hanot Mambres et al. 2016). By microscopic analysis in highly susceptible IL-12p40$^{-/-}$ BALB/c mice, it has been shown that *B. melitensis*, during the chronic phase of infection, resides in reservoir cells, express a specific phenotype (CD11c$^+$ CD205$^+$ Arginase$^+$) (Hanot Mambres et al. 2015). Nevertheless, a microscopic analysis of infected cells in resistant mice is impossible due to the low number of *Brucella* persisting in their spleen. As an alternative, we used DTR-CD11c C57BL/6 mice. These mice express diphtheria toxin (DT) receptor under the control of CD11c promoter. When those mice are injected with DT, depletion of CD11c$^+$ cells is induced by apoptosis and the number of CD11c$^+$ cells remain low for 2 days. This specific depletion is allowed by the fact that murine cells are not sensitive to DT contrary to primate cells (Van Blijswijk et al. 2013; Jung et al. 2002). In order to determine if *Brucella* resides in CD11c$^+$ reservoir cells, DTR-CD11c C57BL/6 mice were infected by an i.n route with $2x10^4$ CFU of mCherry-*B. melitensis* and 26 days post infection, they were injected intraperitoneally with 10 ng of DT in 500 μl of PBS while the control group was injected with 500 μl of PBS alone. 48 hours later, at day 28 post infection with *Brucella*, mice were sacrificed by cervical dislocation and the spleens were harvested. Half of them were used to perform a flow cytometry analysis and the other half were used to count the bacterial load remaining in the spleens. The flow cytometry analysis confirmed that the injection of DT induces a drastic elimination of CD11c$^+$ in infected mice (**Figure 7.A**). The depletion of those cells leads to a drastic drop of the bacterial load when compared to the control group (**Figure 7.B**). A comparable effect is also observed at 50 days post infection (**Figure 7.B**). Those results suggest that *Brucella melitensis* resides in CD11c$^+$ reservoir cells in the spleen of wild type C57BL/6 mice during the chronic phase of infection.

Following an i.n. infection with *Brucella* in wild type C57BL/6 mice, a protective memory T cell response is set up. When a second infection with *Brucella* occurs, this response is able to rapidly control the newly arrived bacterial load and to eliminate it (Hanot Mambres et al. 2016). In order to determine if the protective memory T cell response impact the bacterial load from the first infection that persists in reservoir cells in the spleen, we compared the CFU level of a wild type *B. melitensis* with the CFU level of mCherry-*B. melitensis* in the spleens of mice from 3 different group. As described in **Figure 8.A**, the first group was only infected by an i.n. route with mCherry-*B. melitensis* at day 45, the second group was firstly infected intranasally at day 0 with wild type *B. melitensis* and was challenged at day 45 with mCherry-*B. melitensis*. The third group was only infected at day 0 by an i.n. route with wild

type *B. melitensis*. At day 73, all mice were sacrificed and spleens harvested in order to count the bacterial load. As expected, mCherry-*B. melitensis* challenge is well controlled by immunized mice from the second group when compared to non-immunized mice from the first group. Interestingly, the bacterial load of wild type *B. melitensis* was comparable between the challenged group and the unchallenged one, meaning that the memory immune response is able to control a second infection but does not impair *Brucella* when it is already established in CD11c$^+$ reservoir cells (**Figure 8.B**). Those results suggest that the CD11c$^+$ reservoir cells confer a protection to *Brucella* against the protective memory response allowing the persistence of the bacteria in the host.

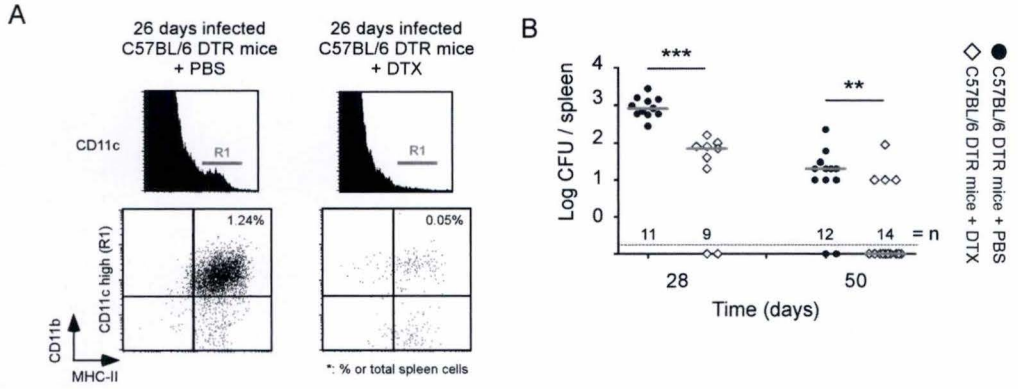


Figure 7:* Brucella *persists in CD11c$^+$ reservoir cells in spleen of chronically infected wild type C57BL/6 mice. DTR-CD11c C57BL/6 mice were infected by an i.n. route with 2x10^4 CFU of mCherry-*B. melitensis*. 26 days post infection, they were injected intraperitonally with 10 ng of diphtheria toxin in 500 $\mu\ell$ of PBS or with PBS alone for the control group. Two days after DT injection, mice were sacrificed by cervical dislocation to harvest the spleen. **A.** Flow cytometry analysis of the expression of CD11c and MHCII on spleen cells. Data show a representative dot plot from individual mice. **B.** The data represent the number of CFU per gram of spleen for each group of mice at indicated time post infection. n denotes the number of mice used in each group. The grey bar represents the means. **, $p < 0.01$; ***, $p < 0.001$. Detection threshold = 10 CFU.

1.2 *T. brucei* infection reduces the number of Brucella persisting in spleen

The previous results show that the protective immune response induced by a second exposition to *Brucella* is able to control this second infection but this immune response is not strong enough to impair the bacterial load from the first infection established in reservoir cells in the spleen of the mice. We wanted to determine if a strongest inflammatory response is able to destabilized *B. melitensis* when already established in its reservoir cells. To do so, we used *T. brucei* which is a parasite known to induce a strong inflammatory immune in order to test this hypothesis. In order to determine whether *T. brucei* impacts the course of infection of *B. melitensis*, wild type C57BL/6 mice were infected by an intranasal (i.n.) route with 2x10^4 CFU of mCherry-*B. melitensis*. At 7 days post *Brucella* infection, mice were injected intraperitoneally with 5000 parasites. At selected time post infection, mice were sacrificed by cervical dislocation and spleen were harvested, crushed and plated in order to determine the bacterial load of each organ by plating. The results show that *T. brucei* infection induces a significant decrease of the bacterial load in the spleen. At 5 days post co-infection with the parasite, a decrease of 1-1,5 log of the CFU in co-infected mice is already observable when compared to mice infected with *B. melitensis* alone. This result is still observable at days 12 and 28 post infection with *B. melitensis* when co-infected. At 21 days post co-infection, the bacterial load observed a serious drop of 3 log and *B. melitensis* CFU levels are under detection threshold (10 CFU / spleen) in the majority of co-infected mice. Those results

suggest that the immune response induced by *T. brucei* infection is able to destabilize *Brucella*'s reservoir cells and thus, allowed the elimination of the bacteria in the spleen (**Figure 9**). When taking into account that the localization of *B. melitensis* in spleen is different depending on the route of infection (Hanot Mambres et al. 2016), we performed a similar experiment to compare the impact of *T. brucei* on *B. melitensis* persistence when injected in an i.p. mode and similar results were obtained (**Figure 9.C**).

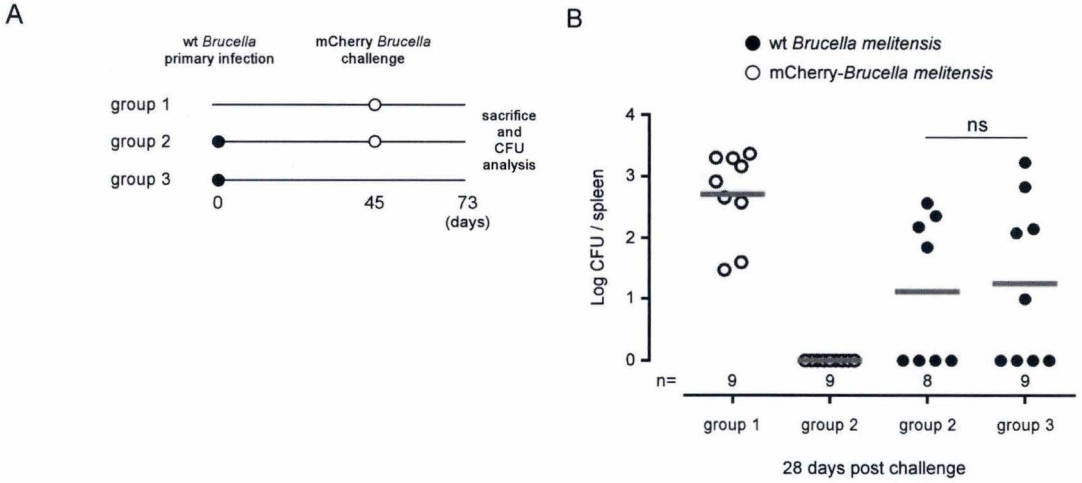


Figure 8: reservoir cells confer a protection to* Brucella *against the protective immune response. Wild type C57BL/6 mice were infected intranasally with $2x10^4$ CFU of wild type *B. melitensis* at day 0 and / or mCherry-*B. melitensis* at day 45. At selected time, mice were sacrificed to harvest the spleens as described in **A**. **B.** The data represents the CFU per spleen for each group at selected time post infection. n denotes the number of mice used in each group. The grey bar represents the means. **, $p < 0.01$; ***, $p < 0.001$. Detection threshold = 10 CFU.

In order to determine if the elimination of *B. melitensis* following an infection with *T. brucei* observed is due to an antigenic cross-reaction between *B. melitensis* and *T. brucei*, we tested if *T. brucei* was able to impact the control of the infection in the spleen with two other *Brucella* species that also affect humans: *B. abortus* and *B. suis*; as for *B. melitensis*, *T. brucei* induces a drop of the bacterial load when the mice are infected with *B. abortus* but also when infected with *B. suis* and the decrease of the bacterial load is as important as it is for *B. melitensis*. We also tested the impact of an infection with *T. cruzi* on the control of an infection with *B. melitensis*. *T. cruzi* is an intracellular protozoan parasite presenting only one peak of parasitaemia 20 days post infection in mice and presents a rate mortality of about 30% whereas *T. brucei* is characterized by parasitaemia every 5 days corresponding to the switch of its VSG (Rudenko 2011; Lalonde & Holbein 1984). The vector of those two parasites are also different and they present their own pathogenicity. Following a co-infection with *T. cruzi* a drastic drop of *B. melitensis* in the spleen of co-infected mice was also observable when compared to mice infected with *B. melitensis* alone (**Figure 10**).
These results demonstrate that the elimination of *Brucella* following *Trypanosoma* infection is not due to an antigenic cross-reaction.

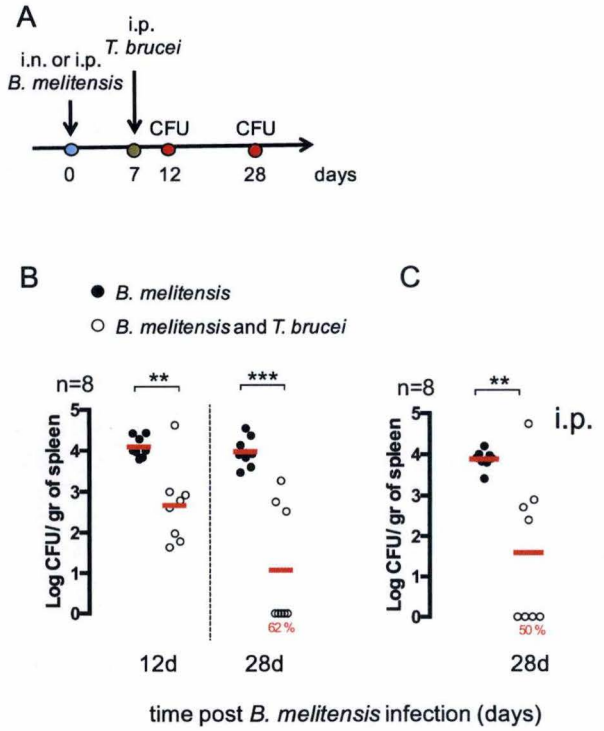


Figure 9: impact of* T. brucei *co-infection on the course of intranasal* B. melitensis *infection in mice. Wild type C57BL/6 mice were infected intranasally with $2x10^4$ CFU of mCherry-*B. melitensis* and were injected 7 days later intraperitoneally with 5000 *T. brucei* in 200 $\mu\ell$ of PBS or with PBS alone for the control group. At the selected times post infection, mice were sacrificed by cervical dislocation to harvest the spleens (see panel **A.**). **B.** The data represents the CFU per gram of spleen for each group at selected time post infection. **C.** Wild type C57BL/6 mice were infected intraperitoneally with $4x10^4$ CFU of mCherry-*B. melitensis* and were injected in i.p. 7 days later with 5000 *T. brucei* 200 $\mu\ell$ of PBS or with PBS alone. n denotes the number of mice used in each group. The red bar represents the means. **, $p < 0.01$; ***, $p < 0.001$. Detection threshold = 10 CFU.

1.3 Elimination of *Brucella* following *T. brucei* infection require functional IL-12 / IFNγ signaling pathways

IFNγ – producing $CD4^+$ T cells plays a central role in the protective immune response against *B. melitensis*. The impact of *T. brucei* infection on the control of *B. melitensis* was compared in wild type and in multiple deficient mice: $CD3^{-/-}$, $\gamma\delta TCR^{-/-}$ and $IL\text{-}1R^{-/-}$ were performed before I arrived in the team and $MHCII^{-/-}$, $IL\text{-}12p35^{-/-}$ and $TAP1^{-/-}$. It has been shown that *T. brucei* was able to induce an elimination of *B. melitensis* in $\gamma\delta TCR^{-/-}$, $IL\text{-}1R^{-/-}$ and $TAP1^{-/-}$ mice but not in $CD3^{-/-}$, $MHCII^{-/-}$ and $IL\text{-}12p35^{-/-}$ mice (**Figure 11. A**). Those results suggest that the elimination of *B. melitensis* by *T. brucei* depends on the host immune response and required a functional IL-12 / IFNγ signaling pathways and the $CD4^+$ T cells.

We also analyzed by flow cytometry the frequency of IFNγ and iNOS-producing cells in the spleen of wild type and deficient mice co-infected with *T. brucei* and *B. melitensis* or infected with *B. melitensis* alone, as nitric oxide produced by iNOS/NOS2 is well known to be produced in response to IFNγ but also described as disadvantaging *B. melitensis* growth in the spleen (Copin et al. 2007) (**Figure 11.B-C**).The results show that a strong increase of IFNγ and iNOS – producing cells is induced by the presence of *T. brucei*. The production of IFNγ is principally due to $CD3^+$ $CD4^+$ T cells. In $IL12p35^{-/-}$ mice, a significant reduction of $IFN\gamma^+$ and $iNOS^+$ cells frequency is observable when compared to wild type mice. An interesting point is that there is no significant difference between the frequency of IFNγ and iNOS –

producing cells in the spleen of TAP1$^{-/-}$ mice (deficient for CD8$^+$ T cells and able to clear *Brucella* during co-infection) and MHCII$^{-/-}$ mice (deficient for CD4$^+$ T cells and unable to clear the bacteria), showing that the control of *B. melitensis* infection when co-infected with *T. brucei* required a functional IL12p35-IFNγ signaling pathway but also to the presence of CD4$^+$ T cells.

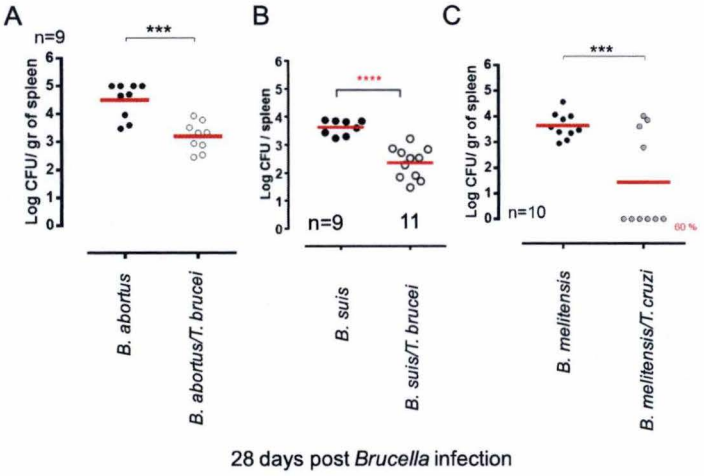


Figure 10: impact of* T. brucei *co-infection on* B. suis *and* B. abortus *infection and impact of* T. cruzi *on* B. melitensis *infection. Wild type C57BL/6 mice were infected intranasally with $2x10^3$ CFU of *B. abortus* (**A**) or *B. suis* (**B**) and were injected 7 days later intraperitoneally with 5000 *T. brucei* in 200 $\mu\ell$ of PBS or with PBS alone for the control group. **C.** Wild type C57BL/6 mice were infected i.n. with $2x10^4$ CFU of mCherry-*B. melitensis* and were injected in i.p. 7 days later with 5000 *T. cruzi* in 200 $\mu\ell$ of PBS or with PBS alone. Mice were sacrificed 28 days post *Brucella* infection to harvest the spleens. The data represent the number of CFU per gram of spleen. n denotes the number of mice used in each group. The red bar represents the means. ***, $p < 0.001$; ****, $p < 0.0001$. Detection threshold = 10 CFU.

1.4 Attenuated *T. brucei* mutant but not killed parasite is able to reduce *Brucella* infection

We have shown previously that *T. brucei* induces a strong Th1 inflammatory response infected mice. This response allowed the control of *B. melitensis* infection in co-infected mice. In order to determine if the intensity and the duration of the Th1 response are essential parameters, we compared the ability of wild type *T. brucei* and a dominant-negative adenylate cyclases (DNc) *T. brucei* mutant. The DNc *T. brucei* mutant shows a lower parasitaemia inducing a weaker inflammatory immune response. This less strong immune response allowed an increase of the survival time of infected mice when compared to mice infected with wild type *T. brucei* (Salmon et al. 2012).

The elimination of *B. melitensis* in co-infected mice was observable in both cases: when co-infected with wild type but also when co-infected with the mutant (**Figure 12.A**). Those results suggest that a low persistence of parasite is able to complete the elimination of *B. melitensis*. In previous experiment, mice were repeatedly injected with a lysate of *T. brucei* and no effect on the control of *B. melitensis* infection were observable meaning that living parasites seem to be needed.

At early time post co-infection, the frequency of IFNγ – producing cells in the spleen is lower in mice infected with DNc than those infected with wild type *T. brucei*. This might be translated by a slower effect of the mutant on the control of *B. melitensis* in the spleen due to less IFNγ (**Figure 12.B**).

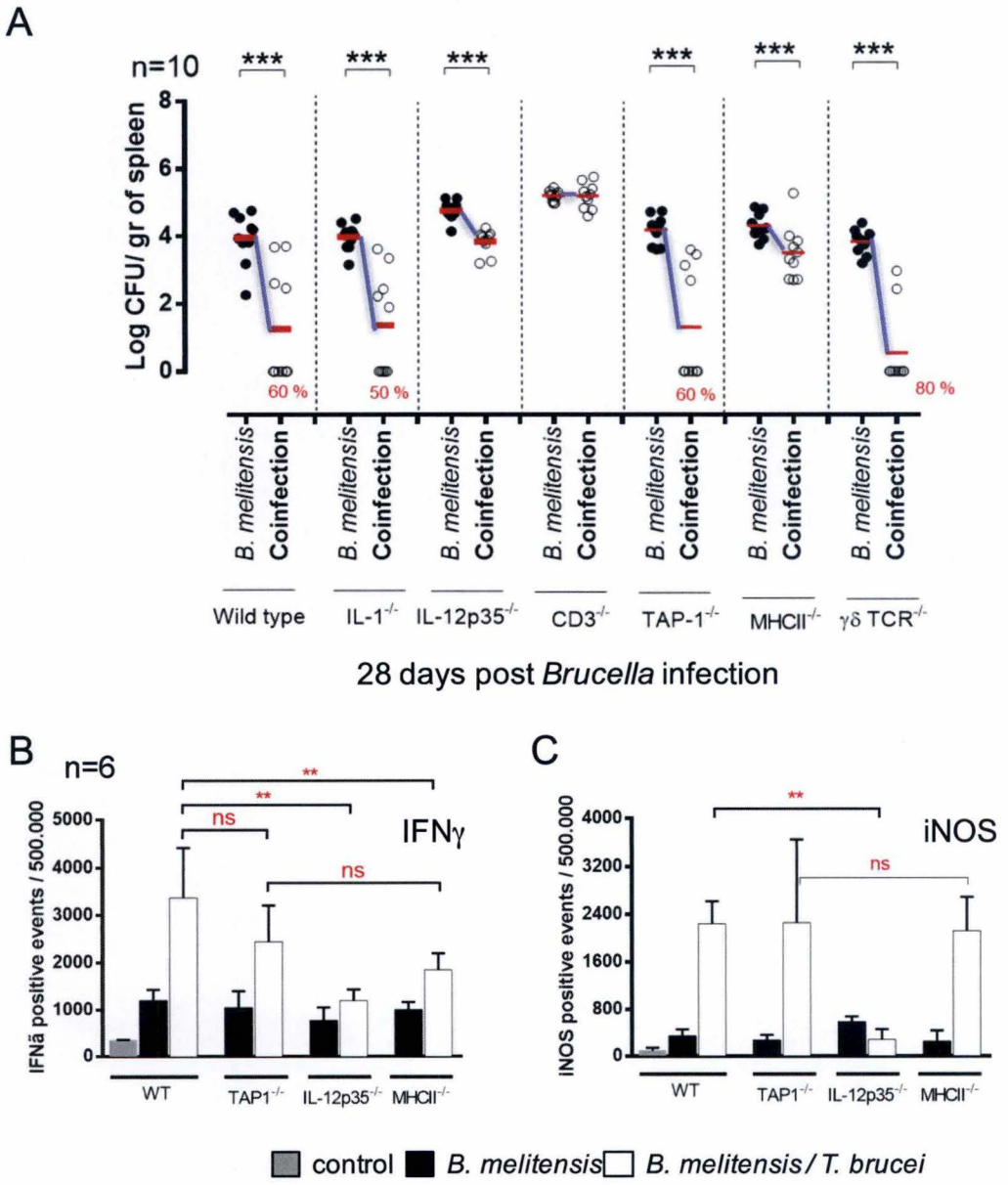


Figure 11: elimination of* Brucella *during* T. brucei *infection requires IL-12 and CD4⁺ T cells. Wild type and multiple deficient C57BL/6 mice were infected intranasally with $2x10^4$ CFU of *B. melitensis* and were injected 7 days later intraperitoneally with 5000 *T. brucei* in 200 $\mu\ell$ of PBS or with PBS alone for the control group. Mice were sacrificed 28 days post *Brucella* infection to harvest the spleens. **A.** The data represent the number of CFU per gram of spleen. n denotes the number of mice used in each group. The red bar represents the means. ***, $p < 0.001$. Detection threshold = 10 CFU. **B, C.** The data represents the frequency of IFNγ and iNOS producing cells in spleen as determined by flow cytometry analysis. Control group corresponds to naïve mice receiving PBS only. **, $p < 0.01$; ***, $p < 0.001$.

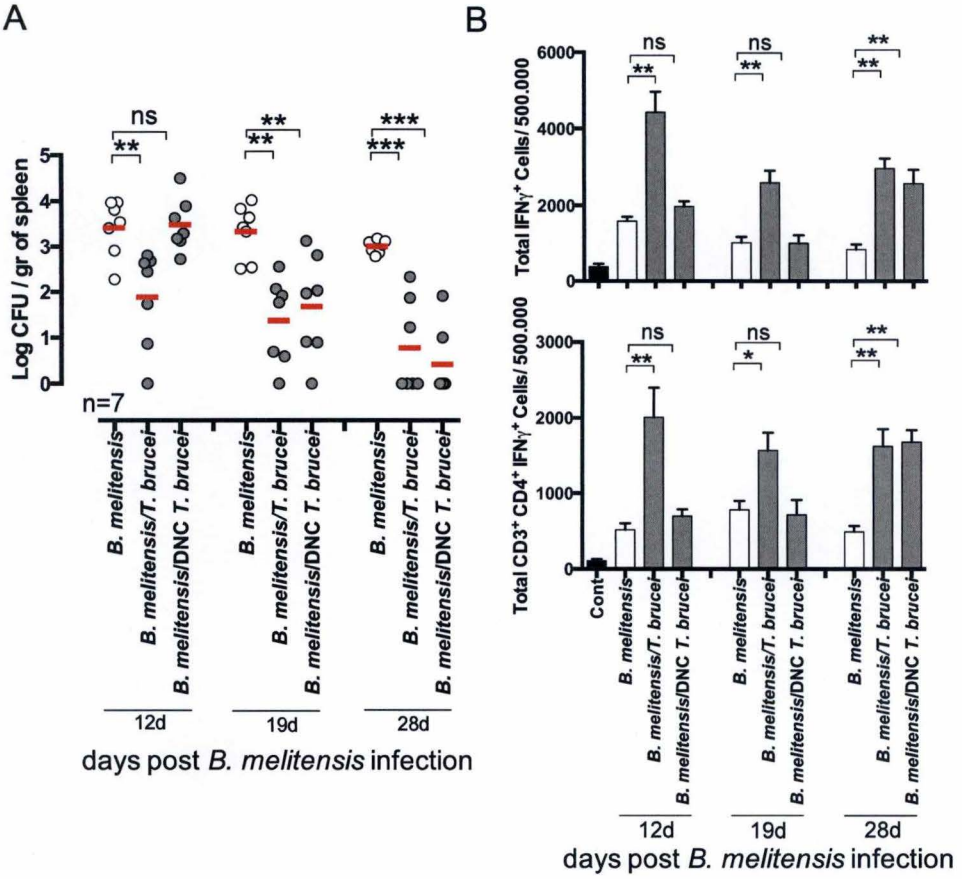


***Figure 12: attenuated dominant negative adenylate cyclase (DNc) mutant of* T. brucei *induces elimination of* Brucella.** Wild type C57BL/6 mice were infected intranasally with $2x10^4$ CFU of mCherry-*B. melitensis* and were injected 7 days later intraperitoneally with 5000 wild type *T. brucei* or attenuated DNc mutant of *T. brucei* in 200 $\mu\ell$ of PBS or with PBS alone for the control group. Mice were sacrificed at selected times post *Brucella* infection to harvest the spleens. **A.** The data represent the number of CFU per gram of spleen. n denotes the number of mice used in each group. The red bar represents the means. Detection threshold = 10 CFU. **B.** The data represent the mean of the frequency of IFNγ producing cells in spleen from 5 individual spleens as determined by flow cytometry analysis for each group. Control group corresponds to naïve mice receiving PBS only. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

1.5 Confirmation of the implication of IL-12p35 pathway in the elimination of *Brucella* following the injection of wild type *T. brucei*, attenuated *T. brucei* mutant and *T. cruzi*

Previous experiments show that the protective memory immune response is not strong enough to destabilized *Brucella* when it is already established in its reservoir cells in spleen. However, we show that a strongest inflammatory response induced by the injection of wild *T. brucei*, attenuated *T. brucei* mutant but also that the injection of *T. cruzi*, indicating that it is not an antigenic cross-reaction, allowed the elimination of the bacteria in the spleen of co-infected mice.

Here we confirm the implication and the requirement of a functional IL-12p35 pathway in the elimination of *B. melitensis* in mice regardless of the inducer of the strong inflammatory response. To do so we infected by an i.n. route wild type C57BL/6 mice and IL-12p35$^{-/-}$ C57BL/6 mice with $2x10^4$ CFU of mCherry-*B. melitensis*. Wild type C57BL6 and IL-12p35$^{-/-}$

C57BL/6 mice were both divided into 4 groups that were injected in i.p. 7 days after *Brucella* infection respectively with: PBS alone for the control group, 5000 wild type *T. brucei*, 5000 attenuated *T. brucei* mutant or 1000 *T. cruzi*. As expected, the elimination of *Brucella* is observable in wild type mice regardless of the parasite injected when compared to the control group. In mice deficient for the IL-12 pathway, the bacterial load remains equal to the control group in co-infected mice. Those results confirm the central and even essential role of the IL-12p35 pathway in the control of *Brucella*'s infection regarding that IL-12p35$^{-/-}$ mice are not able to batter control the bacterial infection when co-infected. **(Figure 13)**.

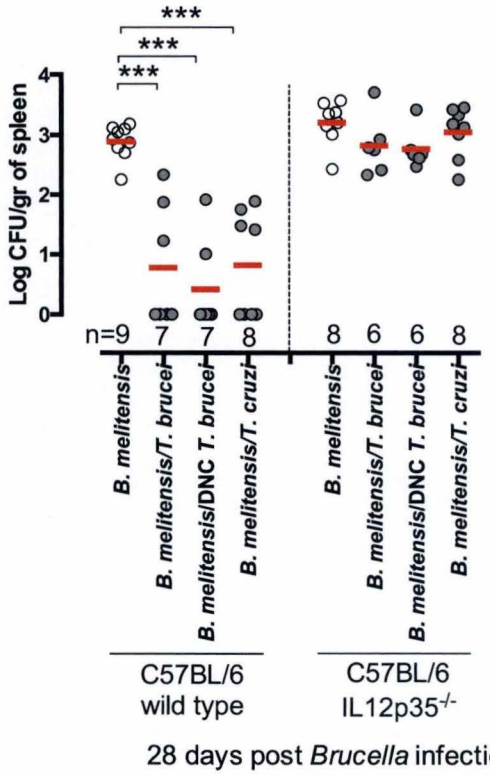


Figure 13: wild type* T. brucei, *attenuated dominant negative adenylate cyclase (DNc) mutant of* T. brucei *and* T. cruzi *induce elimination of* Brucella *on a IL-12p35-depending pathway. Wild type and IL-12p35$^{-/-}$ C57BL/6 mice were infected intranasally with $2x10^4$ CFU of mCherry-*B. melitensis* and were injected 7 days later intraperitoneally with, respectively, PBS alone for the control group, 5000 wild type *T. brucei*, attenuated DNc mutant of *T. brucei* in 200 $\mu\ell$ of PBS or 1000 *T. cruzi* in 200 $\mu\ell$ of PBS. Mice were sacrificed 28 days post *Brucella* infection to harvest the spleens. The data represent the number of CFU per gram of spleen. n denotes the number of mice used in each group. The red bar represents the means. Detection threshold = 10 CFU. ***, $p < 0.001$.

All these results show the behavior of *Brucella* in the spleen of mice following an i.n. infection but the spleen is not the only organ colonized by the bacteria. It could be then interesting to characterized the dissemination of this pathogen in order to target other organs in which *Brucella* is also able to establish itself to persist for a long time.

2. The route of infection affect the dissemination of *Brucella* in mice

It has been shown that following an i.p. infection in mice, *Brucella* spreads in a systemic way and is rapidly found in almost all organs (Rajashekara et al. 2005). This mode of infection is the most used in laboratory because of its ease of use and the rapidity of the bacteria's spreading but it is not the most representative of what happens in nature. Here we compared with i.p. two other route of infection in mice that are more representative of some physiological routes of infection: the intradermal (i.d.) and the intranasal (i.n.) infection, to put them on parallel with the i.p. model. The i.d. and i.n. models present a local dissemination of *Brucella* in the host whereas the i.p. model presents a systemic dissemination (**Figure 14**).

In order to compare those 3 routes of infection, we divided wild type C57BL/6 mice into 3 different groups and each group corresponds to one route of infection. The i.p. group and i.n. group received approximatively the same infectious dose of mCherry-*B. melitensis*. 1 hour post infection, in the i.p. model, all the organs tested were positive for *Brucella* with a bacterial load from 1 to 3 log except the muscle from the posterior leg, the tail, the popliteal draining lymph node and the footpad where the i.d. group was injected (**Figure 15**). While in the i.d. the footpad, the blood and surprisingly, the spleen and the liver were also positives. In the i.n. models, only the lungs: the way of entry of the bacteria, were positives and are the only organs colonized until the day 5 post infection when the bronchial draining lymph node starts to be colonized by *Brucella*. At day 1 post infection, following an i.p. infection, the blood, the spleen and the liver are highly colonized with up to 5 log of bacteria and only the muscle and the popliteal lymph node remain free of bacteria or at least with a bacterial load under the threshold of detection (10 CFU). In the i.d. model, the paw remains highly infected and the popliteal draining lymph node becomes colonized. The bacterial load in spleen and the liver is 1 log higher than at 1 hour post infection while the bacterial load in the blood drops to 1 log. At day 5 post infection, in the i.p. model, all the organs tested are colonized and the blood is the only one where a drastic drop of 3 log is observable whereas the bacterial load in other organs is either stable either higher. Following an i.d. route of infection, about 4 log of *B. melitensis* are detectable in the spleen, the liver, the paw and the popliteal draining lymph node. *Brucella* is also detectable in the tail and the muscle of the mice but at a lower level and in the blood, the bacteria is now undetectable. After 12 days of infection, following an i.p. route of infection, a slight decrease of the bacterial load is observable in every organs and in the blood and the muscle, it drops under the threshold. In the i.d. model, the spleen and the popliteal draining lymph node remain highly colonized while a decrease is observed in the liver and the paw of the mice and the bronchial draining lymph node is newly infected with about 2 log. Following the i.n. route of infection, the spleen, the liver and the thymus are newly colonized and in the thymus, the bacterial load persist until the day 50 where it becomes undetectable while in the spleen the bacterial load remain stable even at day 50 post infection and the same is observable for the bronchial draining lymph node. In the liver, it is the only transitory: it is no more observable at day 28 post infection. Following an i.d. infection at 28 days post infection, the thymus is colonized but only transiently because at day 50 post infection, no bacteria remain detectable, the bacterial load in the spleen and the bronchial draining lymph node stabilized and remain equal at day 50 post infection whereas it drops in the paw and it decreases steadily in the popliteal draining lymph node until the day 50. At day 28 post infection following an i.p. route of infection, the spleen remains highly colonized with a bacterial log of about 4 log that is still detectable at day 50, for the thymus, the bronchial draining lymph node and the popliteal draining lymph node it is the same

schema but with, respectively, a bacterial load of 3 log, 2 log and 1 log (for more data, see **Supplementary data 1**).

In summary, these results demonstrated that if *Brucella* disseminate throughout the organism in each model, its profile of persistence in organs is strongly depending on the way of infection. As the pathology associated to brucellosis is generally depending on local inflammation induced by the persistence of the bacteria, our work suggests that the way of infection can influence the pathology induced by *Brucella* in host.

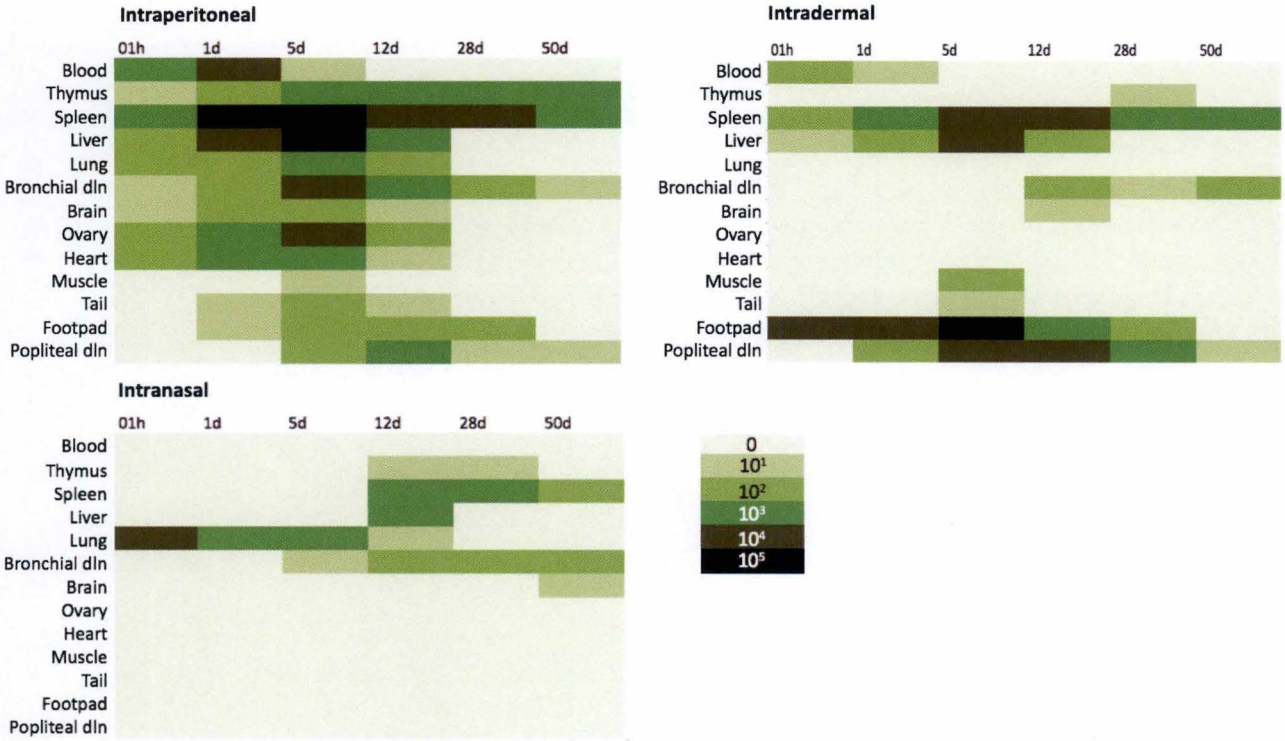


Figure 14:* Brucella *dissemination depends on its route of infection in C57BL/6 mice. C57BL/6 mice infected were infected by an i.n. or i.p. route with $2x10^4$ CFU and on i.d. route with $4x10^4$ CFU of mCherry-*B. melitensis*. At selected times post infection mice were anesthetized to take blood and were then sacrificed by cervical dislocation to harvest multiple organs: the thymus, the spleen, one lobe of the liver, one lung, the bronchial draining lymph node, the brain, one ovary, the heart, 1 cm of the muscle of the posterior leg, 1 cm at the base of the tail, the lesion at the footpad and the popliteal draining lymph node.

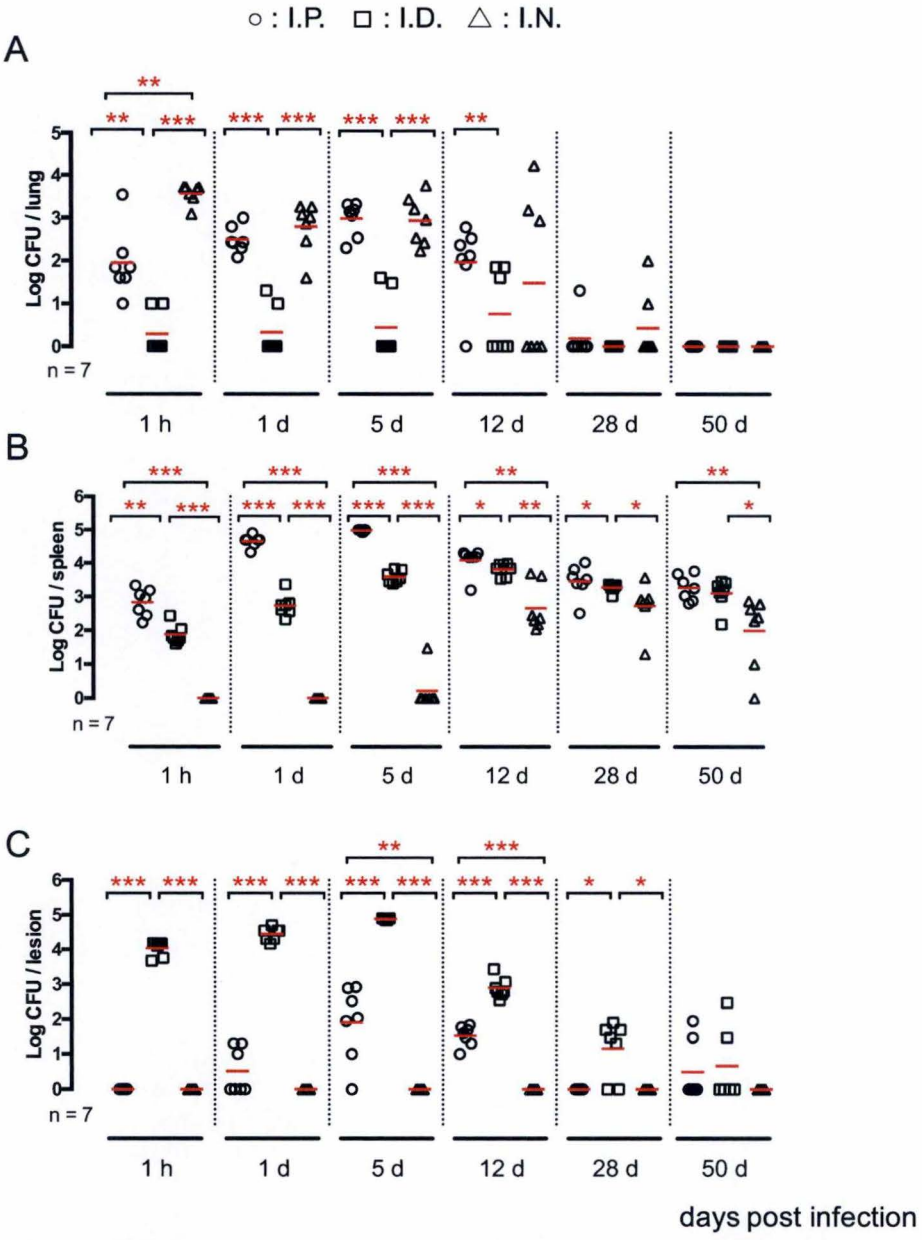


Figure 15:* Brucella *dissemination on the lung, spleen and footpad of C57BL/6 mice following i.p., i.n. and i.d. infection. C57BL/6 mice infected were infected by an i.n. or i.p. route with $2x10^4$ CFU and on i.d. route with $4x10^4$ CFU of mCherry-*B. melitensis*. At selected times post infection mice were anesthetized to take blood and were then sacrificed by cervical dislocation to harvest multiple organs: the thymus, the spleen, one lobe of the liver, one lung, the bronchial draining lymph node, the brain, one ovary, the heart, 1 cm of the muscle of the posterior leg, 1 cm at the base of the tail, the lesion at the footpad and the popliteal draining lymph node. The data represent the number of CFU per **A.** lung, **B.** spleen, **C.** footpad. n denotes the number of mice used in each group. The red bar represents the means. Detection threshold = 10 CFU. *, $p < 0.05$; **, $p < 0.01$ ***, $p < 0.001$.

DISCUSSION

Discussion

Immunological experimental models are often very reductionist by studying the effect of only one parameter on the dynamic of a complex system and thus providing a vision far from the reality. In reality, the ability of the host to face an infection is depending on so many parameters that the outcome of the battle is unpredictable at individual level. Identifying those parameters constitutes a real challenge and could offer a better follow-up of the patient and a better control of the spreading of the disease. In the literature, many factors are described to modulate the immune response of the host, such as genetic, alimentation, microbiota, psychological stress, past immune history, chronic infection... All those factors can influence positively or negatively the control of an infection (Muraille 2016)

During my master thesis, we got interested on the impact of a co-infection on the ability of the host to control an infection with *Brucella*. To do so, we developed a mice model in which we study the impact of *T. brucei* infection on the course of *B. melitensis* during its chronic phase of infection. The long term persistence of the bacteria renders it a good model to study the impact of a co-infection on a chronical infectious disease.

Brucella is an intracellular Gram negative bacteria responsible for a worldwide zoonosis: brucellosis. 500.000 new human cases are diagnosed each year and it causes huge economical issue by causing abortion and infertility in animals (Pappas et al. 2006). Without treatment, brucellosis turns into a chronic disease allowing the bacteria to persist in the host for his lifetime. This persistence makes it likely that a host infected with *Brucella* has to face several other infections. *Brucella* has acquire strategies to interfere with its recognition by the immune by the immune system and to neutralize immune effectors. As soon as it enters into the host cell, *Brucella* is able to divert the intracellular traffic to avoid degradation by the phagolysosome and then to turn to its advantage de metabolism of the host cell.

Our research group has recently developed an almost physiological model of intranasal (i.n.) *Brucella* infection in mice (Hanot Mambres et al. 2016). Mice receive intranasally $2x10^4$ CFU of *B. melitensis*, mimicking the infection by the inhalation of aerosol. *B. melitensis* has been chosen among all *Brucella*'s species because it is the most frequently isolated in human (Young 2002). Following an i.n. infection, bacteria persist in the lungs of the mice until 12 days post infection and the spleen and the liver are infected from 5 days post infection. *Brucella* is able to persist in the spleen for a long period, at least until 50 days post infection, but not in the liver. An early immune response controlling bacteria growth has been identified in the lungs of infected mice as being dependent on IL-17RA, $\gamma\delta^+$ T cells, $CD8^+$ T cells. The late immune response in the lung and the spleen is mainly depending on IFNγ – $CD4^+$ T cells response (Hanot Mambres et al. 2016). As it has been shown by Hanot Mambres in the spleen of highly susceptible IL-12p40$^{-/-}$ BALB/c mice, *Brucella* resides in reservoir cells displaying a specific phenotype. These cells are $CD11c^+$ $CD205^+$ arginase 1^+ and are lipid rich (Hanot Mambres et al. 2015). We hypothesized that these reservoir cells could confer a protection against the protective memory response to *Brucella* and allowed the bacteria to persist in the host through its lifetime.

During my master thesis, we have first confirmed that the reservoir cells of *Brucella* expressed $CD11c^+$ in resistant C57BL/6 mice by using CD11c-DTR mice. We used those mice as an alternative because the remaining level of *Brucella* in the spleen of resistant mice

is too low to be observed by microscopy analysis. CD11c-DTR mice express a diphtheria toxin (DT) receptor under the control of CD11c promoter. An intraperitoneal injection of DT induces the depletion of $CD11c^+$ cells by inducing their apoptosis (Van Blijswijk et al. 2013; Jung et al. 2002). The depletion of those cells is translated by a serious drop of the bacterial load in the spleen of the mice. Using this technique could allow us to identify the phenotype of reservoir cells in other organs where *Brucella* is able to persist such as the bronchial draining lymph node where the bacteria is also able to persist for a long time. But the adverse effect of CD11c-DTR mice is that the injection of DT in mice induces a small inflammatory response that could modify the physiology of the mice. The mechanism by which *Brucella* dies is still unknown but we can hypothesized that when the reservoir cells die by apoptosis, bacteria are released making them accessible to the immune system and killed by the inflammation generated by the apoptosis. But we can also hypothesized that the death of the cell leads to the death of the bacteria because of a lack of nutrients.
We also demonstrated that reservoir cells provide a resistance against the protective *Brucella* specific memory response as *Brucella* challenge strain but not established strain is eliminated in chronically infected mice. Thus, infected mice develop a specific immune response able to control and eliminate a new infection by *Brucella* but unable to eliminate established infection.

As *Brucella* is well known to display various mechanisms allowing to escape both innate and adaptive immune response, we develop a co-infection model with *T. brucei* in order to analyze the effect of a strong, non antigen, specific inflammatory immune response on the persistence of *B. melitensis*. We observed that a co-infection with *T. brucei* in mice previously infected with *B. melitensis* strongly increase their ability to control *Brucella* infection. This result is very surprising as *Trypanosoma's*, are well known to induce an immunosuppressive effect (Murray et al. 1974) It might be due to a stronger Th1 immune response than a Th2 or the immunosuppressive effect could appear later in the kinetic of infection of *T. brucei* and then could be visible later. This last hypothesis is, at that time, impossible to confirm or invalidate due to the fact that mice end up dying when infected with *T. brucei*. Cytofluorometry analysis highlighted that this elimination following the co-infection by the parasite is associated a high frequency of IFNγ producing $CD4^+$ T (Th1) cells. Co-infection of mice deficient for IL-12, which is required for the production of IFNγ, are not able to clear the bacteria confirming that the Th1 immune response is well required for the elimination of *Brucella*. However, co-infection does not eliminate *Brucella* in $MHCII^{-/-}$ mice in despite of a high frequency of IFN-γ^+ $CD8^+$ T cells, suggesting that a functional IL-12/IFNγ signaling axis is required but not sufficient to clear *Brucella*. Additional unknown effector mechanisms, provided by $CD4^+$ T cells, appear also necessary. It could be interesting to transfer IFNγ – producing $CD4^+$ T cells induced by a *T. brucei* infection in mice infected with *Brucella* to determine if these cells mediating the Th1 response are able alone to increase the control of the infection by *Brucella*. It is interesting to remark that co-infection with a strongly attenuated *T. brucei* mutant (DNc *T. brucei* mutant), which induces a weaker inflammatory response than the wild type *T. brucei*, is also able to eliminate *Brucella* in spleen, suggesting that the level of the inflammatory response is not a limiting factor.

In order to better characterize the mechanism of the elimination of *B. melitensis* by *T. brucei*, we performed the same co-infection experiment but with another *Trypanosoma* species: *T. cruzi*. This protozoan parasite has a completely different life cycle than *T. brucei* and shares no common antigen. The only common point between those two species is that they both induce a strong Th1 inflammatory immune response. We observed that mice co-infected with *T. cruzi* were also able to better control the bacterial infection by inducing an important

production of IFNγ by the $CD4^+$ T cells. The similar reduction of the bacterial load that was observed in the spleen when mice were co-infected with *T. cruzi* means that the elimination of *Brucella* is not due to an antigenic cross-reaction but well due to an " antigen unspecific" effect depending of the Th1 response induced by the parasite. We also tested the impact of a co-infection with *T. brucei* on the control of an infection with two other species of *Brucella* that can also affect humans: *B. abortus* and *B. suis*, in order to determine if we could generalize the effect of *T brucei* to *Brucella* in general. The elimination of those two species was not as important as with *B. melitensis*. This partial eradication might be due to the fact that the bacterial load in the spleen of mice was higher with *B. abortus* and *B. suis* than with *B. melitensis*.

In summary, previous studies (Copin et al. 2012; Hanot Mambres et al. 2015) and the present work showed that *Brucella* resides in spleen, in a specific $CD11c^+$ reservoir cells that is little accessible to immune effectors activated during a re-infection with the bacteria. The infection by *T. brucei* or *T. cruzi* might induce a strong Th1 immune response able to destabilize *Brucella*'s niche making the bacteria accessible to the immune effectors. This could due to the fact the inflammatory response is so important that it induces a tissue alteration such that the reservoir cells become accessible. A microscopy analysis of the spleen of the co-infected mice could confirm or invalidate this hypothesis. As shown by Copin, *Brucella* resides in granuloma that acts like fortress against immune effectors, making the access to the infected cells more complicated (Copin et al. 2012). Granulomas are dynamic structures set-up by the host when unable to clear the pathogen. It is composed of macrophages surrounded by T cells lymphocytes in order to reduce the dissemination of the pathogen but in some cases it could also facilitate the proliferation of the pathogen before its dissemination (Davis & Ramakrishnan 2009; Ramakrishnan 2012). In the case of an intranasal infection with *Brucella*, granulomas are localized in the white pulp in the spleen and are composed of macrophages and infected dendritic cells surrounded by T cells (Hanot Mambres et al. 2016). *T. brucei* might be able to destroy this fortress, opening a way to the infected cells for the immune effectors, allowing the elimination of *Brucella* in co-infected mice.
Our co-infection model constitutes an opportunity to identify mechanisms involved in the elimination of *Brucella*'s reservoir cells and maybe could lead to the discovery of new therapeutic strategies for brucellosis not dependent on antibiotic treatment, which is heavy at this time. But one thing to keep in mind is that mice are not the natural host neither for *Brucella* nor for *T. brucei*. However, those two pathogens infect cattle as well as humans. Brucellosis is a widespread pathology in human but also in animals which maximize the chance to find host infected with *Brucella* affected with another pathology. If we take a look at the distribution of brucellosis and the African trypanosomiasis, we can see that endemic regions for *Trypanosoma* are not endemic for brucellosis. That could be explained by our results showing that an infection with *T. brucei* drastically reduces *Brucella*. But the prevalence of brucellosis in regions touched by trypanosomiasis is also probably reduced due to the fact that breeding is less spread in those regions because of the acute risk of transmission of the parasite.

By comparing the dissemination *Brucella* following different route of infection (intraperitoneal, intranasal, intradermal), we observed that the infectious model has a great impact on the nature of chronically infected organs and by consequence, potentially, on the pathology of brucellosis. Following an intraperitoneal infection, the dissemination of the bacteria follows a systemic way when it was a local dissemination when infecting in an intranasal or in an intradermal way. During this master thesis, we focused on the impact of the co-infection with *T. brucei* on the bacterial load in the spleen. It could be interesting to

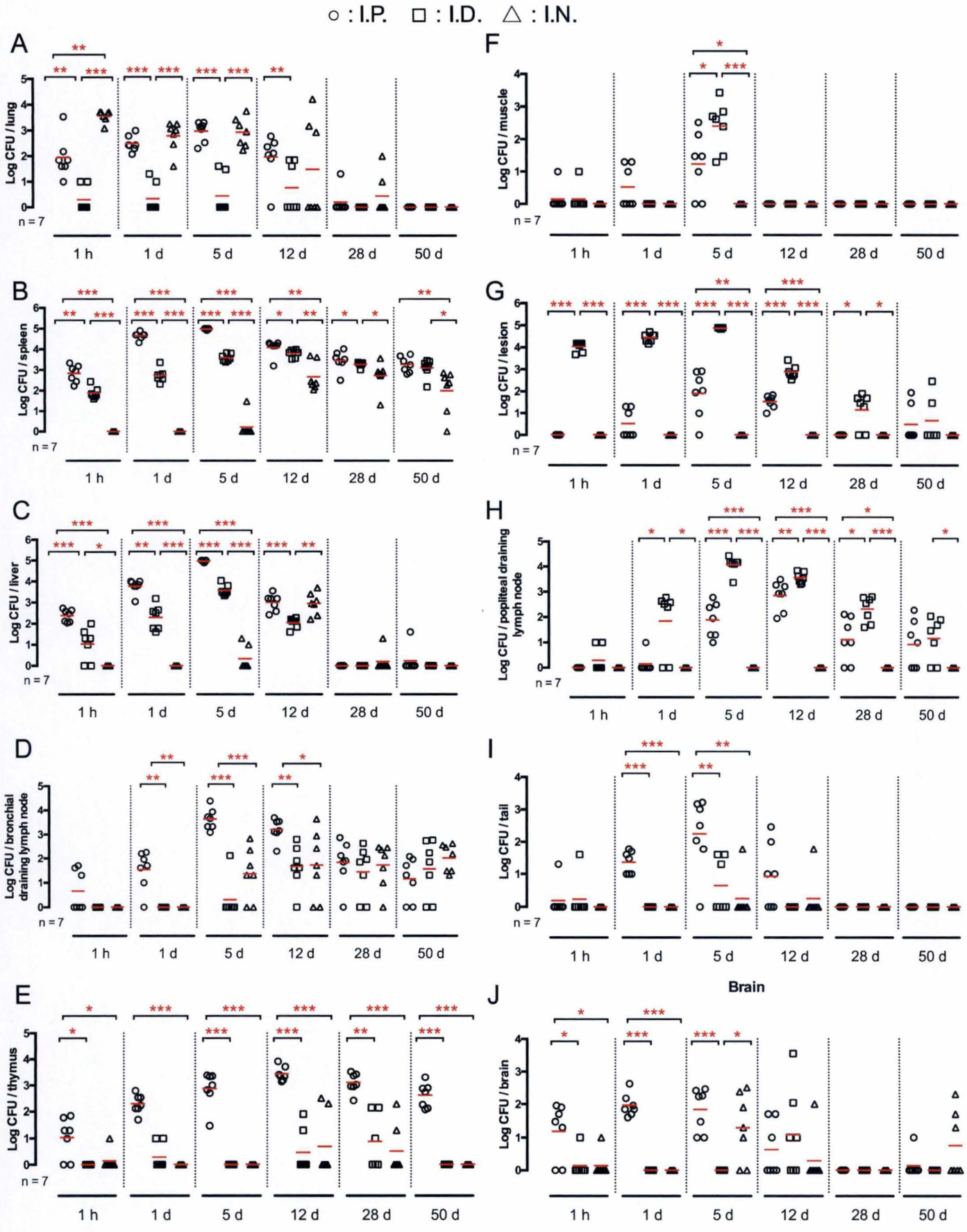
determine if those results can be generalized to other organs. Following an intranasal infection, we observed that *Brucella* was able to persist for a long time in the spleen and the bronchial draining lymph node. The bacteria were also able to persist in those organs following and intradermal infection but also in the popliteal draining lymph node which is coherent knowing that it drains the paw which was the way of entry of the bacteria in this model. Whereas following an intraperitoneal infection with *Brucella*, in addition to the lymph nodes and the spleen, the thymus remains infected on a long period. The impact of an infection with *T. brucei* on *Brucella* in those other organ could be interesting to study.

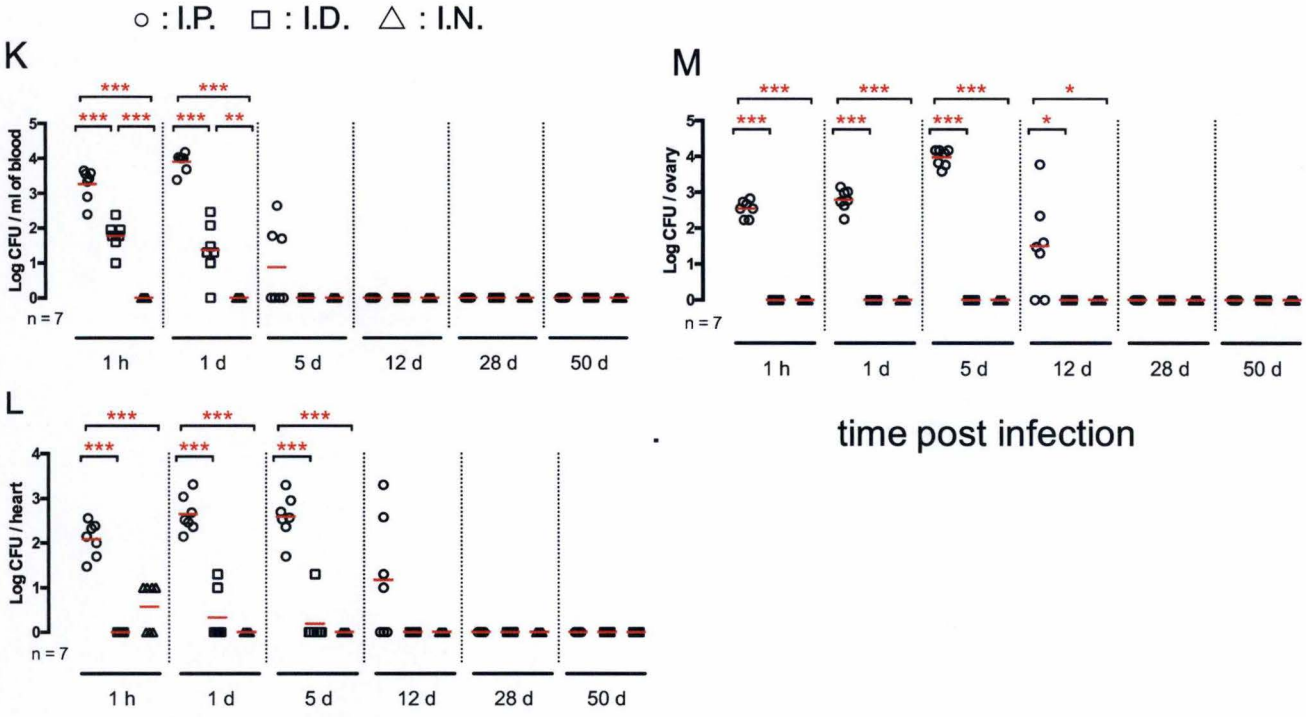
In conclusion, we can say that those results show a real effect of a co-infection and that cross-pathologies have a real impact on the immune response and it is important to take them into account. Discover the mechanisms could lead to the identification of therapeutic strategies and not only for *Brucella*. Those co-infection model could be extended to other infectious model such as *Mycobacterium tuberculosis* or other pathologies presenting public health issues. It could be a new approach to identify therapeutic targets or, in the case of a negative effect of the cross-pathology, a marker of higher susceptibility.
It could be also interesting to be able to predict the future of an affected patient and to be able to personalize its treatment by taking into account its immunological status.

ACKNOWLEDGEMENTS

First of all, I would like to thank my promoter Eric Muraille and my co-promoter Jean-Jacques Letesson for their patience and for the numerous re-lecture of this manuscript and without who all of this would be impossible.
I also want to thank my supervisor, Arnaud Machelart, who teaches me everything ranging from manipulations to the love of Maitre Gims. I have to admit that I listened a lot to his "songs" lately… And thank for all your phone calls to encourage me.
A special thanks to Françoise, Aurélie and Mathieu without whom the life in the lab would be more difficult and disorganized.
Aurore, thank you for your precious help when Arnaud left us to live with our Ch'tis neighbors but also to laugh to my not always funny jokes.
Also thank to the mice that helped me a lot in the realization of these manipulations, for the small talking even if they rarely answered me and for all the good moments we spend together even if they bitted my fingers more than once.
I am thankful to my friends and family for supporting me despite my mood swings and for all the supporting messages they send me lately. And a special thanks to Hubert and Zoé for making me in a good mood just by their presence.
To all the URBM team: thank you for the good atmosphere in the lab and the chats during long day in the P3.
And to finish, thank you to all my jury members for your time: D. Coupeau, B. Hespeels, T. Marcotty and J-Y Matroule

ANNEXES


○ : I.P. □ : I.D. △ : I.N.
A
Log CFU / lung
B
Log CFU / spleen
C
Log CFU / liver
D
Log CFU / bronchial draining lymph node
E
Log CFU / thymus
F
Log CFU / muscle
G
Log CFU / lesion
H
Log CFU / popliteal draining lymph node
I
Log CFU / tail
J
Brain
Log CFU / brain
n = 7
1 h
1 d
5 d
12 d
28 d
50 d
time post infection



Supplementary data 1: Course of* Brucella *infection in C57BL/6 mice following i.p., i.n. and i.d. infection. C57BL/6 mice infected were infected by an i.n. or i.p. route with $2x10^4$ CFU and on i.d. route with $4x10^4$ CFU of mCherry-*B. melitensis*. At selected times post infection mice were anesthetized to take blood and were then sacrificed by cervical dislocation to harvest multiple organs: the thymus, the spleen, one lobe of the liver, one lung, the bronchial draining lymph node, the brain, one ovary, the heart, 1 cm of the muscle of the posterior leg, 1 cm at the base of the tail, the lesion at the footpad and the popliteal draining lymph node. The data represent the number of CFU per **A.** lung, **B.** spleen, **C.** one lobe of the liver, **D.** bronchial draining lymph node, **E.** thymus, **F.** 1 cm of muscle, **G.** footpad lesion, **H.** popliteal draining lymph node, **I.** 1 cm at the base of the tail, **J.** brain, **K.** a ml of blood, **L.** heart, **M.** one ovary. n denotes the number of mice used in each group. The red bar represents the means. Detection threshold = 10 CFU. *, $p < 0.05$; **, $p < 0.01$ ***, $p < 0.001$.

REFERENCES

References

Acquatella, H., 2007. Echocardiography in Chagas heart disease. *Circulation*, 115(9), pp.1124–1131.

Atluri, V.L. et al., 2011. Interactions of the Human Pathogenic *Brucella* Species with Their Hosts. *Annual Review of Microbiology*, 65(1), pp.523–541. Available at: http://dx.doi.org/10.1146/annurev-micro-090110-102905.

Baker, N. et al., 2013. Europe PMC Funders Group Drug resistance in African trypanosomiasis : the melarsoprol and pentamidine story Chemotherapy against African trypanosomiasis Europe PMC Funders Author Manuscripts The diamidines. , 29(3).

Bern, C. et al., 2009. Congenital *Trypanosoma cruzi* transmission in Santa Cruz, Bolivia. *Clin Infect Dis*, 49(11), pp.1667–1674. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19877966\nhttp://cid.oxfordjournals.org/content/49/11/1667.full.pdf.

Bern, C. et al., 2011. *Trypanosoma cruzi* and chagas' disease in the united states. *Clinical Microbiology Reviews*, 24(4), pp.655–681.

Billard, E., Dornand, J. & Gross, A., 2008. VirB type IV secretory system does not contribute to *Brucella suis*' avoidance of human dendritic cell maturation. *FEMS Immunology and Medical Microbiology*, 53(3), pp.404–412.

Blanden, R. V, Lefford, M.J. & Mackaness, G.B., 1969. The host response to Calmette-Guérin bacillus infection in mice. *The Journal of experimental medicine*, 129(5), pp.1079–107. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2138639&tool=pmcentrez&rendertype=abstract.

Van Blijswijk, J., Schraml, B.U. & Sousa, C.R. e, 2013. Advantages and limitations of mouse models to deplete dendritic cells. *European Journal of Immunology*, 43(1), pp.22–26.

Brown, B.N. et al., 2009. Macrophage phenotype and remodeling outcomes in response to biologic scaffolds with and without a cellular component. , 100(2), pp.130–134.

Buffie, C.G. & Pamer, E.G., 2013. Microbiota-mediated colonization resistance against intestinal pathogens. *Nat Rev Immunol*, 13(11), pp.790–801. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24096337.

Burleigh, B.A. & Andrews, N.W., 1995. The mechanisms of *Trypanosoma Cruzi* invasion of Mammalian Cells. *Annual Reviews in Microbiology*, 49(1), pp.175–200. Available at: http://resolver.scholarsportal.info/resolve/00664227/v49i0001/175_tmotciomc.

Cardoso, P.G. et al., 2006. *Brucella* spp noncanonical LPS: structure, biosynthesis, and interaction with host immune system. *Microbial cell factories*, 5, p.13.

Castro, J.A., Montalto de Mecca, M. & Bartel, L.C., 2006. Toxic side effects of drugs used to treat Chagas' disease (American trypanosomiasis). *Human & experimental toxicology*, 25(8), pp.471–479.

Cerf-Bensussan, N. & Gaboriau-Routhiau, V., 2010. The immune system and the gut microbiota: friends or foes? *Nature reviews. Immunology*, 10(10), pp.735–44. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20865020.

Chappuis, F. et al., 2005. Options for Field Diagnosis of Human African Trypanosomiasis. *Clinical Microbiology Reviews*, 18(1), pp.133–146.

Cirl, C. et al., 2008. Subversion of Toll-like receptor signaling by a unique family of bacterial Toll/interleukin-1 receptor domain-containing proteins. *Nature medicine*, 14(4), pp.399–406. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18327267.

Clapp, B. et al., 2016. Nasal vaccination stimulates CD8(+) T cells for potent protection

against mucosal *Brucella melitensis* challenge. *Immunology and cell biology*, 94(5), pp.496–508. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4879022&tool=pmcentrez&rendertype=abstract.

Clayton, J., 2010. Chagas disease 101. *Nature*, 465(7301), pp.S4–S5.

Copin, R. et al., 2012. In situ microscopy analysis reveals local innate immune response developed around *Brucella* infected cells in resistant and susceptible mice. *PLoS Pathogens*, 8(3).

Copin, R. et al., 2007. MyD88-dependent activation of B220-CD11b+LY-6C+ dendritic cells during *Brucella melitensis* infection. *Journal of immunology (Baltimore, Md: 1950)*, 178(8), pp.5182–5191. Available at: papers2://publication/uuid/6AC09FD4-B603-467B-91BB-F5398EC6BE06.

Corbel, M.J., 1997. Brucellosis: An Overview. *Emerging Infectious Diseases*, 3(2), pp.213–221.

Davis, J.M. & Ramakrishnan, L., 2009. The Role of the Granuloma in Expansion and Dissemination of Early Tuberculous Infection. *Cell*, 136(1), pp.37–49. Available at: http://dx.doi.org/10.1016/j.cell.2008.11.014.

Deghelt, M. et al., 2014. G1-arrested newborn cells are the predominant infectious form of the pathogen *Brucella abortus*. *Nature communications*, 5, p.4366. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25006695.

Díaz Aparicio, E., 2013. Epidemiology of brucellosis in domestic animals caused by *Brucella melitensis*, *Brucella suis* and *Brucella abortus*. *Revue scientifique et technique (International Office of Epizootics)*, 32(1), pp.43–51, 53–60. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23837364.

Ducrotoy, M.J. et al., 2016. A review of the basis of the immunological diagnosis of ruminant brucellosis. *Veterinary Immunology and Immunopathology*, 171, pp.81–102. Available at: http://dx.doi.org/10.1016/j.vetimm.2016.02.002.

De Figueiredo, P. et al., 2015. Pathogenesis and immunobiology of brucellosis: Review of *Brucella*-host interactions. *American Journal of Pathology*, 185(6), pp.1505–1517. Available at: http://dx.doi.org/10.1016/j.ajpath.2015.03.003.

Furman, D. et al., 2015. *Cytomegalovirus* infection enhances the immune response to influenza. *Science Translational Medicine*, 7(281), p.281ra43-281ra43. Available at: http://stm.sciencemag.org/content/7/281/281ra43.full.

Garcia-Yoldi, D. et al., 2006. Multiplex PCR assay for the identification and differentiation of all *Brucella* species and the vaccine strains *Brucella abortus* S19 and RB51 and *Brucella melitensis* Rev1. *Clinical Chemistry*, 52(4), pp.778–779.

Gordon, S. & Taylor, P.R., 2005. Monocyte and macrophage heterogeneity. *Nature reviews. Immunology*, 5(12), pp.953–64. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16322748.

Gorvel, J.P., 2008. *Brucella*: a Mr "Hide" converted into Dr Jekyll. *Microbes and Infection*, 10(9), pp.1010–1013. Available at: http://dx.doi.org/10.1016/j.micinf.2008.07.007.

Hakansson, A. et al., 1994. *Adenovirus* infection enhances in vitro adherence of *Streptococcus pneumoniae*. *Infect. Immun.*, 62(7), pp.2707–2714. Available at: http://iai.asm.org/cgi/content/long/62/7/2707 [Accessed November 22, 2016].

Hanot Mambres, D. et al., 2016. Identification of Immune Effectors Essential to the Control of Primary and Secondary Intranasal Infection with *Brucella melitensis* in Mice. *The Journal of Immunology*, 196(9), pp.3780–3793. Available at: http://www.jimmunol.org/cgi/doi/10.4049/jimmunol.1502265.

Hanot Mambres, D. et al., 2015. In situ characterization of splenic *Brucella melitensis* reservoir cells during the chronic phase of infection in susceptible mice. *PLoS ONE*,

10(9), pp.1–20. Available at: http://dx.doi.org/10.1371/journal.pone.0137835.
Horn, D., 2014. Antigenic variation in African trypanosomes. *Molecular and Biochemical Parasitology*, 195(2), pp.123–129. Available at: http://dx.doi.org/10.1016/j.molbiopara.2014.05.001.
Inojosa, W.O. et al., 2006. Diagnosing human African trypanosomiasis in Angola using a card agglutination test: observational study of active and passive case finding strategies. *BMJ (Clinical research ed.)*, 332(July), p.1479.
De Jong, M.F. et al., 2008. Identification of VceA and VceC, two members of the VjbR regulon that are translocated into macrophages by the *Brucella* type IV secretion system. *Molecular Microbiology*, 70(6), pp.1378–1396.
Jung, S. et al., 2002. In vivo depletion of CD11c+ dendritic cells abrogates priming of CD8+ T cells by exogenous cell-associated antigens. *Immunity*, 17(2), pp.211–220.
Kennedy, P.G.E., 2013. Clinical features, diagnosis, and treatment of human African trypanosomiasis (sleeping sickness). *The Lancet Neurology*, 12(2), pp.186–194. Available at: http://dx.doi.org/10.1016/S1474-4422(12)70296-X.
Kitano, H. & Oda, K., 2006. Robustness trade-offs and host-microbial symbiosis in the immune system. *Mol Syst Biol*, 2, p.2006 0022. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16738567.
Lalonde, R.G. & Holbein, B.E., 1984. Role of iron in *Trypanosoma cruzi* infection of mice. *The Journal of clinical investigation*, 73(2), pp.470–476.
Langousis, G. & Hill, K.L., 2014. Motility and more: the flagellum of *Trypanosoma brucei*. *Nature reviews. Microbiology*, 12(7), pp.505–18. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24931043.
Liu, G. et al., 2015. Distinct Contributions of CD4+ and CD8+ T Cells to Pathogenesis of *Trypanosoma brucei* Infection in the Context of Gamma Interferon and Interleukin-10. *Infection and immunity*, 83(7), pp.2785–95. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25916989.
Lokken, K.L. et al., 2014. Malaria Parasite Infection Compromises Control of Concurrent Systemic Non-typhoidal *Salmonella* Infection via IL-10-Mediated Alteration of Myeloid Cell Function. *PLoS Pathogens*, 10(5).
Makala, L.H. et al., 2002. The current status of major tick borne diseases in Zambia. *Veterinary Research*, 33(3), pp.239–250.
Marcello, L. & Barry, J.D., 2007. Analysis of the VSG gene silent archive in *Trypanosoma brucei* reveals that mosaic gene expression is prominent in antigenic variation and is favored by archive substructure. *Genome Research*, 17(9), pp.1344–1352.
Marchesini, M.I. et al., 2011. In search of *Brucella abortus* type iv secretion substrates: Screening and identification of four proteins translocated into host cells through virb system. *Cellular Microbiology*, 13(8), pp.1261–1274.
Martirosyan, A. & Gorvel, J.-P., 2013. *Brucella* evasion of adaptive immunity. *Future microbiology*, 8(2), pp.147–54. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23374122.
Matsumoto, S. et al., 2000. *Mycobacterium bovis* bacillus calmette-guerin induces protective immunity against infection by *Plasmodium yoelii* at blood-stage depending on shifting immunity toward Th1 type and inducing protective IgG2a after the parasite infection. *Vaccine*, 19(7–8), pp.779–787.
Medzhitov, R., 2007. Recognition of microorganisms and activation of the immune response. *Nature*, 449(7164), pp.819–26. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17943118.
Mugnier, M.R., George A. M., C. & Papavasiliou, F.N., 2015. The in vivo dynamics of antigenic variation in *Trypanosoma brucei*. , 33(4), pp.395–401.

Muraille, E., 2016. The unspecific side of acquired immunity against infectious disease: Causes and consequences. *Frontiers in Microbiology*, 6(JAN), pp.1–11.

Murray, P.K. et al., 1974. The Nature of Immunosuppression in *Trypanosoma brucei* Infections in Mice. *Source*, 27, pp.825–840.

Mutolo, M.J. et al., 2012. Osteological and molecular identification of brucellosis in ancient Butrint, Albania. *American Journal of Physical Anthropology*, 147(2), pp.254–263.

Namangala, B. et al., 2001. Relative contribution of interferon-gamma and interleukin-10 to resistance to murine African trypanosomosis. *The Journal of infectious diseases*, 183(12), pp.1794–800. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11372033.

Pansaerts, R. et al., 1998. Increased sensitivity of the card agglutination test CATT/*Trypanosoma brucei gambiense* by inhibition of complement. *Acta Tropica*, 70(3), pp.349–354.

Pappas, G. et al., 2006. The new global map of human brucellosis. *The Lancet infectious diseases*, 6(2), pp.91–99.

Pasquevich, K.A. et al., 2011. An oral vaccine based on U-Omp19 induces protection against *B. abortus* mucosal challenge by inducing an adaptive IL-17 immune response in mice. *PloS one*, 6(1), p.e16203. Available at: http://dx.doi.org/10.1371/journal.pone.0016203 [Accessed November 6, 2016].

Pays, E. et al., 2006. The trypanolytic factor of human serum. *Nature Reviews Microbiology*, 4(6), pp.477–486. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20398209\nhttp://www.nature.com/doifinder/10.1038/nrmicro1428.

Potian, J.A. et al., 2011. Preexisting helminth infection induces inhibition of innate pulmonary anti-tuberculosis defense by engaging the IL-4 receptor pathway. *The Journal of experimental medicine*, 208(9), pp.1863–1874. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3171086&tool=pmcentrez&rendertype=abstract.

Radwanska, M. et al., 2008. Trypanosomiasis-induced B cell apoptosis results in loss of protective anti-parasite antibody responses and abolishment of vaccine-induced memory responses. *PLoS Pathogens*, 4(5).

Rajashekara, G. et al., 2005. Temporal analysis of pathogenic events in virulent and avirulent *Brucella melitensis* infections. *Cellular Microbiology*, 7(10), pp.1459–1473.

Ramakrishnan, L., 2012. Revisiting the role of the granuloma in tuberculosis. *Nature reviews. Immunology*, 12(5), pp.352–66. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22517424.

Rassi, A., Rassi, A. & Marin-Neto, J.A., 2010. Chagas disease. *The Lancet*, 375(9723), pp.1388–1402. Available at: http://dx.doi.org/10.1016/S0140-6736(10)60061-X.

Rudenko, G., 2011. African trypanosomes: the genome and adaptations for immune evasion. *Essays in biochemistry*, 51, pp.47–62. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22023441.

Sacks, D.L. & Askonas, B.A., 1980. *Trypanosome*-induced suppression of anti-parasite responses during experimental African trypanosomiasis. *European Journal of Immunology*, 10(12), pp.971–974.

Saenz, S.A., Taylor, B.C. & Artis, D., 2008. Welcome to the neighborhood: Epithelial cell-derived cytokines license innate and adaptive immune responses at mucosal sites. *Immunological Reviews*, 226(1), pp.172–190.

Salcedo, S.P. et al., 2008. *Brucella* control of dendritic cell maturation is dependent on the TIR-containing protein Btp1. *PLoS Pathogens*, 4(2).

Salmon, D. et al., 2012. Adenylate Cyclases of *Trypanosoma brucei* Inhibit the Innate Immune Response of the Host. *Science*, 337(6093), pp.463–466.

Schneider, M.C. et al., 2011. Importance of animal/human health interface in potential Public Health Emergencies of International Concern in the Americas. *Revista panamericana de salud publica = Pan American journal of public health*, 29(1), pp.371–379.

Seleem, M.N., Boyle, S.M. & Sriranganathan, N., 2010. Brucellosis: A re-emerging zoonosis. *Veterinary Microbiology*, 140(3–4), pp.392–398.

Sher, N.A. et al., 1975. Effects of BCG, *Corynebacterium parvum*, and methanol extraction residue in the reduction of mortality from *Staphylococcus aureus* and *Candida albicans* infections in immunosuppressed mice. *Infection and Immunity*, 12(6), pp.1325–1330.

Starr, T. et al., 2008. *Brucella* intracellular replication requires trafficking through the late endosomal/lysosomal compartment. *Traffic*, 9(5), pp.678–694.

Starr, T. et al., 2012. Selective subversion of autophagy complexes facilitates completion of the *Brucella* intracellular cycle. *Cell Host and Microbe*, 11(1), pp.33–45.

Terwagne, M. et al., 2013. Innate immune recognition of flagellin limits systemic persistence of *Brucella*. *Cellular Microbiology*, 15(6), pp.942–960.

Torrico, F. et al., 2004. Maternal *Trypanosoma cruzi* infection, pregnancy outcome, morbidity, and mortality of congenitally infected and non-infected newborns in Bolivia. *American Journal of Tropical Medicine and Hygiene*, 70(2), pp.201–209.

Verreck, F. a W. et al., 2006. Phenotypic and functional profiling of human proinflammatory type-1 and anti-inflammatory type-2 macrophages in response to microbial antigens and IFN-γ- and CD40L-mediated costimulation. *Journal of Leukocyte Biology*, 79(2), pp.285–293.

Vickerman, K., 1985. Developmental cycles and biology of pathogenic trypanosomes. *British medical bulletin*, 41(2), pp.105–14. Available at: http://www.ncbi.nlm.nih.gov/pubmed/3928017.

Vitry, M.-A. et al., 2014. Humoral Immunity and CD4+ Th1 Cells Are Both Necessary for a Fully Protective Immune Response upon Secondary Infection with *Brucella melitensis*. *Journal of immunology (Baltimore, Md. : 1950)*, 192, pp.3740–52. Available at: http://www.jimmunol.org/content/192/8/3740.full.

Welsh, R.M. & Selin, L.K., 2002. No one is naive: the significance of heterologous T-cell immunity. *Nature reviews. Immunology*, 2(6), pp.417–426.

Young, E.J., 2002. An Overview of Human Brucellosis. *Mmwr. Morbidity And Mortality Weekly Report*, 21(2), pp.283–290.

Zeng, M.Y. et al., 2016. Gut Microbiota-Induced Immunoglobulin G Controls Systemic Infection by Symbiotic Bacteria and Pathogens. *Immunity*, 44(3), pp.647–658.