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On the components of adaptive immune response involved in the control of a primary and secondary infection by *Brucella melitensis*

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Summary

Brucellosis is a bacterial zoonose caused by organisms belonging to the genus *Brucella*. These bacteria are facultative intracellular pathogens that cause abortion in domestic (cattle, goats, sheeps, etc.) and wild (deers, bisons, etc.) animals and a febrile chronic illness in humans. The disease exists worldwide and continues to have a great health significance and economic importance in developing countries. Despite past and current efforts to eradicate brucellosis by vaccination and culling within cattle and herds, as many as 500 000 new human cases are reported annually worldwide. Vaccines against brucellosis were initially developed on an empirical basis. The current studies are moving towards a more rational design but are hindered by an incomplete knowledge of the *in vivo* life style of *Brucella* and the immune mechanisms involved in the establishment of a protective memory. Despite progresses, several fields of host adaptive immunity remain highly controversial such as the implication of B cells, $CD4^+$ and $CD8^+$ T cells. While it is commonly accepted that *Brucella* infection induces a Th1 immune response characterized by the production of IFN-γ that activates bactericidal mechanisms of macrophages, little has been described with regards to a potential implication of the Th2 response or the recently described Th17 response.

Objectives of this thesis were therefore to determinate the implication of lymphocyte subsets and signaling pathways after primary infection with *Brucella melitensis* and characterize their roles in the development of a protective secondary immune response in the murine experimental model.

In the first part of this study we clearly confirm the central role of MHC-II-dependent antigen presentation to $CD4^+$ T cells and the IFN-γ-mediated Th1 response in the control of *B. melitensis* primary infection. We also report that the absence of B cells, MHC-I-dependent antigen presentation, Th2 and Th17 responses appears to have no important positive or negative impact on the course of infection.

In the second part of this work, we show that *Brucella* is able to persist several weeks in the blood of infected mice. Surprisingly, we found that bacteria are initially localized extracellularly and then infect erythrocytes where they are already detectable after 24h.

Finally, the last part demonstrates that humoral immunity and $CD4^+$ Th1 cells are both necessary and complementary for a sterilizing immune response upon a secondary infection with *B. melitensis*. Circulating specific antibodies and IFN-γ production by $CD4^+$ T cells activated at the site of infection after the re-call infection appear as key immunological markers of protection in the murine experimental model of *Brucella* infection.

In conclusion, this work improves our understanding of the nature of murine immune response developed following *B. melitensis* infection and tries to provide correlates of protection that could help to define rational strategies for designing new vaccines against brucellosis. This study also reveals for the first time that erythrocytes constitute an unexpected niche for *Brucella*, and maybe a currently undescribed way of transmission by blood-sucking arthropods.

Composants de la réponse immunitaire adaptative impliqués dans le contrôle d'une infection primaire et secondaire par *Brucella melitensis*

par Marie-Alice Vitry

Résumé

La brucellose est une zoonose bactérienne causée par des organismes appartenant au genre *Brucella*. Ces bactéries sont des pathogènes intracellulaires facultatifs qui causent l'avortement tant chez les animaux domestiques (bovins, chèvres, moutons, etc.) que sauvages (cerfs, bisons, etc.) et une maladie chronique chez l'homme. La brucellose est présente dans le monde entier et constitue un problème majeur de santé publique qui affecte également l'économie dans les pays en voie de développement. Malgré les efforts passés et présents visant à éradiquer la brucellose, notamment par la vaccination et l'abattage systématique des troupeaux infectés, plus de 500 000 nouveaux cas humains sont signalés chaque année dans le monde. À l'origine, les vaccins contre la brucellose ont été développés empiriquement. Les études vaccinales actuelles s'orientent vers une conception plus rationnelle, mais sont freinées par une méconnaissance du cycle infectieux de *Brucella in vivo* et des mécanismes immunitaires impliqués dans l'établissement d'une mémoire immunitaire protectrice. Malgré de récents progrès, plusieurs domaines de la réponse adaptative développée en modèle murin suite à l'infection par *Brucella* sont encore fort controversés, comme l'importance de l'implication des lymphocytes B, T $CD4^+$ et T $CD8^+$. Bien qu'il soit communément admis que l'infection par *Brucella* induit une réponse immunitaire de type Th1, caractérisée par la production d'IFN-γ qui active les mécanismes bactéricides des macrophages, peu de choses ont été décrites concernant une implication potentielle des réponses Th2 ou Th17.

En conséquence, les objectifs de cette thèse étaient de déterminer l'implication des différentes populations de lymphocytes et des voies de signalisation induites après infection par *Brucella melitensis* en modèle expérimental murin et de caractériser leurs rôles dans le développement d'une réponse immunitaire protectrice lors d'une ré-infection.

Dans la première partie de ce travail, nous avons clairement confirmé le rôle central de la voie de présentation des antigènes dépendante du MHC-II aux lymphocytes T $CD4^+$ ainsi que la nécessité de la production d'IFN-γ par les lymphocytes Th1 dans le contrôle d'une infection primaire par *B. melitensis*. Nous avons également démontré que l'absence de lymphocytes B, de la voie de présentation dépendante du MHC-I ou des réponses Th2 et Th17 n'a pas d'impact majeur sur le déroulement de l'infection.

Dans la seconde partie de ce travail, nous avons montré que *Brucella* est capable de persister plusieurs semaines dans le sang de souris infectées. De manière surprenante, nous avons constaté que, dans un premier temps, les bactéries étaient libres dans le sang et qu'elles infectaient ensuite les globules rouges où elles sont déjà détectables après 24h.

Enfin, la dernière partie de ce travail démontre que les réponses humorale et cellulaire sont toutes deux complémentaires et nécessaires à l'établissement d'une réponse immune stérilisante lors d'une ré-infection par *B. melitensis*. Les anticorps circulants et la production d'IFN-γ par les lymphocytes T $CD4^+$ activés sur le site d'infection lors d'une réponse secondaire apparaissent comme des effecteurs clés de la protection en modèle murin.

En conclusion, ce travail améliore notre compréhension de la réponse immunitaire induite par *B. melitensis* en modèle murin. Il essaye également de proposer des marqueurs immunologiques de protection qui pourraient aider à définir des stratégies plus rationnelles pour la conception de nouveaux vaccins contre la brucellose. Enfin, cette étude révèle pour la première fois que les globules rouges constituent une niche d'infection inattendue qui pourrait assurer la transmission de la brucellose par des insectes suceurs de sang.

Abbreviations

AID	Activation-induced cytidine deaminase
APC	Antigen presenting cell
BCR	B cell receptor
BCV	*Brucella*-containing vacuole
Bregs	B régulateurs
CCL3	CC Chemokine ligand 3, also named Macrophage inflammatory protein-1α (MIP-1α)
CCR2	CC chemokine receptor 2
CD	Cluster of differentiation
CFU	Colony forming unit
CLRs	C-type lectin receptors
CpG	Cytosine-phosphate-guanosine
CX3CR1	CX3 Chemokine receptor 1
DAMPs	Damage-associated molecular patterns
DC	Dendritic cell
FcR	Fc receptor
GC	Germinal center
HC	Heavy chain
HK	Heat-killed
HKBA	*Brucella abortus* HK
i.n.	(voie d'infection) intra-nasale
i.p.	(voie d'infection) intra-péritonéale
i.v.	(voie d'infection) intra-veineuse
ICSBP	Interferon consensus sequence binding protein
IFN	Interferon
IFN-γR	Interferon-γ receptor
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRAK	IL-1 Receptor-associated kinase
IRF	Interferon regulatory factor
IRG	Immune-related GTPases

LC	Light chain
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
MPC	Mononuclear phagocytic cells
MuMT	μM mutation (B-cell deficient mice)
MyD88	Myeloid differentiation factor 88
NALP3	NACHT, LRR and PYD domains-containing protein 3
NETs	Neutrophil extracellular traps
NF-κB	Nuclear factor-kappa B
NK	Natural killer
NKSF	NK stimulatory factor (IL-12)
NKT	Natural killer T(cell)
NLRs	NOD-like receptors
NO	Nitric Oxide
NOD	Nucleotide oligomerization domain
Omp	Outer membrane protein
p. Ch.	Post challenge
PAMP	Pathogen-associated molecular patterns
phox	Phagocyte oxidase
POPs	Patterns of pathogenesis
PRR	Pattern recognition receptor
RAG	Recombination activating gene
RLRs	RIG1-like receptors
ROS	Reactive oxygen species
s.c.	(voie d'infection) sous-cutanée
SCID	Severe combined immunodeficient
SI	Système immunitaire
SLAT	SWAP-70-like adapter of T cells
STAT6	Signal transducer and activator of transcription-6
TAP-1	Transporter associated with antigen processing-1
T_{CM}	Central memory T cells
TcpB / Btp1	TIR domain-containing protein / Brucella TIR-containing protein
TCR	T cell receptor
T_{EM}	Effector memory T cell

TGF	Transforming growth factor
Th	T helper
TI/TD	(activation) thymo-(in)dépendante
TipDC	TNF-iNOS producing dendritic cells
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Tregs	T régulateurs
TRIF	Toll/interleukin-1 receptor domain-containing adapter inducing IFN-β
T4SS	Type IV secretion system
WT	Wild-type

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Table of contents

INTRODUCTION 1

1 Avant propos : la relation hôte / pathogène 1

1.1 Définition du système immunitaire, d'un paradigme à l'autre 1

1.2 Le pathogène 3

1.3 Le pathogène et son hôte 4

2 Le système immunitaire 6

2.1 La réponse immunitaire innée 6
2.1.1 Senseurs de l'envahisseur 7
2.1.2 Senseurs de l'invasion 9
2.1.3 Senseurs instructeurs 10
2.1.4 Effecteurs instructeurs : les cellules dendritiques 12

2.2 Réponse immunitaire adaptative 17
2.2.1 Lymphocytes T 17
2.2.1.1 Lymphocytes T cytotoxiques ($CD8^+$) 19
2.2.1.2 Lymphocytes T $CD4^+CD25^+$ régulateurs (Tregs) 19
2.2.1.3 Lymphocytes NK T 20
2.2.1.4 Lymphocytes T $\gamma\delta$ 20
2.2.2 Lymphocytes B 21
2.2.2.1 Lymphocytes B2 : activation thymo-dépendante (TD) 23
2.2.2.2 Lymphocytes B1 et B2 MZ : activation thymo-indépendante (TI) 26
2.2.2.3 Lymphocytes B régulateurs 28

2.3 Sous-classes de lymphocytes T helper : un choix de réponse adapté 29
2.3.1 Trilogie Th1, Th2, Th17 29
2.3.2 Réponse Th1 : lutte contre les pathogènes intracellulaires 31
2.3.3 Réponse Th2 : lutte contre les pathogènes extracellulaires 35
2.3.4 Réponse Th17 : lutte contre les bactéries extracellulaires 36

3 Mémoire immunitaire et vaccination 38

3.1 Jenner et Pasteur, les pionniers en vaccination 39

3.2 Développement d'une mémoire immunitaire T et B 41
3.2.1 Lymphocytes T – mémoire à médiation cellulaire 41
3.2.2 Lymphocytes B - mémoire à médiation humorale 43

3.3 **Combiner une meilleure compréhension du SI et du pathogène pour élaborer de meilleurs vaccins 44**
3.3.1 Compréhension du SI 44
3.3.2 Compréhension du monde microbien 45

4 La brucellose 48

4.1 Épidémiologie 48

4.2 Transmission 49

4.3 Pathologie 49
4.3.1 Brucellose humaine 49
4.3.2 Brucellose animale 50

4.4 Traitements et vaccination 50

4.5 Les différentes espèces de *Brucella* 53

4.6 *Brucella* et la cellule hôte **54**
4.6.1 Brucella, une bactérie extracellulaire facultative 54
4.6.2 Cellules hôtes 55

5 La réponse immunitaire contre *Brucella* **56**

5.1 Modèle d'étude : la souris **56**
5.1.1 Du cobaye à la souris 56
5.1.2 Les souris, des BALB/c aux C57BL/6 56
5.1.3 Modes d'infection expérimentaux 58
5.1.4 Déroulement de l'infection par voie intrapéritonéale dans le modèle murin 59

5.2 Réponse immunitaire innée **63**
5.2.1 La détection de Brucella : TLRs et MyD88 63
5.2.2 L'activation des DCs – manipulation du système 67
5.2.3 La réponse innée effectrice anti-Brucella - les armes du SI 68

5.3 Réponse immunitaire adaptative **72**
5.3.1 Lymphocytes T 72
5.3.1.1 Lymphocytes T $CD4^+$ ou T $CD8^+$, histoire d'une controverse 72
5.3.1.2 Lymphocytes T et production d'IFN-γ 74
5.3.1.3 Réponses des lymphocytes T non conventionels 75
5.3.1.4 Sous classes de réponse immunitaire 75
5.3.2 Lymphocytes B 77
5.3.2.1 Lymphocytes B : armés contre Brucella 77
5.3.2.2 Lymphocytes B : victimes d'une manipulation insidieuse 79

6 Vaccination contre la brucellose **81**

6.1 **Problématique de la vaccination contre la brucellose** **81**

6.2 **Vaccin vivant, tué ou inactivé ?** **81**
6.2.1 Extraits bactériens, faibles inducteurs d'une réponse Th1... 82
6.2.2 ... Brucella HK, bon inducteur d'une réponse Th1 82
6.2.3 Brucella irradiée aux rayons gamma, une solution ? 83

6.3 Vers un meilleur vaccin, pour l'homme et l'animal ? **83**

OBJECTIVES 85

RESULTS 86

1 Part I : Crucial role of gamma interferon-producing $CD4^+$ Th1 cells but dispensable function of $CD8^+$ T cell, B cell, Th2 and Th17 responses in the control of *Brucella melitensis* infection in mice **86**

2 Part II : *Brucella melitensis* invades murine erythrocytes during infection **89**

3 Part III : Humoral immunity and $CD4^+$ Th1 cells are both necessary for a sterilizing immune response in the spleen upon secondary infection with *Brucella melitensis* **92**

DISCUSSION & CONCLUSIONS 96

1 Context **96**

2 Primary immune response against *B. melitensis* **98**

2.1 Lymphocyte subsets controlling brucellosis **98**

2.1.1 IFN-γ production must be associated with activated $CD4^+$ T cells rather than $CD8^+$ T cells to be protective 98
2.1.2 $CD8^+$ T cells are not able to sustain IFN-γ production during the plateau phase of infection 101
2.1.3 On the different ability of IL-12p35- and IL-12p40-deficient C57BL/6 mice to control Brucella infection 103

2.2 *B. melitensis* persists in the blood of infected mice and infects erythrocytes 105
2.2.1 Intraerythrocytic infection is not so scarce 105
2.2.2 The mystery of invasion process 107

3 Secondary immune response against *B. melitensis* 111

3.1 On differences between primary and secondary immune response 111
3.1.1 B cells are dispensable for controlling primary B. melitensis infection but are essential in a secondary infection 111
3.1.2 $CD4^+$ but not $CD8^+$ T cells are activated to produce IFN-γ during secondary infection 114
3.1.3 IFN-γ production correlates with bacterial clearance but does it make it the mediator of protection? 114
3.1.4 IL-4 receptor signaling pathways negatively impact on the sterilizing immunity in the spleen after challenge without affecting primary response to Brucella infection in BALB/c mice 116

3.2 On differences between live and HK immunization 116

3.3 Manipulation of the model: on the need for antibiotic treatment prior to the challenge 117

4 Limitations of our model: organ, timing, inoculation route and mice 118

5 Final conclusion 120

APPENDIX 124

1 Réplication et persistance de *Brucella* dans la rate de souris C57BL/6 ou BALB/c génétiquement déficientes pour des composants de la réponse immunitaire. 125

2 Additional results on genetically deficient mice tested during this thesis 131

3 In situ microscopy analysis reveals local innate immune response developed around *Brucella* infected cells in resistant and susceptible mice 135

BIBLIOGRAPHIE 136

Introduction

1 Avant propos : la relation hôte / pathogène

1.1 Définition du système immunitaire, d'un paradigme à l'autre

Définir le rôle du système immunitaire est devenu au fil des ans une démarche particulièrement ardue et complexe tant les premiers concepts ne cessent d'évoluer afin d'intégrer de nouvelles dimensions insoupçonnées quelques années auparavant.

Le terme « immunité » dérive du latin *immunitas* qui signifie « exempt ». On définirait donc l'immunité d'un individu comme l'art de le préserver des infections. Le système immunitaire (SI) comprend l'ensemble des tissus, organes, cellules et molécules qui agissent de manière coordonnée dans le but de protéger l'organisme contre les agents pathogènes, les substances étrangères et les cellules anormales.

Toutefois, notre vision du système immunitaire ne peut se limiter à le considérer comme un gardien à l'encontre des organismes étrangers. En effet, tandis que la détection de quelques bactéries dans les tissus périphériques résulte en une rapide activation du SI, les milliards de bactéries commensales, champignons et virus en contact permanent avec les muqueuses digestives et respiratoires ne déclenchent aucune réponse immunitaire. Cependant, la présence de cette flore microbienne (nommée microbiote) et son interaction avec le SI apparaissent essentiels au développement et à la régulation de ce dernier, comme l'ont notamment montré de récentes études portant sur les maladies du système digestif (Mazmanian *et al.*, 2008; Round & Mazmanian, 2009).

« We propose that the mammalian genome does not encode for all the functions required for immunological development but rather that mammals depend on critical interactions with their microbiome for health » (Mazmanian *et al.*, 2008)

Au-delà de ses fonctions « défensives », il a également été suggéré que le SI pouvait s'apparenter à un organe sensoriel. En effet, le système immunitaire et le système neuro-hormonal partagent un langage biochimique commun et communiquent via un circuit bidirectionnel impliquant des ligands communs tels que des neurotransmetteurs, des hormones neuroendocriniennes, des cytokines, et leurs récepteurs respectifs. Le système immunitaire agirait dès lors comme un organe sensoriel, un sixième sens en quelque sorte (Blalock, 2005; Shepherd *et al.*, 2005). Il a par exemple été montré que la stimulation ou l'ablation de certaines zones du cerveau pouvait inhiber ou stimuler la réponse immunitaire (Felten & Felten, 1994).

« Lymphocytes and natural killer cells can be viewed as cell-sized sensory organs, continuously sampling the internal environment for things that don't belong there or for cellular stress or aberrations. Just as rod cells in the eye can detect even a single photon, cytotoxic T cells can kill on the advice of only three peptide-MHC ligands (Davis, 2004). »

Chez les vertébrés, le système immunitaire est arbitrairement divisé en deux ensembles : le SI inné, phylogénétiquement ancien et fortement conservé chez tous les organismes multicellulaires, et le SI adaptatif, propre aux métazoaires supérieurs tels que les vertébrés et hautement spécifique (Moser & Leo, 2010). L'efficacité du SI des vertébrés repose sur la collaboration étroite de ces deux systèmes qui utilisent des stratégies fondamentalement différentes pour reconnaître les pathogènes.

Le SI inné utilise un répertoire de récepteurs encodés dans le génome permettant notamment la détection de structures moléculaires invariantes typiques des micro-organismes (virus, bactéries, protozoaires, champignons, helminthes), les PAMPs (Pathogen Associated Molecular Patterns). Ces récepteurs sont nommés PRRs (Pattern Recognition Receptors) et ont la capacité de reconnaître plusieurs PAMPs différents. Ils ont été sélectionnés au cours de l'évolution et ne nécessitent donc pas un apprentissage de la discrimination entre le soi et le non soi (Janeway & Medzhitov, 2002). Les PRRs sont exprimés par les cellules du SI inné mais également par les lymphocytes (SI adaptatif) et de nombreuses cellules résidant dans les tissus, comme les fibroblastes, les cellules épithéliales et endothéliales.

Le SI adaptatif des vertébrés utilise un répertoire de récepteurs hautement spécifiques qui sont exprimés par des cellules spécialisées, les lymphocytes T et B. Ces récepteurs, nommés T cell receptor (TCR) et B cell receptor (BCR), sont générés par des réarrangements aléatoires complexes d'ADN survenant au cours de la maturation des lymphocytes T et B dans les organes lymphoïdes primaires (moelle osseuse pour les lymphocytes B et thymus pour les lymphocytes T). Chaque lymphocyte exprime un seul récepteur et dispose donc d'une seule spécificité antigénique. Etant produit de manière aléatoire par recombinaison de gènes, le répertoire des TCRs et BCRs peut potentiellement reconnaître n'importe quelle structure et doit donc impérativement subir une sélection destinée à éliminer les lymphocytes exprimant un récepteur reconnaissant le soi avec une haute affinité (sélection négative). Contrairement aux cellules du SI inné, les lymphocytes disposant d'un récepteur spécifique du pathogène peuvent proliférer (amplification clonale) après reconnaissance de ce pathogène, lutter contre celui-ci et former une mémoire immunologique.

Selon le type de récepteur exprimé, les acteurs du SI sont donc « classés » dans l'une ou l'autre catégorie. Cette division en deux entités est toutefois fortement réductrice et la limite entre l'immunité innée et adaptative apparaît de plus en plus floue (Criscitiello & de Figueiredo, 2013; Netea *et al.*, 2011). Ainsi, les études de Paust (Paust *et al.*, 2010) ont par exemple démontré que des cellules appartenant au SI inné (les cellules natural killer (NK)) présentaient certaines propriétés du système immunitaire adaptatif, tels que la formation d'une mémoire immunologique (Biron, 2010; Sun *et al.*, 2009). De même, les lymphocytes expriment de nombreux PRRs en plus du récepteur antigénique et peuvent donc être activés indépendamment de ce dernier.

1.2 Le pathogène

Le terme « pathogène » trouve son origine dans le grec ancien et signifie « qui engendre la douleur ». On définit dès lors un pathogène comme un micro-organisme capable, en altérant l'organisme hôte, d'induire une maladie.

Plusieurs caractéristiques peuvent le différencier d'un micro-organisme non pathogène, parmi lesquelles on peut citer (i) la génération de « POPs » (Patterns of Pathogenesis, définis plus loin dans l'exposé), (ii) dans le cas d'un pathogène intracellulaire,

son habilité à délivrer des molécules microbiennes dans le cytosol des cellules hôtes qui permettent, dans de nombreux cas, de (iii) détourner ou perturber les fonctions du cytosquelette de la cellule infectée (Vance *et al.*, 2009).

On distingue traditionnellement 4 grandes classes de pathogènes qui, selon leur cycle infectieux, déclencheront des réponses immunitaires différentes, activant des effecteurs aptes à les éliminer :

- bactéries, protozoaires et champignons extracellulaires
- bactéries et protozoaires intracellulaires
- virus (intracellulaires)
- helminthes (extracellulaires)

Les micro-organismes, qu'ils soient pathogènes ou non, jouent un rôle crucial dans « l'éducation » du SI. Dès la venue au monde du nouveau né, le développement et la maturation de son système immunitaire sont dépendants des micro-organismes avec lesquels ses muqueuses entrent en contact (Martin *et al.*, 2010). Par la suite, la présence d'une flore microbienne s'avère nécessaire au développement et au maintien de nombreuses structures (Macpherson & Harris, 2004). Les animaux élevés dans un environnement totalement stérile présentent un SI immature (Gill & Finlay, 2011). De plus, il est généralement reconnu que la pression de sélection imposée par les agents infectieux au cours de l'évolution a « modelé » les éléments du système immunitaire et défini son organisation (Lee & Mazmanian, 2010; Medzhitov & Janeway, 1997).

1.3 Le pathogène et son hôte

On qualifie de « pathogènes professionnels » les micro-organismes qui ont acquis d'importantes stratégies d'échappement à la réponse immunitaire, pouvant mener à une infection chronique. Ces stratégies visent à neutraliser les différents effecteurs de la réponse immunitaire qui sont activés spécifiquement par le pathogène en question, elles diffèrent donc selon son cycle infectieux et sa niche de réplication. Par exemple, un pathogène se répliquant au sein d'une vacuole intracellulaire peut dévier le trafic de cette vacuole pour éviter sa fusion avec le lysosome.

Toutefois, la persistance d'un pathogène au sein d'un organisme peut parfois présenter certains avantages pour l'hôte. En effet, il a déjà été démontré que la présence d'un pathogène pouvait avoir un effet protecteur contre l'envahissement d'autres espèces pathogènes (Barton *et al.*, 2007; Sieve *et al.*, 2009). Les études les plus anciennes sont celles de Mackaness qui a pu mettre en évidence l'existence de mécanismes de résistance non spécifiques à l'infection. Dans ses expériences, des souris infectées par la bactérie à Gram négatif *Brucella abortus* montraient une résistance plus élevée à l'infection par *Listeria monocytogenes* (bactérie à Gram positif) (Araya *et al.*, 1989; Mackaness, 1964). Mackaness a observé que l'activité bactéricide développée envers *L. monocytogenes* chez les souris infectées par *B. abortus* suivait de manière linéaire la réplication de ces bactéries à Gram négatif dans la rate. De plus, il a mis en évidence que la fin de cette activité bactéricide coïncidait avec le moment où le nombre de *B. abortus* chez les souris infectées était fortement réduit. Par la suite, les souris conservaient une immunité protectrice contre *B. abortus*, mais pas contre *L. monocytogenes*. Ce phénomène s'explique par la persistance de *B. abortus* chez les souris infectées, ce qui conduit à la présence continue d'effecteurs activés de l'immunité innée. Ceux-ci, bien que spécifiquement induits par *B. abortus*, sont capables de conférer une protection non-spécifique à l'encontre d'un autre pathogène durant une période limitée (Mackaness, 1964). Ce phénomène a également été observé pour d'autres agents infectieux (Barton *et al.*, 2007; Moreira *et al.*, 2009; Perry *et al.*, 2010; Sieve *et al.*, 2009; Sutherland *et al.*, 2011) mais ne doit pas être généralisé à toutes les infections. En effet, certaines infections chroniques peuvent augmenter la susceptibilité de l'hôte lors d'une infection par un autre pathogène ou même réduire l'efficacité d'un vaccin (Radwanska *et al.*, 2008; Stelekati & Wherry, 2012).

2 Le système immunitaire

2.1 La réponse immunitaire innée

La réponse immunitaire innée apparaît depuis quelques décennies comme une stratégie de défense complexe dont le rôle dans l'activation et le contrôle de la réponse immunitaire adaptative est primordial (Palm & Medzhitov, 2009). Présentés dans le passé comme des entités distinctes, l'inné et l'adaptatif font aujourd'hui figure de partenaires indissociables.

Les effecteurs de l'immunité innée comprennent des facteurs solubles (complément, protéines de la phase aigue) et des cellules parmi lesquelles on distingue les granulocytes (neutrophiles, éosinophiles, basophiles et mastocytes), les monocytes (qui génèrent, après différenciation, les macrophages et les cellules dendritiques) et des cellules cytotoxiques (cellules Natural Killer, NK) (Moser & Leo, 2010) (**Table 1**). À l'exception des cellules dendritiques, les autres effecteurs du SI inné ne seront peu ou pas abordés dans cet exposé.

Table 1. Cellules de l'immunité innée et fonctions (d'après (Janeway *et al.*, 2003). Cette table présente brièvement les principaux effecteurs de l'immunité innée. Les fonctions mentionnées sont parmi les plus représentatives, mais ne constituent pas une liste exhaustive du mode d'action de ces effecteurs.

EFFECTEURS	PRINCIPALES FONCTIONS
Complément	Opsonisation, recrutement des macrophages/neutrophiles et destruction du pathogène
Protéines de la phase aigue	Opsonisation et activation du complément
Macrophages	Phagocytose et activation des mécanismes bactéricides du phagolysosome Présentation de l'antigène
Cellules dendritiques	Capture de l'antigène dans les sites périphériques et migration vers les ganglions drainant, présentation de l'antigène
Neutrophiles	Phagocytose, granules, filets extracellulaires (neutrophil extracellular traps, NETS) et activation

	des mécanismes bactéricides extracellulaires
Eosinophiles	Destruction des parasites recouverts d'anticorps, granules
Basophiles	Libération de granules contenant de l'histamine et d'autres médiateurs
Mastocytes	Libération de granules contenant de l'histamine et d'autres médiateurs, production massive d'IL-4 (induction Th2)
Cellules Natural Killer (NK)	Libération de granules lytiques, induction d'apoptose par Fas, destruction des cellules infectées, production massive d'IFN-γ (induction Th1)

2.1.1 Senseurs de l'envahisseur

A la fin des années 1980, Charles Janeway a proposé un modèle conceptuel (« stranger model ») du fonctionnement du système immunitaire traitant notamment de la collaboration entre l'inné et l'adaptatif (Janeway, 1989). La réponse adaptative des lymphocytes T et B est incapable de distinguer le soi du non-soi car ses récepteurs sont produits de manière aléatoire durant la maturation des lymphocytes. Janeway a donc proposé un modèle dans lequel le système immunitaire inné, grâce aux PRRs sélectionnés au cours de l'évolution pour reconnaître les PAMPs, constituerait un instructeur fiable du SI adaptatif sur l'origine infectieuse de l'antigène.

Suite à l'énonciation de ce modèle, la caractérisation de la réponse immunitaire innée et notamment de ses récepteurs a connu un important engouement. Les PRRs ont été classés fonctionnellement en 3 familles, selon qu'ils soient sécrétés, transmembranaires ou cytosoliques (**table 2**).

Les **PRRs sécrétés** (collectines, ficolines et pentraxines) se lient à la surface des pathogènes, activent le complément et induisent la phagocytose de ceux-ci par les macrophages et neutrophiles (Garlanda *et al.*, 2005; Holmskov *et al.*, 2003). Bien que propres à l'immunité innée, leur action en tant qu'opsonines[1] les rend

[1] Les opsonines comprennent également le complément, les protéines de la phase aiguë et les anticorps. Ces molécules sont capables de se fixer à la surface des antigènes et facilitent leur ingestion par les cellules phagocytaires. On parle d'opsonisation.

fonctionnellement semblables aux anticorps sécrétés par les lymphocytes B (SI adaptatif) (Palaniyar, 2010).

Les **PRRs transmembranaires** comprennent les TLRs (Toll-like receptors), dont 10 sont décrits à ce jour chez l'humain et 13 chez la souris, et peuvent détecter une large gamme de PAMPs au niveau de la membrane plasmique ou au sein des endosomes, et les CLRs (C-type lectin receptors) qui détectent des composants fongiques (Takeuchi & Akira, 2010).

Les **PRRs cytosoliques** comprennent les RLRs (RIG1-like receptors), qui reconnaissent les virus, et les NLRs (NOD-like receptors) qui détectent les pathogènes mais aussi les signaux issus de conditions de stress (Creagh & O'Neill, 2006).

Table 2. PRRs et leurs ligands (d'après (Takeuchi & Akira, 2010))

	PRRs	LIGAND	ORIGINE DU LIGAND
PRRs sécrétés	Collectines	Sucres	Bactéries, virus, protozoaires et champignons
	Ficolines	Glycoprotéines	
	Pentraxines	Composants microbiens	Certaines bactéries, virus et champignons
PRRs transmembranaires	**TLRs**		
	TLR1	Peptidoglycans, lipoprotéines	Bactéries
	TLR2	Lipoprotéines	Bactéries, virus, parasites, soi
	TRL3	dsRNA	Virus
	TLR4	LPS	Bactéries, virus, soi
	TLR5	Flagelline	Bactéries
	TLR6	Diacyl lipoprotéines	Bactéries, virus
	TLR7/8	ssRNA	Virus, bactéries, soi
	TLR9	CpG-DNA	Virus, bactéries, protozoaires, soi
	TLR10	Non connu	Non connu
	TLR11	Profilin-like molecule	Protozoaires
	CLRs		
	Dectin-1	β-glucan	Champignons
	Dectin-2	β-glucan	Champignons
	MINCLE	SAP130	Champignons, soi
PRRs cytosoliques	**RLRs**		
	RIG-1	Short dsRNA, 5'triP dsRNA	Virus à ARN, à ADN
	MDA5	Long dsRNA	Virus à ARN (Picornaviridae)
	LGP2	Non connu	Virus à ARN
	NLRs		
	NOD1	IE-DAP	Bactéries
	NOD2	MDP	Bactéries

2.1.2 Senseurs de l'invasion

Le modèle de Janeway a depuis été revisité par plusieurs auteurs (Medzhitov, 2009). En 1994, Polly Matzinger a proposé une théorie (« danger model », (Matzinger, 1994; Matzinger, 2002)) selon laquelle le rôle du SI inné serait principalement de détecter le danger pour protéger l'organisme. Le SI inné serait activé suite à la détection par les PRRs de « DAMPs » (Damage-Associated Molecular Patterns), des signaux signifiant au SI que des tissus ont été endommagés, que cela soit d'origine infectieuse ou endogène (Beg, 2002).

En 2001, Dangl et Jones ont décrit chez les plantes la théorie du gardien qui implique que la détection du pathogène pourrait dépendre de « gardiens de l'intégrité des processus cellulaires ». Une perturbation cellulaire signifierait l'activité de facteurs de virulence et pourrait activer le SI sans passer par une détection via les PRRs (Dangl & Jones, 2001). Cette théorie s'applique également dans le monde animal. Par exemple certains inflammasomes (complexes protéiques comprenant certains membres de la famille des NLRs) fonctionnent en contrôlant l'intégrité de la membrane cellulaire (Ogura *et al.*, 2006).

Réconciliant les différentes théories, Russel Vance a suggéré en 2009 que le système immunitaire inné ne se contentait pas de percevoir les pathogènes comme de simples « paquets de PAMPs ». En effet, les PAMPs à eux seuls ne renseignent pas nécessairement sur la viabilité du pathogène envahisseur. Il faudrait considérer l'existence de PAMPs différents selon qu'ils sont issus de bactéries vivantes (PAMPs pro-vita) ou de bactéries mortes (PAMPs post-mortem). Selon cet auteur, le système immunitaire inné serait capable de percevoir les signaux issus du contexte infectieux, liés par exemple aux stratégies utilisées par les pathogènes pour envahir, manipuler et se répliquer au sein de l'hôte. L'ensemble de ces signaux incluant les PAMPs et les DAMPs pourrait dès lors être désigné sous le terme de « POPs » (Patterns of Pathogenesis) (Vance *et al.*, 2009).

La prise en considération des PAMPs pro-vita ou post-mortem est essentielle dans la recherche de nouveaux vaccins. Il est indéniable que certains aspects de la « vie » microbienne, absents lors de l'utilisation de vaccins tués, sont particulièrement efficaces dans l'induction d'une réponse immunitaire protectrice (Sander *et al.*, 2011).

« The immune system gauges the infectious risk by searching PAMPs for signatures of microbial life and thus infectivity. Detection of PAMPs pro-vita triggers a state of alert not warranted for dead bacteria. Vaccine formulation that incorporates PAMPs pro-vita could thus combine the superior protection of live vaccines with the safety of dead vaccines ». (Sander *et al.*, 2011)

2.1.3 Senseurs instructeurs

Parmi les PRRs, certains jouent le rôle d'instructeur du SI adaptatif, notamment les TLRs, NLRs, Dectin-1, RIG-I and MDA-5 (Fearon & Locksley, 1996; Iwasaki & Medzhitov, 2010; Palm & Medzhitov, 2009). Selon Noah Palm (Palm & Medzhitov, 2009), les signaux induits par ces PRRs contrôlent la réponse adaptative à différents niveaux de son activation. Ces points de contrôle dictent l'initiation de la réponse, son amplitude et sa durée, ainsi que la mise en place d'une mémoire immunologique (**figure 1**). Il s'agirait en quelque sorte de soupapes de sécurité qui empêchent l'activation inopportune du SI adaptatif, préservant de cette façon l'organisme du développement de réponses auto-immunes ou excessives (hypersensibilités).

Ces points de contrôle sont d'autant plus importants que les TLRs sont également exprimés par les cellules du SI adaptatif, tels que les lymphocytes B (Dasari *et al.*, 2005), T $CD4^+$ (Imanishi *et al.*, 2007; Komai-Koma *et al.*, 2004; Xu *et al.*, 2005), T $CD8^+$ (Cottalorda *et al.*, 2006), T $\gamma\delta$ (Martin *et al.*, 2009) et les lymphocytes T régulateurs (Caramalho *et al.*, 2003; Liu *et al.*, 2006). Par exemple, chez les lymphocytes T activés, les TLRs peuvent exercer une fonction de co-stimulation en augmentant leur prolifération et leur production de cytokines (Kabelitz, 2007; Komai-Koma *et al.*, 2004).

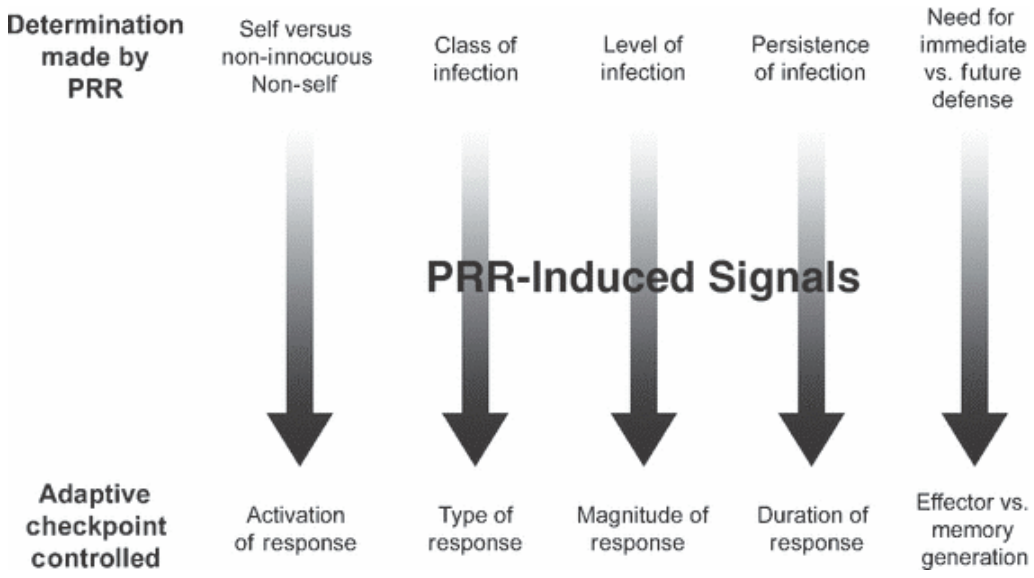


Figure 1. Les PRRs contrôlent la réponse adaptative à différents niveaux de son activation (Palm & Medzhitov, 2009). Par l'identification des caractéristiques propres à l'envahisseur, qu'elles soient morphologiques ou « comportementales », les PRRs informent efficacement le SI adaptatif et évitent une activation inadaptée de la réponse adaptative. Tout manquement à l'un des niveaux de ce système de régulation pourrait conduire au développement de pathologies, comme les maladies auto-immunes.

2.1.4 Effecteurs instructeurs : les cellules dendritiques

Les cellules dendritiques (DCs) jouent un rôle central dans la détection du pathogène et elles sont uniques dans leur capacité à initier une réponse immunitaire adaptative.

Diversité des cellules dendritiques

Il existe plusieurs sous-populations de DCs, définies sur base de leur phénotype et de leur localisation tissulaire. En 2007, Shortman a proposé un classement de ces différentes sous-populations (Shortman & Naik, 2007) :

Pré-DCs. Ces **précurseurs de DCs** ont la capacité de se développer rapidement en DCs fonctionnelles en présence d'un stimulus inflammatoire ou microbien. Parmi les pré-DCs, on peut citer les monocytes ainsi que les cellules dendritiques plasmacytoïdes (pDCs), productrices d'interféron (IFN) de type I (α et β) et spécialisées dans la réponse anti-virale (Colonna *et al.*, 2004).

DCs conventionnelles. Pleinement fonctionnelles, ces DCs comprennent les ***DCs migratoires*** (par exemple les cellules de Langerhans) et les ***DCs résidants dans les organes lymphoïdes*** (par ex. les DCs spléniques ou thymiques). Les premières sont comparables à des sentinelles de l'organisme, circulant dans les tissus périphériques et ingérant continuellement les différents composants présents dans leur environnement. La reconnaissance d'un pathogène constitue un signal de maturation qui les incite à rejoindre les organes lymphoïdes où elles activeront les lymphocytes T. Les secondes résident en permanence dans les organes lymphoïdes où elles capturent et présentent les antigènes, qu'ils soient endogènes ou d'origine étrangère. Chez les souris, selon l'expression des marqueurs membranaires CD4, CD8α et CD11b, ces DCs peuvent être divisées en plusieurs sous-populations dont les fonctions immunologiques diffèrent (Vremec *et al.*, 1992; Vremec *et al.*, 2000), notamment au niveau de la production de cytokines et de la présentation des antigènes (Heath *et al.*, 2004; Hochrein *et al.*, 2001). Par conséquent, ces diverses sous-populations jouent un rôle déterminant dans l'orientation de la réponse immunitaire adaptative (Maldonado-Lopez *et al.*, 1999; Pulendran *et al.*, 1999; Shortman & Heath, 2010).

DCs inflammatoires. Absentes en conditions normales, ces cellules se différencient à partir des monocytes suite à la présence d'un stimulus inflammatoire ou microbien.

Plusieurs études ont décrit leur présence en souris suite à une infection par *Listeria monocytogenes* (Serbina *et al.*, 2003) ou *Brucella melitensis* (Copin *et al.*, 2007). Ces DCs inflammatoires ont initialement été dénommées TipDCs (TNF-iNOS producing DCs) car elles produisaient du TNF-α et du NO dans les modèles infectieux étudiés. On soupçonne à présent que les DCs inflammatoires constituent un ensemble hétérogène de cellules (Geissmann *et al.*, 2010).

Présentation des antigènes

L'expression conjointe des molécules du complexe majeur d'histocompatibilité de type I (MHC-I) et II (MHC-II) par les cellules dendritiques leur confère la capacité de présenter les peptides dérivés de protéines intra (MHC-I) ou extracellulaires (MHC-II) au récepteur des lymphocytes T (TCR). Cette capacité d'activer les lymphocytes T ainsi que leur présence dans tous les tissus de l'organisme fait que les DCs sont désignées comme des cellules présentatrices d'antigènes (Antigen Presenting Cells, APC) professionnelles et se distinguent des autres cellules nucléées de l'organisme capables d'exprimer le MHC (monocytes, macrophages, lymphocytes B, etc.) (Moser & Leo, 2010).

En contexte infectieux, les cellules dendritiques phagocytent[1] les pathogènes mais contrôlent rarement l'infection. Elles constituent donc fréquemment un réservoir pour les pathogènes. La phagocytose du pathogène déclenche un processus de différenciation et de maturation de la cellule dendritique (**Figure 2**). Les antigènes vont être apprêtés (dégradés en peptides) pour être présentés à la surface cellulaire en association avec les molécules du MHC (Moser & Leo, 2010). L'instruction du SI adaptatif sur l'identité du pathogène est notamment réalisée par le type de MHC présenté en surface. Si l'antigène est présent dans le cytosol de l'APC (origine intracellulaire), il est présenté par un MHC-I, constitué d'une chaîne α et d'une chaîne β2-microglobuline. Si l'antigène est confiné dans une vésicule suite à son internalisation par phagocytose (origine extracellulaire), il est présenté par un MHC-II, constitué d'une chaîne α et d'une chaîne β. La cellule dendritique activera donc des lymphocytes T fonctionnellement différents selon le type de MHC présenté en surface. Par

[1] Phagocytose : Ingestion de particules, d'origine endogène ou microbienne, par des cellules phagocytaires (habituellement des macrophages, cellules dendritiques et neutrophiles). Le matériel ingéré est contenu dans une vésicule, le phagosome, qui fusionne avec un ou plusieurs lysosomes pour former le phagolysosome. Le micro-environnement du phagolysosome est essentiel pour assurer la destruction des pathogènes ou antigènes internalisés.

conséquent, la réponse immunitaire adaptative déclenchée sera différente. Ce sujet sera abordé en détail plus loin dans le manuscript. Les modes de présentation des antigènes décrits ci-dessus ne sont pas exclusifs. Dans certains cas, des antigènes d'origine extracellulaire quittent la vacuole de phagocytose et se retrouvent dans le cytoplasme, ce qui conduit à leur présentation par un MHC-I. On parle de « cross-presentation » (Kovacsovics-Bankowski & Rock, 1995). De la même manière, les cellules dendritiques sont capables de séquestrer au sein d'une vacuole des constituants du cytoplasme par un processus connu sous le nom d'autophagie. Ce procédé permet la présentation d'antigènes cytosoliques en MHC-II (Dengjel *et al.*, 2005).

Parallèlement à la présentation antigénique, les cascades de signalisation intracellulaires déclenchées par les PRRs mènent à l'expression de molécules de co-stimulation et à la production de médiateurs pro-inflammatoires (Takeuchi & Akira, 2010) (**Figure 3**). Les DCs activées perdent alors leur capacité de phagocytose, augmentent la présentation antigénique et quittent la périphérie pour atteindre les organes lymphoïdes secondaires, tels que les ganglions lymphatiques (Banchereau & Steinman, 1998).

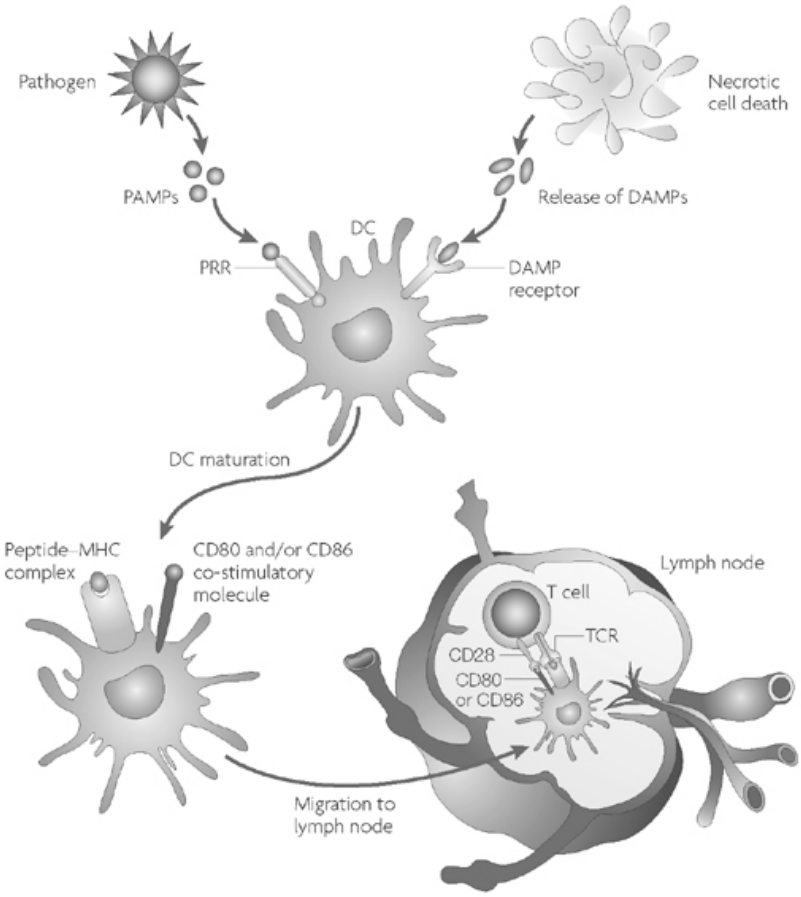


Figure 2. Les cellules dendritiques (DCs) sont les intermédiaires entre l'immunité innée et adaptative (d'après (Kono & Rock, 2008)). Plusieurs signaux d'alerte peuvent être détectés par les DCs. Ceux-ci peuvent dériver soit du pathogène (PAMPs), soit des cellules de l'hôte qui subissent une situation de stress (DAMPs), soit des deux situations. La capture des antigènes par les DCs constitue un signal de maturation qui les incite à quitter le site d'infection pour rejoindre les ganglions lymphatiques. En tant que cellules présentatrices d'antigènes (APC) professionnelles, les DCs activées présentent les antigènes sous la forme d'un complexe peptide-MHC aux lymphocytes T, ce qui mène à l'activation de la réponse adaptative.

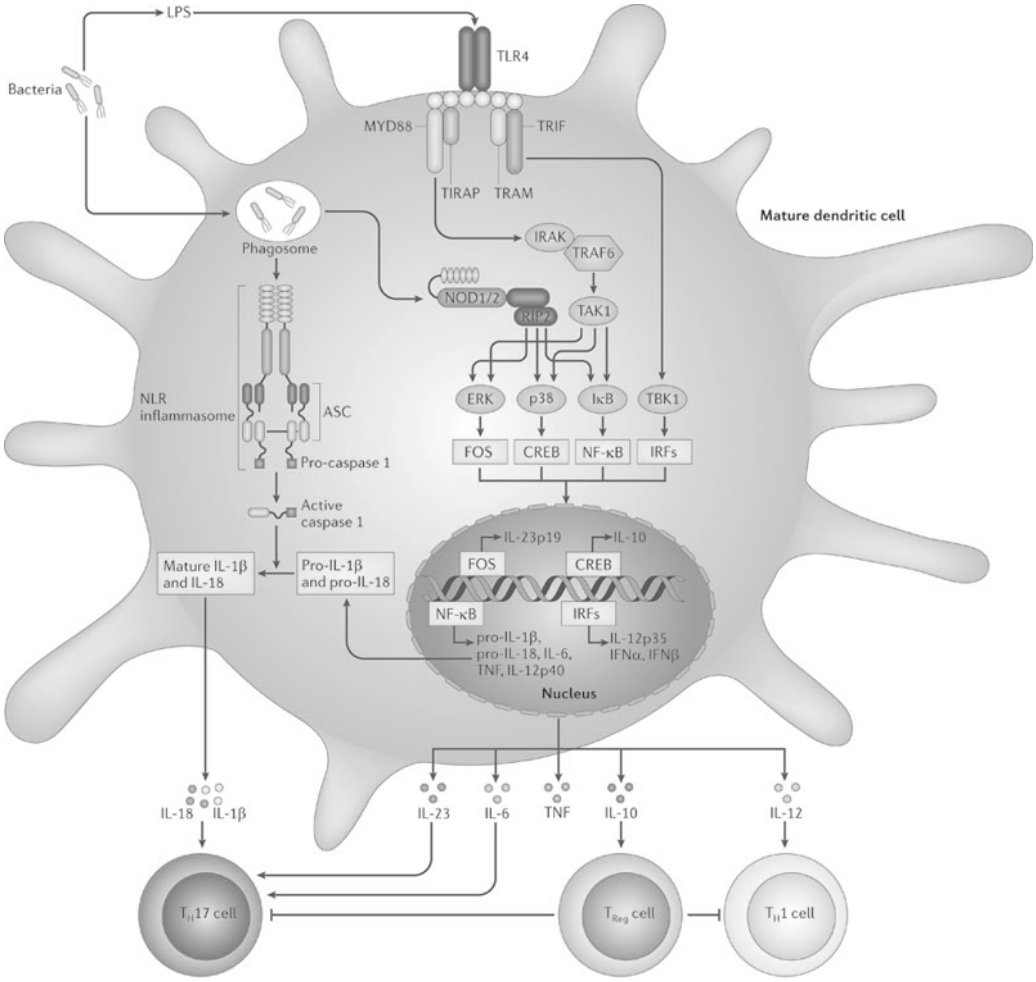


Figure 3. La cascade de signalisation intracellulaire induite par les pathogènes dans les cellules dendritiques mène à la production de cytokines et à l'expression de signaux de costimulation (non représentés ici) qui assureront l'activation des lymphocytes T (Mills, 2011). À l'exception du TLR3, tous les TLRs initient une cascade de signalisation qui utilise la protéine adaptatrice MyD88. De plus, les TLR2 et TLR4 utilisent la protéine TIRAP (TIR domain-containing adaptor protein, aussi connue sous le nom de MAL), tandis que TRIF (TIR domain-containing adaptor protein inducing IFNβ) et TRAM (TRIF-related adaptor molecule) sont requis pour la production d'interférons (IFNs) de type I (α et β) dépendante des TLR3 et TLR4. La figure représente certaines des voies de signalisation induites dans une cellule dendritique en réponse à une bactérie à Gram négatif. La liaison du LPS bactérien au TLR4 initie une voie de signalisation utilisant les protéines adaptatrices Myd88, TIRAP, TRIF et TRAM. Cette cascade de signalisation mène notamment à l'activation de NF-κB (Nuclear Factor-κB) qui induit la transcription d'une série de gènes codant pour des cytokines pro-inflammatoires, comme le TNF-α (Tumor Necrosis Factor) et l'IL-6. Les IFNs de type I ainsi que l'IL-12p35 sont produits suite à l'activation des facteurs de transcription IRFs (Interferon Regulatory Factors). Les molécules bactériennes se lient aussi aux NLRs intracellulaires (NOD1, NOD2 et NLRP3) dont l'activation mène à l'expression d'autres médiateurs inflammatoires. La variété de cytokines produites activera différentes sous-populations de lymphocytes T.

2.2 Réponse immunitaire adaptative

La réponse immunitaire adaptative fait intervenir les lymphocytes T et B dont les récepteurs antigéniques ont été produits par un processus de recombinaisons d'ADN survenu au cours de la maturation de ces cellules. La génération aléatoire d'un très large répertoire de récepteurs (propre à chaque individu) confère potentiellement aux populations de lymphocytes la capacité de reconnaître avec une haute affinité tout type de structure moléculaire, une propriété absente chez les récepteurs de l'immunité innée qui sont directement encodés en nombre limité dans le génome. Au cours de la réponse adaptative, les lymphocytes spécifiques de l'antigène rencontré sont sélectionnés et subissent une expansion clonale. Une partie d'entre eux se différencieront pour assurer la mise en place d'une mémoire immunologique. Ces caractéristiques impliquent que la réponse adaptative intervient plus tardivement dans la cinétique d'infection.

2.2.1 Lymphocytes T

Au début des années 1960, l'immunologie a connu un important essor suite aux études de Miller sur les fonctions immunologiques du thymus (Miller, 1959; Miller, 1960). Ses découvertes ont permis une meilleure compréhension du fonctionnement des lymphocytes T et de leur implication dans l'activation des lymphocytes B (Miller, 2011). Issus de la moelle osseuse, les précurseurs des lymphocytes T atteignent le thymus où sera générée la diversité de récepteurs (TCR, T Cell Receptor) suite à des recombinaisons somatiques. C'est également au sein du thymus qu'ils seront sélectionnés sur base de leur tolérance aux antigènes du soi. Les lymphocytes T migrent ensuite vers les organes lymphoïdes secondaires où pourront avoir lieu leur activation et leur différenciation au contact avec des cellules présentatrices d'antigènes. Au sein de ces organes lymphoïdes, les lymphocytes T activés peuvent à leur tour induire la différenciation des lymphocytes B. Ces lymphocytes T, identifiés par l'expression du récepteur $\alpha\beta$ et du co-récepteur CD4, sont qualifiés de T « helper » pour leur implication dans l'activation des autres lymphocytes (T et B). Les différentes classes de lymphocytes T helper seront abordées au paragraphe 2.3. Il existe d'autres types de lymphocytes T, décrits ci-après: les lymphocytes T $CD8^+$, T régulateurs (Tregs), T Natural Killer (NKT) et les T $\gamma\delta$.

De manière générale, les lymphocytes T naïfs requièrent plusieurs signaux pour leur activation. Ceux-ci sont fournis par les cellules dendritiques activées (Banchereau & Steinman, 1998) (**figure 4**) :

- l'antigène (**signal 1**) dont la reconnaissance par le récepteur membranaire du lymphocyte T (TCR) ne peut être réalisée que s'il est présenté sous forme de peptide par le complexe majeur d'histocompatibilité (MHC). Les antigènes présentés en MHC-I (issus du cytosol) activent les lymphocytes T $CD8^+$ tandis que ceux présentés en MHC-II (confinés dans une vacuole) activent les lymphocytes T $CD4^+$.
- les molécules de co-stimulation (**signal 2**) dont l'expression par la DC indique qu'elle a été activée par des antigènes microbiens. Ceux-ci peuvent être activateurs ou inhibiteurs. A titre d'exemple, une fois activées, les DCs expriment des protéines de la famille B7 (notamment CD80 et CD86) qui reconnaissent la protéine CD28 exprimée constitutivement par les lymphocytes T. Cette liaison mènera à l'activation du lymphocyte T.
- des facteurs dits polarisants ou de différenciation (**signal 3**) qui détermineront quel phénotype de T effecteur sera produit. Il s'agit généralement de cytokines pro-inflammatoires.

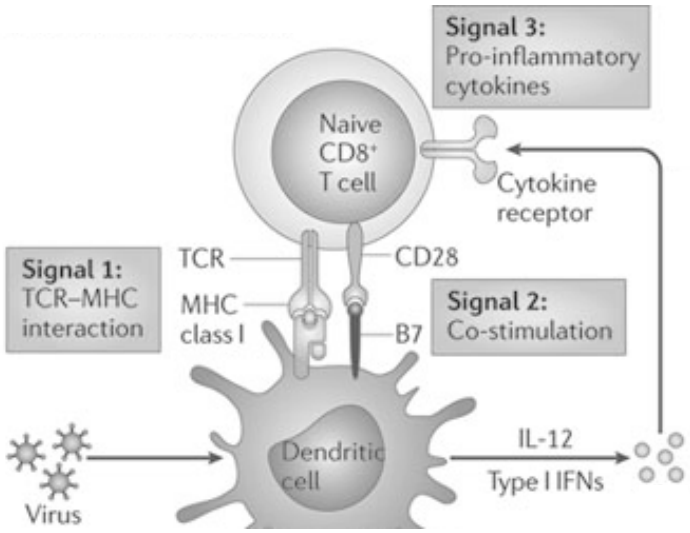


Figure 4. Activation du lymphocyte T (ici T $CD8^+$) par une cellule dendritique activée (d'après (Sun & Lanier, 2011)). Trois signaux sont requis pour l'activation complète du lymphocyte T : l'interaction du complexe MHC-peptide avec le récepteur lymphocytaire (TCR) (signal 1), le signal costimulateur (signal 2) et des facteurs solubles tels que les cytokines (signal 3).

Après leur activation, les lymphocytes T effecteurs vont se diviser, c'est la phase d'expansion clonale, qui sera suivie par une phase de contraction. Celle-ci est caractérisée par l'élimination par apoptose d'une grande partie des cellules effectrices et permet de réduire le risque de développement de pathologies auto-immunes. Enfin, certains lymphocytes se différencieront en cellules mémoire.

2.2.1.1 Lymphocytes T cytotoxiques ($CD8^+$)

Les lymphocytes T $CD8^+$, exprimant le co-récepteur CD8, sont activés par des antigènes cytosoliques présentés en MHC-I. On les désigne également par le terme de lymphocytes T cytotoxiques car ils sont capables d'induire directement la lyse des cellules infectées par un pathogène ou anormales. Ces lymphocytes sécrètent également des cytokines (TNF-α, IFNs) capables d'entraver la survie intracellulaire des pathogènes (Bartee *et al.*, 2008).

La cytotoxicité des lymphocytes T $CD8^+$ implique d'une part la sécrétion de facteurs solubles altérant la surface membranaire de la cellule infectée, ou induisant son apoptose (perforine, granzyme, granulysine) et d'autre part l'expression du facteur membranaire FasL, dont la liaison avec la protéine Fas sur la cellule infectée conduit également à l'apoptose (Henkart, 1994).

2.2.1.2 Lymphocytes T $CD4^+CD25^+$ régulateurs (Tregs)

L'activation et les fonctions effectrices des lymphocytes T sont finement régulées par un autre type de cellules : les lymphocytes T $CD4^+CD25^+$ régulateurs (Tregs). Ces lymphocytes exercent un effet immunosuppresseur sur les autres lymphocytes et assurent le maintien de la tolérance aux antigènes du soi (Sakaguchi *et al.*, 2008). On distingue les Tregs naturels, présents dans l'organisme de manière intrinsèque, et les Tregs induits, qui acquièrent leurs fonctions régulatrices dans un contexte infectieux, notamment en présence de TGF-β (Li *et al.*, 2006; Reiner, 2007). De manière générale, leur action suppressive s'exerce par la sécrétion de cytokines anti-inflammatoires telles que l'IL-10 et le TGFβ, ou

par contact membranaire via la molécule CTLA-4 (Cytotoxic T-Lymphocyte-associated Antigen-4) (Bluestone & Abbas, 2003).

L'équilibre entre lymphocytes Tregs et T effecteurs est déterminant pour le devenir de la réponse adaptative. Il s'agit d'une caractéristique dont certains pathogènes tirent parti, par exemple en activant préférentiellement les fonctions immunosuppressives des Tregs (Belkaid *et al.*, 2002; Mills, 2004; Taylor *et al.*, 2005).

2.2.1.3 Lymphocytes NK T

Les lymphocytes Natural Killer (NK) T sont des lymphocytes T non conventionnels, exprimant un TCR$\alpha\beta$ mais également des récepteurs typiques des cellules cytotoxiques de l'immunité innée, les cellules NK. Ils sont activés suite à la reconnaissance d'antigènes conservés (PAMPs) de type glycolipides présentés par un complexe protéique structurellement proche du MHC-I (comprenant aussi une chaîne β2-microglobuline) et nommé CD1 (Bendelac *et al.*, 2007).

2.2.1.4 Lymphocytes T $\gamma\delta$

Les lymphocytes T $\gamma\delta$ sont des lymphocytes T dits non conventionnels car ils expriment un TCR composé des chaînes γ et δ. Ils sont activés suite à la reconnaissance d'antigènes non peptidiques (antigènes phosphorylés, alkylamines, etc.) encore peu caractérisés à ce jour (Eberl *et al.*, 2003). On considère ces lymphocytes comme des cellules reliant l'immunité innée et adaptative, notamment parce qu'ils sont présents à proximité des épithéliums cutanés et des muqueuses respiratoires et digestives (Carding & Egan, 2002; Havran & Jameson, 2010). Un intérêt croissant est porté aux lymphocytes T $\gamma\delta$ car, en contexte infectieux, ils peuvent constituer une source importante de cytokines pro-inflammatoires telles que l'IFN-γ (Wang *et al.*, 2003) et l'IL-17 (Roark *et al.*, 2008; Shibata *et al.*, 2007) et sont dotés d'une mémoire immunologique (Hoft *et al.*, 1998; Spencer *et al.*, 2008).

2.2.2 Lymphocytes B

Tandis que le TCR des lymphocytes T ne reconnaît les antigènes peptidiques que s'ils sont présentés par les MHC en surface des cellules infectées (on parle de « restriction au MHC »), les lymphocytes B expriment des récepteurs antigéniques (BCRs) capables de reconnaître les antigènes sous leur forme native (c'est-à-dire solubles et sans apprêtement). Ces BCRs peuvent être membranaires - ils jouent alors un rôle de détection du pathogène, comme les TCRs - ou sécrétés en tant que molécules effectrices, plus connues sous le nom d'immunoglobulines (Ig) ou anticorps. La particularité de ces molécules effectrices est qu'elles peuvent gagner en affinité pour l'antigène rencontré suite à des processus d'hypermutation de l'ADN codant pour le BCR et exercer de nouvelles fonctions effectrices grâce à des phénomènes de recombinaisons entre séquences d'ADN qui mèneront à la synthèse de nouvelles classes d'anticorps (**figure 5**) (Acosta-Rodriguez *et al.*, 2007; Moser & Leo, 2010).

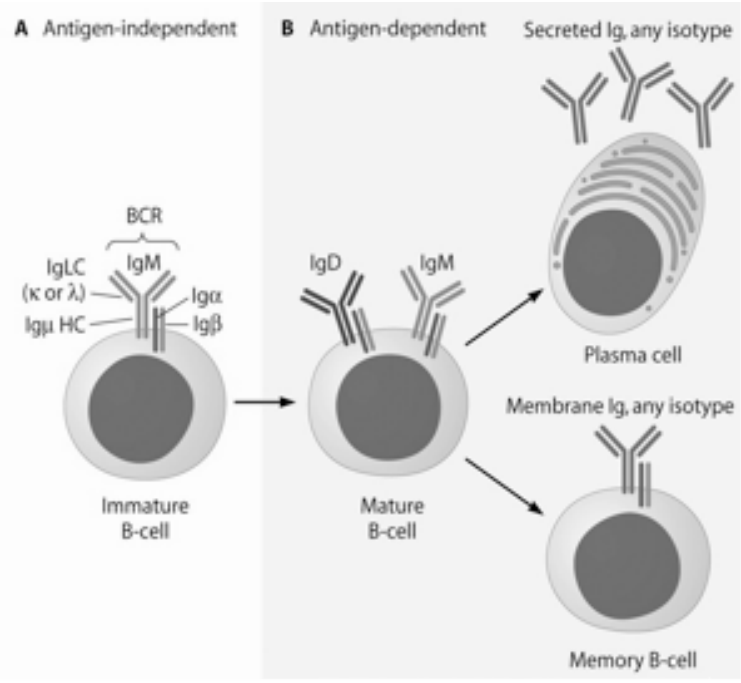


Figure 5. Le récepteur des lymphocytes B (BCR) et la maturation des lymphocytes B (d'après (Fried & Bonilla, 2009; Woodland & Kohlmeier, 2009)). **A,** Les lymphocytes B immatures expriment un BCR de type IgM. Celui-ci est constitué de deux chaînes lourdes (Igμ HC, HC : Heavy Chain) et deux chaînes légères (IgLC, LC : Light Chain). Les sites de liaison antigénique, situés au sommet de chaque bras de la molécule, constituent la zone variable qui confère la spécificité unique de l'anticorps à un antigène donné, tandis que la queue de la molécule constitue la région constante. Cette dernière comprend la partie Fc qui est reconnue par les récepteurs FcR présents à la surface de certaines cellules de l'immunité innée. **B,** Après leur rencontre avec l'antigène, les lymphocytes B se différencient en plasmocytes ou en lymphocytes mémoires et produisent des nouvelles classe d'anticorps de haute affinité.

Les lymphocytes B sont principalement issus de la moelle osseuse. C'est au sein de ce tissu qu'auront lieu les recombinaisons somatiques qui génèreront la diversité de BCR, ainsi que la sélection des cellules sur base de leur tolérance au soi. Leur développement en lymphocytes B matures et producteurs d'anticorps (plasmocytes) se poursuit dans les centres germinatifs au sein des organes lymphoïdes secondaires (Acosta-Rodriguez *et al.*, 2007). Tout comme les lymphocytes T, les B peuvent également se différencier en cellules mémoire à longue durée de vie.

On distingue plusieurs sous-populations de lymphocytes B, dont les principales sont :

Les lymphocytes B2, dits « conventionnels », sont aptes à répondre à la plupart des antigènes protéiques s'ils reçoivent le signal adéquat des lymphocytes T. Ils peuvent produire diverses classes d'anticorps de haute affinité suite aux processus d'hypermutation somatique et de commutation de classe. Selon leur localisation dans la rate, on distingue les lymphocytes B2 folliculaires (FO) et ceux de la zone marginale (MZ).

Les lymphocytes B1, présents dans les cavités péritonéale et pleurale, sont originaires du foie fœtal et semblent avoir été sélectionnés au cours de l'évolution pour répondre rapidement à certains antigènes sans requérir l'aide des lymphocytes T (Baumgarth, 2011). Ils produisent principalement des IgM de basse affinité (Alugupalli *et al.*, 2004) et leurs fonctions aux stades précoces de l'infection les rapprochent des effecteurs du SI inné.

Les lymphocytes B régulateurs (« Bregs »), récemment caractérisés (Mauri & Bosma, 2012) et disposant de fonctions immunosuppressives proches de celles des lymphocytes Tregs. Leur existence nuance quelque peu l'idée préconçue considérant les lymphocytes B comme régulant uniquement positivement les réponses immunitaires et participant activement à la résolution des infections.

Les anticorps naturels

En absence d'infection, ces anticorps sont déjà présents dans l'organisme et reflètent le répertoire spontané d'Igs circulantes. Essentiellement produits par les lymphocytes B1, ces anticorps sont majoritairement des IgM de basse affinité (Ehrenstein *et al.*, 1998), mais la présence d'autres classes d'Igs (IgG et IgA) a aussi été décrite (Baumgarth *et al.*, 2005; Cohen & Norins, 1966). Un nombre croissant d'études démontre un rôle protecteur pour ces anticorps naturels, notamment en début d'infection (Boes *et al.*, 1998; Boes, 2000; Ochsenbein *et al.*, 1999; Quintana *et al.*, 2008; Reid *et al.*, 1997; Rolan *et al.*, 2009).

2.2.2.1 Lymphocytes B2 : activation thymo-dépendante (TD)

Signaux d'activation

Les lymphocytes B2 peuvent être activés par la majorité des antigènes. Deux signaux sont toutefois nécessaires à l'activation de ces cellules : le signal antigénique spécifique fourni par le BCR, et le signal de costimulation fourni par les lymphocytes T $CD4^+$ activés sous la forme de cytokines et/ou ligands membranaires (McHeyzer-Williams & McHeyzer-Williams, 2005). Parmi les ligands membranaires, on peut citer à titre d'exemple l'interaction de CD40, exprimé constitutivement par les lymphocytes B, et de CD40L (ou CD154), surexprimé chez les lymphocytes T activés, et qui est impliquée dans la commutation de classe et la formation du centre germinatif (Armitage *et al.*, 1992) (**figure 6**). Comme pour les lymphocytes T, ces signaux additionnels permettent de limiter le risque de développement d'une réponse autoimmune, mais sont aussi à l'origine du délai d'activation supplémentaire de ce type de réponse.

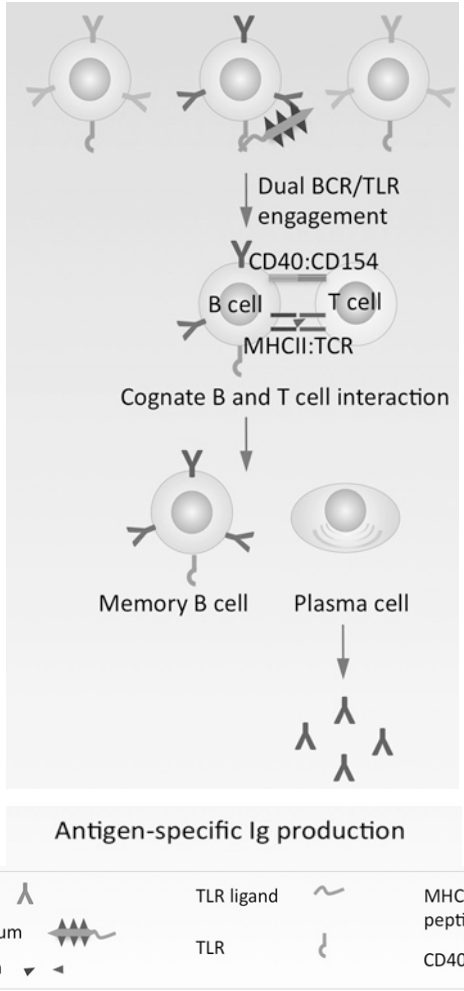


Figure 6. Activation thymo-dépendante des lymphocytes B (d'après (Pone *et al.*, 2012)). Le lymphocyte T $CD4^+$ préalablement activé par une cellule dendritique exprime des molécules de costimulation (CD28, CD40L/CD154) et des cytokines. Le lymphocyte B ayant reconnu le pathogène par l'action conjointe du BCR et TLR (signal 1) présente l'antigène en MHC-II au lymphocyte T activé spécifique du même antigène. Ce lymphocyte T délivre alors le signal de costimulation (signal 2) qui permet d'activer le lymphocyte B. Ce dernier se différencie ensuite en plasmocyte, producteur d'anticorps, ou en cellule mémoire.

Production d'anticorps

Après leur activation, les lymphocytes B prolifèrent et se différencient en cellules productrices d'anticorps, les plasmocytes. Cette première vague effectrice participe directement à la réponse immunitaire en cours et est constituée de plasmocytes qui sécrètent des anticorps de basse affinité, principalement des IgM. En parallèle, une partie des lymphocytes B activés va former le centre germinatif (GC) dans la zone B des organes lymphoïdes secondaires. C'est au sein de cette organisation cellulaire particulière que les gènes codant pour les récepteurs des lymphocytes B subissent des hypermutations somatiques au niveau des zones variables et sont sélectionnés pour leur gain d'affinité envers l'antigène. Cela est réalisé via des contacts étroits avec des lymphocytes T helper folliculaires (Thf) et des cellules dendritiques folliculaires (FDCs). Le contexte infectieux, représenté par les cytokines produites par les lymphocytes T et les DCs, va également mener à l'expression de nouvelles classes d'immunoglobulines (Igs), ou isotypes, aptes à répondre et reconnaître l'antigène rencontré. Ce processus se traduit par un changement de la chaîne lourde de l'anticorps et se nomme commutation de classe (ou switch isotypique) (**figure 7**) (McHeyzer-Williams & McHeyzer-Williams, 2005; Pone *et al.*, 2012). Les lymphocytes B commencent par exprimer des IgM, d'abord en tant que récepteurs, puis sous la forme d'Igs secrétés. Les autres classes d'anticorps sont ensuite produites : IgG, IgA ou IgE et les taux d'anticorps ne cessent de croître jusqu'à atteindre un plateau qui peut se maintenir sur une longue période.

Les hypermutations somatiques et la commutation de classe sont notamment dépendants de l'enzyme nommée « Activation-induced cytidine deaminase » (AID) qui n'est exprimée que par les lymphocytes B activés (Honjo *et al.*, 2004). Les souris déficientes pour cette enzyme disposent donc uniquement d'IgM membranaires ou circulantes et sont totalement déficientes en IgG, IgA et IgE (Muramatsu *et al.*, 2000).

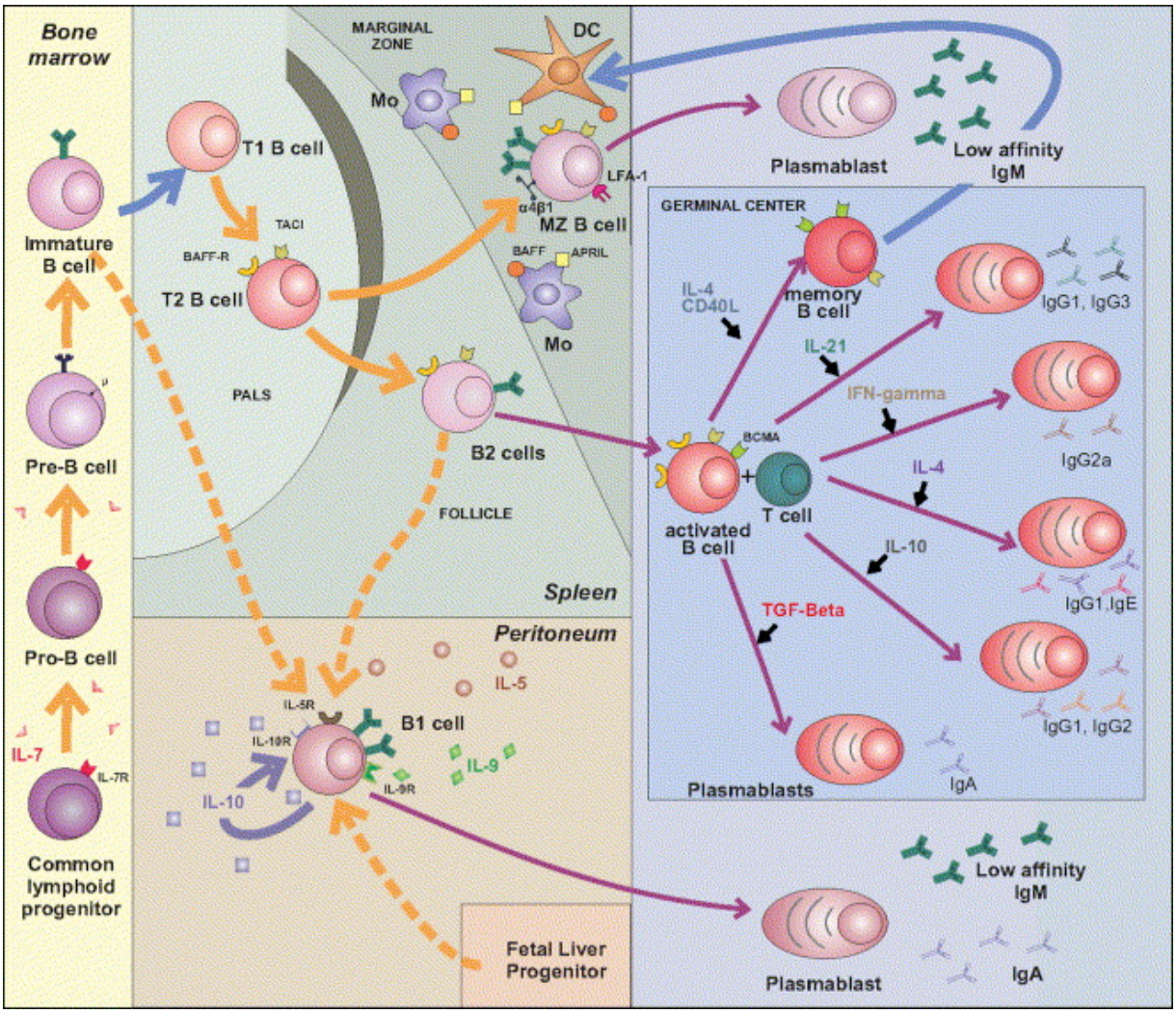


Figure 7. Les cytokines jouent un rôle essentiel dans le processus de différenciation des lymphocytes B et sont déterminantes quant au type d'anticorps produits (Acosta-Rodriguez *et al.*, 2007). Les lymphocytes B subissent les recombinaisons somatiques de leurs récepteurs et sont sélectionnés sur base de leur tolérance au soi dans la moelle osseuse. Leur maturation se poursuit dans les organes lymphoïdes secondaires. Les lymphocytes B1 (cavité péritonéale) et B2 de la zone marginale (MZ) sécrètent des anticorps de basse affinité, principalement des IgM. Les lymphocytes B2 folliculaires sont activés par les lymphocytes T et subissent une expansion clonale. Une partie d'entre eux vont former le centre germinatif, une organisation cellulaire particulière où les lymphocytes B subissent des hypermutations somatiques menant à l'expression de récepteurs de haute affinité, et la commutation de classe menant à l'expression de différentes sous-classes d'anticorps (IgG, IgA, IgE). L'environnement de cytokines, déterminé par le type d'antigène rencontré, oriente la réponse anticorps induite.

2.2.2.2 Lymphocytes B1 et B2 MZ : activation thymo-indépendante (TI)

Certaines molécules (PAMPs) et micro-organismes peuvent directement induire la prolifération et la différenciation des lymphocytes B sans requérir l'aide des lymphocytes T $CD4^+$, on parle d'activation thymo-indépendante (**figure 8**). Ce type d'activation est généralement attribué aux lymphocytes B1 mais aussi aux lymphocytes B2 de la zone marginale (Martin *et al.*, 2001; Martin & Kearney, 2002) et, suite à l'absence de maturation des lymphocytes B dans les centres germinatifs, génère un débat quant à sa capacité à induire une mémoire immunologique (Alugupalli *et al.*, 2004; Bortnick *et al.*, 2012; Defrance *et al.*, 2011).

Selon l'antigène, on distingue 2 catégories de réponses :

- *L'activation thymo-indépendante de type 1 (TI-1)*, où l'antigène est un dérivé saccharidique (PAMPs) qui est directement reconnu par les TLRs. Cette interaction mène à une activation polyclonale et peu spécifique de la plupart des lymphocytes B. Ces antigènes sont dits « mitogènes ». Un antigène TI-1 présent en faible quantité peut toutefois mener à l'activation de lymphocytes B spécifiques suite à sa détection conjointe par les BCRs et TLRs (Pone *et al.*, 2010).

> *Le LPS, un antigène TI-1*
>
> En assurant une réponse anticorps rapide et capable de contenir le pathogène jusqu'à l'établissement d'une réponse T mature, l'activation TI-1 peut jouer un rôle très important dans les conditions physiologiques d'une infection. Les propriétés mitogéniques du LPS sont connues depuis plus de 30 ans (Betts *et al.*, 1993; Spellman & Reed, 1979), mais la récente étude de Quintana a permis une meilleure compréhension des mécanismes moléculaires sous-jacents à l'activation lymphocytaire par le LPS. Cette étude a démontré que le TLR4 et le BCR pouvaient contrôler conjointement l'induction d'anticorps anti-LPS de type IgG3 (Pone *et al.*, 2010; Quintana *et al.*, 2008). Cette activation ne nécessite qu'une faible quantité de LPS circulant et ne peut avoir lieu en absence de MyD88.

- *L'activation thymo-indépendante de type 2*, où c'est la structure particulière de l'antigène (TI-2) qui est reconnue par les BCRs des lymphocytes B matures. Ces antigènes présentent des motifs hautement répétitifs induisant une importante réticulation des BCRs (par exemple des polysaccharides capsulaires). Bien que la réponse développée soit spécifique à l'antigène, les lymphocytes B activés ne

produiront que des IgM. La commutation de classe n'interviendra qu'en présence des signaux issus des lymphocytes T activés (Vos *et al.*, 2000).

Antigènes TI-2 et vaccination

En terme de vaccination, les antigènes TI-2 sont cliniquement importants car ils incluent ceux présents chez *Haemophilus influenza*, *Streptococcus pneumoniae* et *Neisseria meningitidis*, des pathogènes qui sont les principales causes de mortalité chez les enfants (Lesinski & Westerink, 2001). L'utilisation des polysaccharides capsulaires purifiés de ces pathogènes permet la mise au point de vaccins efficaces chez les adultes immuno-compétents. Cependant, comme les antigènes TI-2 ne peuvent activer que des lymphocytes B matures, ces vaccins sont inefficaces chez les enfants de moins de 2 ans, et doivent donc impérativement être couplés à une protéine pour induire une réponse humorale protectrice de longue durée (Lesinski & Westerink, 2001; Siegrist & Aspinall, 2009).

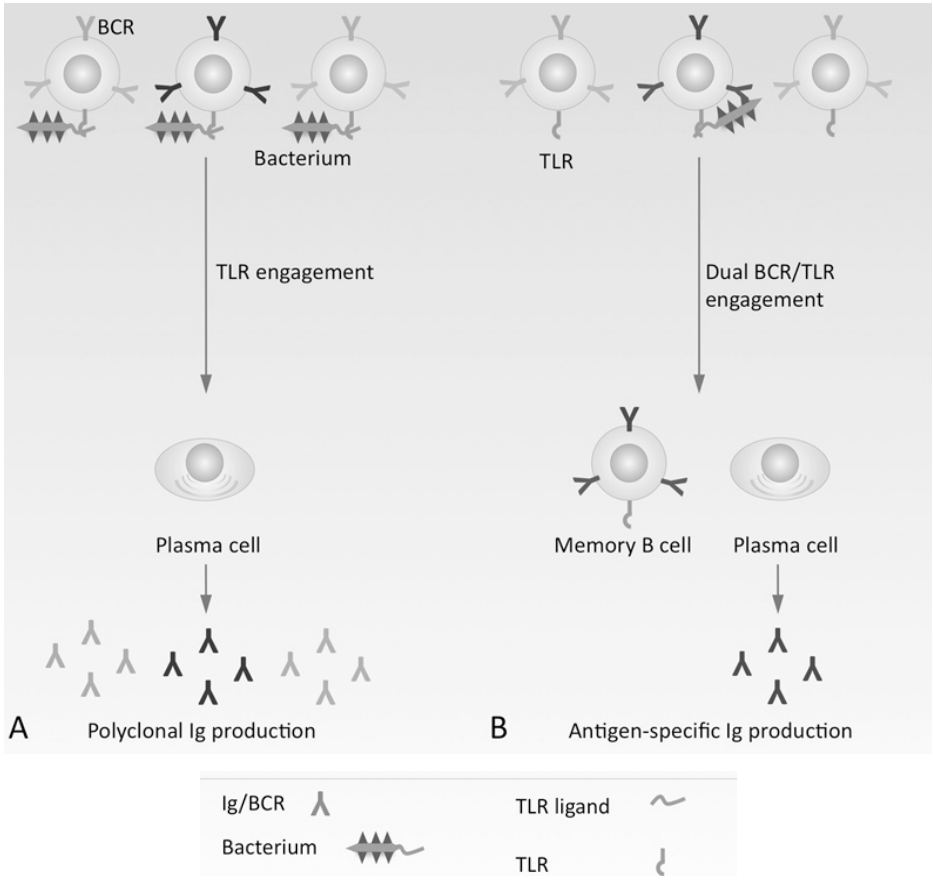


Figure 8. Activation thymo-indépendante des lymphocytes B (d'après (Pone *et al.*, 2012)). A, Les lymphocytes B peuvent être directement activés par la reconnaissance d'un pathogène via le TLR. L'activation est alors polyclonale et par conséquent la réponse anticorps sera également polyclonale. **B**, Lorsque le pathogène est reconnu conjointement par le TLR et le BCR, seuls les lymphocytes B spécifiques du pathogène sont activés, la réponse anticorps générée est donc spécifique de l'antigène.

2.2.2.3 Lymphocytes B régulateurs

Bien que l'existence de lymphocytes B aux propriétés suppressives soit documentée depuis 1974 (Neta & Salvin, 1974), ce n'est que récemment que les lymphocytes B producteurs d'IL-10 ont été désignés par le terme « B régulateurs » (Bregs) (Mizoguchi *et al.*, 2002).

Actions-fonctions

Les Bregs produisent des cytokines anti-inflammatoires telles que l'IL-10 et le TGF-β. Outre leur impact sur l'activation des autres lymphocytes T et B, ces cytokines, et plus particulièrement l'IL-10, seraient essentielles à la différenciation des Tregs (Carter *et al.*, 2011; Tadmor *et al.*, 2011). Plusieurs études ont démontré que les Bregs pouvaient inhiber les réponses inflammatoires dans certains modèles expérimentaux, tels que l'encéphalomyélite auto-immune (EAE), l'arthrite induite au collagène (CIA) et la colite (Fillatreau *et al.*, 2008; Lampropoulou *et al.*, 2008; Mauri *et al.*, 2003; Tian *et al.*, 2001). Toutefois, en modèle infectieux, ces cellules ont parfois un impact négatif sur la résolution de l'infection (Gillan *et al.*, 2005; Mauri & Bosma, 2012; Mizoguchi & Bhan, 2006; Smelt *et al.*, 2000), notamment par l'effet immuno-suppresseur de l'IL-10 et du TGF-β qu'elles produisent. Ainsi, les souris déficientes pour les lymphocytes B résolvent plus efficacement l'infection par *Brucella abortus* (Goenka *et al.*, 2011), mais présentent une réponse exacerbée et néfaste pour l'hôte dans le cas d'une infection par *Leishmania donovani* (Smelt *et al.*, 2000). Dans ce dernier cas, il est à noter que l'administration de sérum immun réduit le nombre de lésions hépatiques chez les souris déficientes ($MuMT^{-/-}$), sans toutefois affecter leur meilleure résistance (Smelt *et al.*, 2000).

2.3 Sous-classes de lymphocytes T helper : un choix de réponse adapté

2.3.1 Trilogie Th1, Th2, Th17

En 1986, Mosmann et Coffman ont décrit l'existence de 2 populations de lymphocytes T CD4$^+$ helper (Th) différant par le type de cytokines produites et la réponse B générée (Mosmann *et al.*, 1986). Pendant près de deux décennies, la réponse des lymphocytes T helper a été définie comme un système binaire : Th1 pour les pathogènes intracellulaires ou Th2 pour les pathogènes extracellulaires. Mais la découverte d'une nouvelle famille de cytokines produites par les lymphocytes T CD4$^+$ – famille de l'IL-17 – a mené à la caractérisation d'une nouvelle population de lymphocytes T helper, les Th17. Ceux-ci sont impliqués dans la réponse aux bactéries extracellulaires (Harrington *et al.*, 2006; Weaver *et al.*, 2007). L'engagement des lymphocytes T CD4$^+$ naifs dans l'une des 3 voies de différenciation dépend du pathogène détecté par le système immunitaire inné (Kaiko *et al.*, 2008) (**figure 9**). C'est pourquoi il est d'usage de considérer que ces voies sont exclusives, du moins localement. Les cytokines sécrétées par les lymphocytes T helper exercent en général une fonction inhibitrice sur l'engagement des lymphocytes T CD4$^+$ naïfs dans une autre voie.

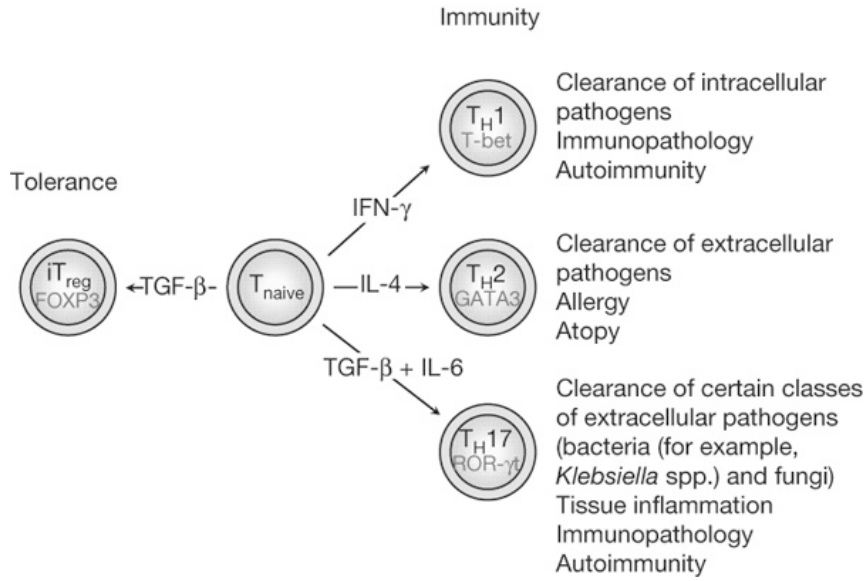


Figure 9. L'engagement des lymphocytes T naïfs dans l'une des 3 voies de différenciation dépend du pathogène détecté par le SI inné (Bettelli *et al.*, 2008). Après la détection du pathogène par le SI inné, les cellules dendritiques vont produire une variété de cytokines qui diffèrent en fonction des caractéristiques propres à l'envahisseur. Les pathogènes intracellulaires vont induire une réponse Th1, les pathogènes extracellulaires (vers, parasites) seront à l'origine du développement d'une réponse Th2, tandis que les pathogènes extracellulaires (bactéries ou champignons) vont initier une réponse Th17. En absence de régulation fine, chaque type de réponse peut conduire à des pathologies immunitaires. Le maintien de l'équilibre entre la protection de l'organisme contre l'envahisseur et les pathologies immunitaires est assumé par les lymphocytes Tregs.

Ces dernières années, le paradigme Th1-Th2-Th17 a de nouveau évolué suite à la découverte de l'existence d'autres types de lymphocytes T helper, dont les profils de cytokines sécrétés diffèrent des 3 populations citées ci-dessus.

Ainsi, les **lymphocytes Th9** produisent de l'IL-9 qui semble jouer un rôle prépondérant dans des maladies inflammatoires comme l'asthme (Dardalhon *et al.*, 2008; Stassen *et al.*, 2012; Veldhoen *et al.*, 2008).

Les **lymphocytes T helper folliculaires (Thf)** sont d'importants régulateurs de la maturation des lymphocytes B dans les centres germinatifs. Par leur production d'IL-21, sont capables de promouvoir la sécrétion de grandes quantités d'anticorps de haute affinité par les lymphocytes B (Breitfeld *et al.*, 2000; Crotty, 2011; Schaerli *et al.*, 2000).

Certaines sous-classes de lymphocytes T sont également capables de produire un large panel de cytokines (dont les principales sont l'IFN-γ, l'IL-2 et le TNF-α), reflétant une qualité de réponse supérieure. On les appelle des **lymphocytes T polyfonctionnels** (Makedonas & Betts, 2006) et ils semblent d'un grand intérêt en vaccination (Darrah *et al.*, 2007; Seder *et al.*, 2008). Les études les plus récentes considèrent cependant que ces lymphocytes T constitueraient un état d'activation particulier, plutôt qu'une nouvelle sous-classe de cellules T helper (Haining, 2012; Han *et al.*, 2012).

2.3.2 Réponse Th1 : lutte contre les pathogènes intracellulaires

La réponse de type Th1 orchestre l'activation d'effecteurs aptes à détruire des pathogènes intracellulaires : les macrophages, les cellules Natural Killer (NK), les lymphocytes T $CD8^+$, les lymphocytes B producteurs d'IgG et le complément. Ce type de réponse est caractérisé par la présence de lymphocytes T $CD4^+$ produisant de l'IFN-γ. On rapproche généralement la réponse Th1 à ce qu'on appelait autrefois la « réponse à médiation cellulaire ».

Leishmania major montre la voie

L'importance du développement d'une réponse Th appropriée a été démontrée dans des études portant sur le protozoaire intracellulaire *Leishmania major* (Heinzel *et al.*, 1989; Locksley *et al.*, 1987). Ces études ont été menées dans deux modèles murins présentant chacun une réponse au pathogène différente. La protection observée chez les souris résistantes C57BL/6 correspondait à la mise en place d'une réponse Th1 protectrice, tandis que la susceptibilité observée chez les souris BALB/c témoignait du développement d'une réponse Th2 inadéquate (Locksley *et al.*, 1987). De nombreuses études ont été menées chez ces 2 souches de souris, l'une d'elles a notamment montré que l'absence de réponse Th2 chez les souris BALB/c génétiquement déficientes pour le facteur de transcription STAT6 avait pour conséquence le développement d'une réponse Th1 assurant le contrôle de l'infection parasitaire (Stamm *et al.*, 1998).

La découverte des facteurs induisant la différenciation des lymphocytes T naïfs en Th1 a été de pair avec l'identification de l'IL-12 en 1989, un facteur soluble produit par les DCs en réponse à certains pathogènes, et qui stimule les cellules NK à produire de l'IFN-γ (Hsieh *et al.*, 1993; Kobayashi *et al.*, 1989).

Initiation dépendante de l'IFN-γ

Dès leur rencontre avec des pathogènes intracellulaires, les macrophages et les cellules dendritiques sont activés via leurs TLRs et sécrètent de l'IL-12 et des IFNs de type I (IFN-α et IFN-β). À son tour, l'IL-12 induit la production d'IFN-γ par les cellules NK (Kobayashi *et al.*, 1989; Trinchieri, 1995). L'IFN-γ sécrété régule alors la différenciation des lymphocytes T $CD4^+$ en Th1 (Martin-Fontecha *et al.*, 2004). En effet, la liaison de l'IFN-γ à son récepteur

lymphocytaire (IFN-γR) initie une voie de signalisation menant à l'expression du facteur de transcription T-bet. Propre aux réponses Th1, T-bet induit la production d'IFN-γ par les lymphocytes T activés et stimule l'expression du récepteur de l'IL-12 (Afkarian *et al.*, 2002). L'IL-12 issu des cellules immunitaires innées peut alors renforcer la différenciation des lymphocytes Th1. Certains auteurs s'opposent toutefois sur la question de savoir qui de l'IL-12 ou de l'IFN-γ est l'initiateur de la différenciation des lymphocytes T (Boehm *et al.*, 1997; Seder & Paul, 1994). L'IFN-γ exerce également une action paracrine en activant la différenciation des lymphocytes T $CD4^+$ naïfs avoisinant (Boehm *et al.*, 1997) (**figure 10**).

L'IFN-γ est le médiateur central de la réponse Th1, sa production stimule les effecteurs de l'immunité innée (Boehm *et al.*, 1997) et accélère la résolution de l'infection :

- il stimule les mécanismes bactéricides des macrophages, comme la production d'oxyde nitrique et des dérivés toxiques de l'oxygène,
- il stimule la présentation des antigènes en augmentant l'expression des MHC-I et MHC-II sur les cellules présentatrices d'antigènes,
- il amplifie la réponse des macrophages et DCs en stimulant leur production d'IL-12,
- il agit comme un inhibiteur de la réponse Th2

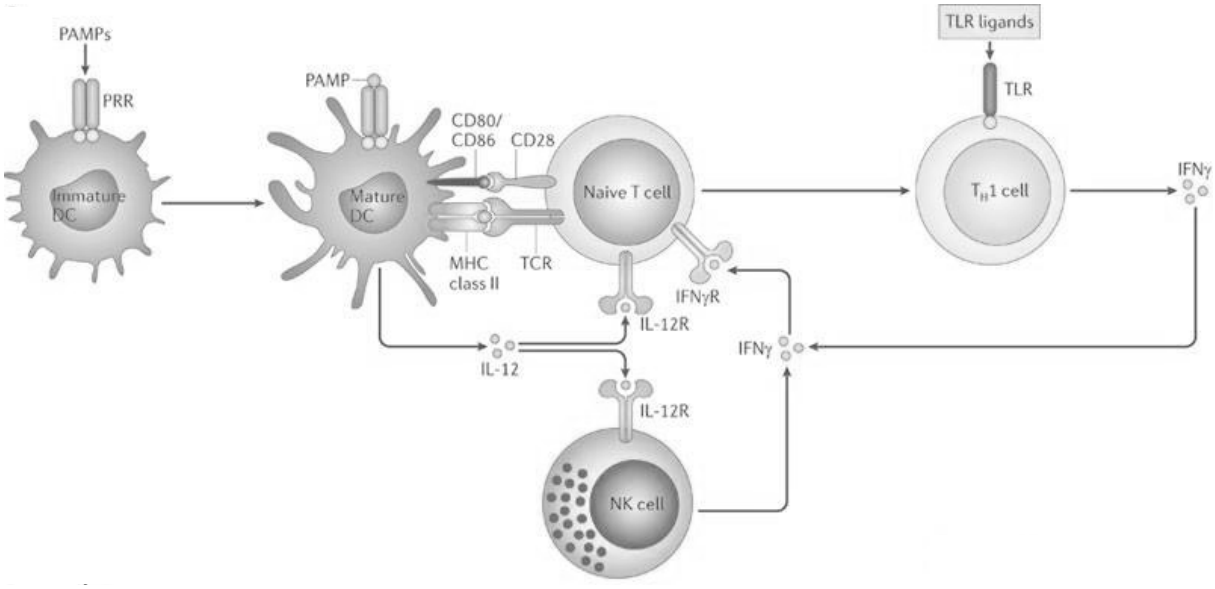


Figure 10. Activation d'une réponse Th1 (d'après (Mills, 2011)). Les PAMPs reconnus par les cellules dendritiques induisent leur maturation, comme en témoignent la présentation de l'antigène par le MHC-II, l'expression de molécules de costimulation (CD80/CD86) et la sécrétion d'IL-12. L'IL-12 induit la différenciation des lymphocytes T naïfs en cellules Th1, soit directement, soit via la production d'IFN-γ par les cellules NK. Les lymphocytes Th1 activés sécrètent à leur tour de l'IFN-γ qui stimulera la différenciation des lymphocytes T naïfs avoisinants.

Cellules Natural Killer (NK), effecteur inné, médiateur de l'adaptatif

Les cellules NK sont des cellules de l'immunité innée pourvues de propriétés cytotoxiques qui les rend aptes à détruire les cellules infectées ou tumorales, indépendamment d'une reconnaissance antigénique. Comme les lymphocytes T $CD8^+$, leur cytotoxicité est réalisée par la production de perforine, granzyme et granulysine et/ou par contact membranaire via l'interaction protéique FasL-Fas.
Activées par les cytokines pro-inflammatoires sécrétées par les macrophages infectés (dont l'IL-12, autrefois appelée « NKSF » pour « NK stimulatory factor », (Kobayashi *et al.*, 1989)), les cellules NK produisent de l'IFN-γ. Dans de nombreux modèles infectieux, il a été montré que les cellules NK constituaient la première source d'IFN-γ (Feng *et al.*, 2006; Orange *et al.*, 1995; Scharton & Scott, 1993). Ces cellules jouent donc un rôle essentiel dans la résistance de l'organisme dès les premières heures suivant l'infection, mais également dans l'orientation de la réponse adaptative (Andoniou *et al.*, 2008).

MyD88, un acteur clé

En amont de la production d'IFN-γ plusieurs éléments de la réponse immunitaire innée sont indispensables à la mise en place d'une réponse Th1 effectrice. Dans de nombreux modèles expérimentaux, l'absence de la protéine adaptatrice MyD88 résulte en une réduction dramatique de la résistance de l'hôte face aux agents infectieux (Copin *et al.*, 2007; Fremond *et al.*, 2004; Kaisho & Akira, 2001). La détection d'un PAMP par les TLRs (à l'exception du TLR3) active la voie de signalisation intracellulaire dépendante de la protéine adaptatrice MyD88. La cascade de signalisation qui est initiée mène à la translocation nucléaire de NF-κB et à l'activation des MAPK (mitogen activated protein kinases), induisant au final la sécrétion de cytokines et chemokines pro-inflammatoires comme l'IL-12 (Takeuchi & Akira, 2010). MyD88 participe aussi à la signalisation des récepteurs de l'IL-1 et de l'IL-18 (Adachi *et al.*, 1998). L'IL-1 induit la production d'une variété de protéines inflammatoires, comme les protéines de la phase aigue, des cytokines et des protéines d'adhésion (Dinarello, 2009) tandis que l'IL-18 induit la production d'IFN-γ et stimule l'activité des cellules NK (Okamura *et al.*, 1995). Ces fonctions sont absentes chez les souris $MyD88^{-/-}$ (Adachi *et al.*, 1998; Baratin *et al.*, 2005). MyD88 est également exprimé par les lymphocytes T et est impliqué dans les processus de différenciation de ces cellules, mais les mécanismes sous-jacents à cette régulation sont encore inconnus à ce jour (Fukata *et al.*, 2008; Zhou *et al.*, 2009). Il a récemment été montré que MyD88 était un régulateur important de la réponse Th17 (Chang *et al.*, 2013). Enfin, chez les lymphocytes B, la voie de signalisation dépendante de MyD88 contrôle l'amplitude de la réponse anticorps induite par les lymphocytes Th1, en

particulier la production des IgG2 (Barr *et al.*, 2009; Barr *et al.*, 2010; Pasare & Medzhitov, 2005; Sudres *et al.*, 2012).

Th1, IFN-γ et réponse humorale

La commutation de classe des lymphocytes B est finement régulée par un ensemble de cytokines (Severinson *et al.*, 1990). L'IFN-γ est le principal régulateur de la production d'IgG2a (Collins & Dunnick, 1993; Finkelman *et al.*, 1988; Snapper & Paul, 1987) et est également impliqué dans la production d'IgG3 (Snapper *et al.*, 1992). En absence du récepteur de l'IFN-γ (IFN-γR) les niveaux d'IgG2a (IgG2c dans le cas des souris C57BL) et d'IgG3 dans le sérum sont fortement réduits en modèle murin (Huang *et al.*, 1993). De même, en absence de MyD88, la production des classes d'anticorps IgM et IgG1 en réponse à des antigènes T-dépendants est réduite, voir absente pour la classe d'anticorps IgG2. Toutefois, les populations de lymphocytes B sont normales et phénotypiquement comparables à celles présentes chez les souris sauvages (Pasare & Medzhitov, 2005).

Les IgG induits par l'IFN-γ agissent comme des opsonines ; par leur fixation à la surface d'un pathogène, ils rendent celui-ci plus vulnérable à la phagocytose par les macrophages. L'expression du récepteur des IgG (FcγR1) à la surface des macrophages est également stimulée par l'IFN-γ (Paquette *et al.*, 1995). Par la détection d'une portion particulière de la chaîne lourde des anticorps (partie Fc), ces récepteurs permettent la capture et la destruction des bactéries opsonisées par les IgG (van de Winkel & Anderson, 1991).

Enfin, après fixation à la surface du pathogène détecté, les IgG peuvent induire l'activation du complément. Ce mécanisme de défense propre à l'immunité innée est réalisé par l'intermédiaire de protéines plasmatiques produites par le foie qui, tout comme les anticorps, peuvent jouer le rôle d'opsonines et stimuler la phagocytose des pathogènes détectés. L'activation du complément aboutit également à la production de médiateurs pro-inflammatoires (Frank & Fries, 1991).

En conséquence du principe d'exclusion mutuelle entre les différentes réponses Th, l'IFN-γ inhibe le switch isotypique induit par l'IL-4, cytokine propre à la réponse Th2, et menant à la production d'IgG1 et d'IgE (Finkelman *et al.*, 1988).

2.3.3 Réponse Th2 : lutte contre les pathogènes extracellulaires

La réponse Th2 est adaptée à l'élimination des pathogènes extracellulaires comme les helminthes, mais elle peut également être induite en présence d'allergènes (Anthony *et al.*, 2007). Les lymphocytes Th2 produisent des cytokines, telles que l'IL-4, l'IL-5 ou l'IL-13. Cette réponse se caractérise par un recrutement important d'éosinophiles, de mastocytes et de basophiles, ainsi que des niveaux élevés d'immunoglobulines de type IgG1 et IgE (Oliphant *et al.*, 2011).

Contrairement à la réponse Th1, fortement pro-inflammatoire, la réponse Th2 assure la destruction du pathogène en limitant les dégâts. L'IL-4 et l'IL-13 induisent notamment la production de cytokines anti-inflammatoires et de facteurs de réparation tissulaires par les macrophages (Gratchev *et al.*, 2001a; Gratchev *et al.*, 2001b).

Initiation dépendante de l'IL-4

La différenciation des lymphocytes Th2 dépend de la présence d'IL-4, toutefois l'origine de cette cytokine n'est pas encore clairement définie (Kaiko *et al.*, 2008). Les lymphocytes Th2 activés produisent à leur tour de l'IL-4 qui d'une part agira de manière paracrine, induisant la différenciation des autres lymphocytes T avoisinants, et d'autre part permettra le recrutement des autres cellules effectrices. La réponse Th2 inhibe l'expression du récepteur à l'IL-12 et de cette façon le développement d'une réponse Th1 (Ouyang *et al.*, 1998).

Th2, IL-4 et réponse humorale

Lors d'une réponse Th2, l'IL-4 induit la commutation de classe menant à la production d'IgE et d'IgG1. De même, l'IL-4 stimule l'expression du récepteur des IgE (FcεRI) sur les mastocytes et basophiles. Ce récepteur est de très haute affinité, dès lors une fraction importante des IgE circulants est fixée à la surface de ces cellules. Les mastocytes et basophiles ne sont activés que lorsque les IgE sont pontés par des antigènes multivalents. Ce pontage active leur dégranulation et la libération de médiateurs solubles pro-inflammatoires (Stone *et al.*, 2010). Réciproquement à l'action de l'IFN-γ lors d'une réponse Th1, l'IL-4 inhibe la commutation de classe menant à la production d'IgG2a et d'IgG3 (Kaiko *et al.*, 2008).

2.3.4 Réponse Th17 : lutte contre les bactéries extracellulaires

La réponse Th17 a été caractérisée suite à la découverte de l'IL-23 (Oppmann *et al.*, 2000), une cytokine promouvant la production d'IL-17 par des lymphocytes T qui présentent des propriétés distinctes des lymphocytes Th1 et Th2 (Aggarwal *et al.*, 2003; Langrish *et al.*, 2004). Ces lymphocytes Th17 agissent au niveau des surfaces épithéliales et assurent l'élimination des bactéries extracellulaires (Aujla *et al.*, 2007). Outre l'IL-17, ils produisent d'autres cytokines pro-inflammatoires parmi lesquelles on peut citer l'IL-21 et l'IL-22 (Bettelli *et al.*, 2007).

L'IL-17 induit un recrutement massif de neutrophiles vers les tissus infectés (Schwarzenberger *et al.*, 1998) et, en coopération avec l'IL-22, stimule la production de peptides antimicrobiens (β-défensine) par les cellules épithéliales (Liang *et al.*, 2006; Wolk *et al.*, 2004). L'IL-21 est quant à elle impliquée dans l'amplification de la réponse Th17 (Bettelli *et al.*, 2007; Nurieva *et al.*, 2007) mais également dans la différenciation des lymphocytes T helper folliculaires et dans la formation du centre germinatif (Eto *et al.*, 2011; Ozaki *et al.*, 2002; Vogelzang *et al.*, 2008). Son absence affecte donc la présence d'anticorps de haute affinité. La réponse Th17 régule également la production d'IgA qui exercent un rôle de défense essentiel au niveau des surfaces muqueuses et dont la commutation de classe dépend essentiellement du TGF-β (Coffman *et al.*, 1989; Konkel & Chen, 2011).

Initiation dépendante du TGF-β et de l'IL-6

La différenciation des lymphocytes Th17 est initiée par la production conjointe de TGF-β et d'IL-6 par les DCs (Reiner, 2007; Veldhoen *et al.*, 2006). En présence de TGF-β seul, les lymphocytes T naïfs se différencient en Tregs induits ; il existe donc une régulation réciproque, déterminée par l'environnement de cytokines, entre la tolérance privilégiée par les Tregs, et la robuste réponse inflammatoire générée par les lymphocytes Th17. Toutefois il a été démontré que la différenciation des lymphocytes Th17 pouvait avoir lieu en absence de TGF-β et reposait sur la présence combinée d'IL-6, IL-23 et IL-1β (Ghoreschi *et al.*, 2010). Cependant, les lymphocytes Th17 ainsi générés semblent constituer une sous-population de cellules Th17 qui expriment les facteurs de transcription T-bet et ROR-γt et sont capables de produire de l'IFN-γ. Ces lymphocytes Th17 induisent des réponses inflammatoires plus exacerbées que ceux générés en présence de TGF-β et pourraient être responsables du

développement de réponses autoimmunes (Kyburz & Corr, 2011). Assez logiquement, le TGF-β inhibe la production d'IFN-γ ou d'IL-4 par les lymphocytes Th1 ou Th2, respectivement (Reiner, 2007). Enfin, l'IL-23, qui a la particularité de partager la sous-unité p40 de l'IL-12 (Oppmann *et al.*, 2000), est également produite par les cellules dendritiques et assure le maintien et la survie des lymphocytes Th17 (Langrish *et al.*, 2004).

3 Mémoire immunitaire et vaccination

L'une des caractéristiques les plus remarquables du système immunitaire adaptatif est certainement sa capacité à garder en mémoire ses rencontres avec les pathogènes, et ce pendant plusieurs dizaines d'années voir durant toute la vie d'un organisme (Yu *et al.*, 2008). Cette propriété fondamentale du système immunitaire est la base de la vaccination, dont le but est d'induire une immunité protectrice de longue durée contre un pathogène donné.

Historiquement, l'immunologie trouve sa source dans une tentative de comprendre et de rationaliser les travaux pionniers menés sur la vaccination par Pasteur, Jenner, etc. Il est intéressant de noter que la plupart de nos vaccins actuels les plus efficaces ont été découverts de manière empirique (Plotkin, 2005) et qu'actuellement nous restons largement ignorants des mécanismes immunologiques sous-jacents.

« We really don't know how to make vaccines in a predictable way.
It's still a little bit of black magic. »
(Tachi Yamada, Keystone Symposium on Immunological Mechanisms of Vaccination, Washington, 2010), (von Bubnoff, 2010)

La compréhension de ces mécanismes est toutefois indispensable au développement de nouveaux vaccins. Par exemple, le vaccin contre la fièvre jaune (YF-17D), mis au point empiriquement en 1937 (Theiler & Smith, 1937), fait partie des vaccins les plus efficaces, avec une persistance des anticorps pendant plus de 35 ans après une seule injection et une puissante réponse T cytotoxique (Pugachev *et al.*, 2005). Jusqu'il y a peu, ses mécanismes d'action étaient totalement inconnus, mais de récentes études ont démontré que YF-17D activait plusieurs sous-populations de cellules dendritiques en stimulant plusieurs TLRs (TLRs 2, 7, 8 et 9). La réponse adaptative mise en place est alors polyvalente, incluant les deux réponses Th1 et Th2 ainsi que des lymphocytes T $CD8^+$ cytotoxiques (Querec *et al.*, 2006).

3.1 Jenner et Pasteur, les pionniers en vaccination

« Les Circassiens s'aperçurent que, sur mille personnes, il s'en trouvait à peine une seule qui fut attaquée deux fois d'une petite vérole bien complète… ; qu'en un mot jamais on n'a véritablement cette maladie deux fois en sa vie. […]
Il restait donc, pour conserver la vie et la beauté de leurs enfants, de leur donner la petite vérole de bonne heure ; c'est ce que l'on fit, en insérant dans le corps d'un enfant un bouton que l'on prit de la petite vérole, la plus complète et en même temps la plus favorable qu'on pût trouver. L'expérience ne pouvait pas manquer de réussir. »
(Voltaire, 11ème Lettre Philosophique, 1734)

Pratiquée dès le XIème siècle par les chinois, la variolisation décrite par Voltaire (ci-dessus) n'a été introduite en Occident qu'au début du 18ème siècle. Mais ce n'est qu'en 1796, suite aux expériences d'un médecin anglais, Edouard Jenner, que la vaccination fut réellement découverte. Celui-ci inocula un enfant de huit ans avec le contenu des vésicules de vaccine qu'il avait prélevé au préalable sur la main d'une laitière. La vaccine (cowpox) ou variole des vaches est une maladie proche de la variole humaine (smallpox), souvent contractée par les laitières. Quelques semaines après cette inoculation, l'administration répétée de la variole au même enfant mit en évidence la résistance générée par la vaccine et démontra l'efficacité de cette méthode.

Il fallut toutefois attendre près d'un siècle pour qu'un lien soit clairement établi entre les maladies infectieuses et les microorganismes. En 1860, Louis Pasteur émis l'hypothèse que la virulence des agents pathogènes pouvait être atténuée par l'exposition à des conditions environnementales défavorables, telles que les hautes températures. Par la suite, ses études et celles de Robert Koch sur le choléra, la maladie du charbon et la rage confirmèrent cette hypothèse et aboutirent au développement de vaccins prophylactiques basés sur l'atténuation (vaccin vivant atténué). Au siècle suivant, Calmette et Guérin utilisèrent des passages en milieu artificiel pour atténuer *Mycobacterium bovis* (Calmette, 1907), et Theiler utilisa des passages en souris et embryons de poulet pour atténuer le virus de la fièvre jaune (Theiler & Smith, 1937) (**table 3**).

Peu après les découvertes de Pasteur, l'utilisation de vaccins complètement inactivés (vaccin mort ou inactivé) fit son apparition et plus tard, avec les nombreuses avancées en biotechnologie, il devint possible de créer des vaccins à partir de composants microbiens tels que les protéines ou les polysaccharides (vaccin sous-unitaire ou recombinant) (**table 4**).

Table 3. Vaccins vivants (d'après (Plotkin, 2005))

Stratégie utilisée	Date	Vaccin ou cible
Utilisation d'un virus apparenté (animal)	1798	Variole
Atténuation chimique	1885	Rage
	1881	Anthrax
Passages in vitro	1927	BCG
	1937	Fièvre jaune
Passages en culture cellulaire	1962	Vaccin oral contre la polio
	1963	Rougeole
	1971	Adénovirus
	1995	Varicelle
	2005	Rotavirus 89-12
Passages en culture cellulaire avec	1969	Rubéole
adaptation au froid	2003	Grippe (vaccin vivant)
Auxotrophie	1989	Fièvre typhoïde Ty21a
Assortiment de plusieurs souches	1970s	Grippe (vaccin inactivé)
	2003	Grippe (vaccin vivant)
	2005	Rotavirus

Table 4. Vaccins tués (d'après (Plotkin, 2005))

Stratégie utilisée	Date	Vaccin ou cible
Inactivation de l'organisme entier	1896	Fièvre typhoïde
	1896	Choléra
	1897	Peste
	1926	Coqueluche
	1938	Grippe
	1955	Polio (vaccin inactivé)
	1995	Hépatite A
Utilisation d'extraits ou sous-unités	1944	Encéphalite japonaise
	1970s	Grippe
	1960	Anthrax
	1976	Rage
Utilisation de toxines	1923	Diphtérie
	1927	Tétanos
	2008	Anthrax
Utilisation de polysaccharides capsulaires	1977	Pneumocoque
	1974	Méningocoque
	1995	Fièvre typhoïde
Utilisation de conjugués protéines-	1987	Grippe (type b)
polysaccharides capsulaires	2002	Pneumocoque
	2002	Méningocoque
Utilisation de protéines purifiées ou	1986	Hépatite B
recombinantes	1996	Coqueluche
	1998	Maladie de Lyme

3.2 Développement d'une mémoire immunitaire T et B

3.2.1 Lymphocytes T – mémoire à médiation cellulaire

Après la résolution d'une réponse immunitaire, les lymphocytes T qui survivent à la phase de contraction vont constituer un pool de lymphocytes T mémoires, spécifiques de l'antigène rencontré et aptes à persister dans les différents tissus de l'organisme. Certains résident préférentiellement dans les tissus périphériques, comme la peau, les intestins et les poumons, où ils fournissent une première ligne de défense ; tandis que d'autres constituent un pool de réserve au sein des organes lymphoïdes (Sallusto *et al.*, 1999). La mémoire T présente cependant une certaine plasticité, avec une re-circulation possible des deux populations au sein des tissus lymphoïdes ou périphériques et une conversion d'un phénotype à l'autre (Kassiotis & Stockinger, 2004; Masopust *et al.*, 2004; Moulton & Farber, 2006).

Les lymphocytes T mémoires qui résident dans les organes lymphoïdes constituent la **mémoire centrale** (T_{CM}, central memory T cells). Leur localisation au sein de ces tissus dépend de l'expression de CD62L et du récepteur CCR7 dont le ligand est sécrété par les cellules endothéliales des veinules post-capillaires. Les cellules T_{CM} ne peuvent exercer une fonction effectrice immédiate lors d'un nouveau contact avec le pathogène, mais elles sont capables de se différencier rapidement en lymphocytes T mémoires effecteurs (T_{EM}) et migrer vers le site d'infection.

La deuxième population de lymphocytes T mémoires persiste dans les tissus périphériques ($CD62L^{-}CCR7^{-}$) après la réponse immunitaire et constitue la **mémoire périphérique** (T_{EM}, effector memory T cells) (Masopust *et al.*, 2001). Lors d'une nouvelle exposition au même antigène, les cellules T_{EM} prolifèrent et peuvent rapidement initier une réponse inflammatoire ou cytotoxique sans nécessiter de processus de différenciation (Sallusto *et al.*, 1999; Sallusto *et al.*, 2004). Ce mode d'action permet de limiter la réplication du pathogène en périphérie en attendant la nouvelle vague de lymphocytes T effecteurs spécifiques du pathogène et générée au sein des organes lymphoïdes (**figure 11**).

Le tropisme des cellules T_{EM} pour les différents sites périphériques est déterminé par l'expression de combinaisons spécifiques de molécules d'adhésion et de récepteurs à chimiokines (Bonder *et al.*, 2006; Sanders *et al.*, 1988; Woodland & Kohlmeier, 2009) et est

fortement dépendant du site primaire d'infection et du type de tissu contaminé (Woodland & Kohlmeier, 2009). Toutefois, ce tropisme peut être re-programmé lors d'une seconde infection, en fonction de la localisation du nouveau site d'infection (Dudda *et al.*, 2005; Masopust *et al.*, 2004).

Alors que les cellules T_{EM} varient en nombre selon le tissu concerné, le nombre de lymphocytes T mémoires (T_{CM}) qui s'établissent dans la rate, le foie ou la moelle osseuse est souvent similaire, quelque soit le site primaire d'infection (Reinhardt *et al.*, 2001; Woodland & Kohlmeier, 2009). Une fois établi, le nombre de lymphocytes T mémoires reste relativement constant et subit une prolifération dite « homéostatique » qui dépend notamment des cytokines IL-7 et IL-15 (Surh *et al.*, 2006).

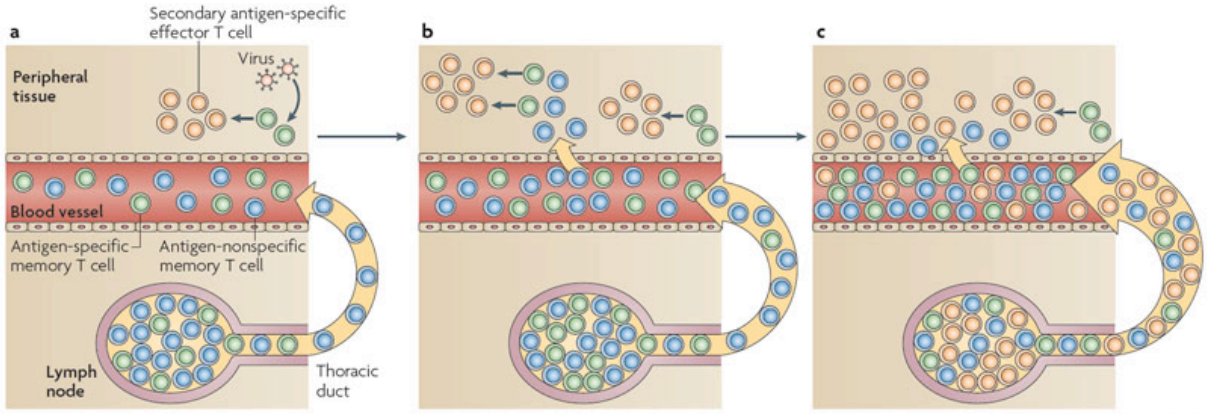


Figure 11. Dynamique de la réponse secondaire des lymphocytes T mémoires dans les tissus périphériques . La réponse de mémoire des lymphocytes T lors d'une réponse secondaire peut être divisée en 3 phases distinctes. **a** Les lymphocytes T mémoires présents dans les tissus périphériques (T_{EM}, en vert) sont les premiers à répondre au pathogène rencontré (ici un virus). Après reconnaissance antigénique, les cellules T_{EM} prolifèrent et génèrent une réponse T effectrice secondaire (en orange). **b** L'expression locale de chimiokines provoquée par l'inflammation du tissu infecté assure le recrutement de lymphocytes T mémoires spécifiques (verts) ou non (bleus) du pathogène rencontré. Au contact avec l'antigène, les lymphocytes T mémoires spécifiques du pathogène (verts) initient eux aussi une réponse T effectrice secondaire (orange). A ce moment (24 à 72h après l'infection), les cellules présentatrices d'antigène (non représentées ici) ont migré vers les ganglions lymphatiques où elles activent les cellules T_{CM}. **c** Les cellules T_{CM} résidant dans les ganglions lymphatiques initient la génération d'un grand nombre de lymphocytes T effecteurs spécifiques du pathogène (orange) qui migrent via la circulation sanguine vers le site d'infection. Ces 3 étapes permettent de limiter la réplication du pathogène lors d'une réponse secondaire et assurent la résolution rapide de l'infection.

3.2.2 Lymphocytes B - mémoire à médiation humorale

L'activation et la sélection de lymphocytes B produisant des anticorps de haute affinité pour l'antigène rencontré a été décrite précédemment (paragraphe 2.2.2). Les lymphocytes B de haute affinité vont constituer le pool de lymphocytes B mémoires qui comprend deux sous-populations (Kalia *et al.*, 2006) :

- ***les lymphocytes B mémoires résidant dans les organes lymphoïdes secondaires***. Bien que ces cellules ne sécrètent pas activement des anticorps, elles expriment un BCR de haute affinité. Lors d'une réponse secondaire, ces lymphocytes B mémoires se divisent rapidement et se différencient en plasmocytes sécrétant des anticorps. La réponse humorale développée est quantitativement et qualitativement meilleure que lors de la première exposition à l'antigène : les anticorps sécrétés sont plus nombreux et présentent une plus grande affinité pour l'antigène.
- ***les plasmocytes mémoires (à longue durée de vie), résidant principalement dans la moelle osseuse.*** Ces cellules produisent et sécrètent en permanence des anticorps qui sont déversés dans le sang et autres fluides corporels. Une des particularités de ces plasmocytes est qu'ils expriment peu de BCR, ils sont donc incapables de répondre à une nouvelle stimulation antigénique et de proliférer. Cela signifie également que ces cellules maintiennent leurs fonctions effectrices (production d'anticorps) en absence de stimulation antigénique.

3.3 Combiner une meilleure compréhension du SI et du pathogène pour élaborer de meilleurs vaccins

3.3.1 Compréhension du SI

Avec l'accroissement de nos connaissances et des technologies en matière d'immunologie, nombre de chercheurs tentent de trouver des pistes pour améliorer les stratégies vaccinales (Ahlers & Belyakov, 2010; Bevan, 2011; Foulds *et al.*, 2006; Kaech *et al.*, 2002; Pulendran & Ahmed, 2006). Parmi ceux-ci, Pulendran *et al* (Pulendran & Ahmed, 2006) a exposé de manière très complète les challenges des futures recherches en matière de vaccination. Quelques pistes visant à améliorer la réponse immunitaire générée par les vaccins ont donc été proposées, parmi lesquelles on peut citer :

- *Optimiser l'activation des cellules dendritiques.* Comme l'information perçue par les DCs sur le pathogène ou le vaccin est transmise aux lymphocytes B et T et modulera l'orientation, la qualité et la persistance de la réponse adaptative, il convient de choisir un adjuvant[1] qui stimulera au mieux les cellules dendritiques (Wille-Reece *et al.*, 2006). Il existe en effet de multiples sous-classes de DCs, auxquelles on attribue la capacité d'induire des réponses immunitaires distinctes (Pulendran, 2005). Par exemple, chez les souris, les DCs $CD8\alpha^+$ stimulent les lymphocytes T à produire de l'IFN-γ et de l'IL-2 tandis que les DCs $CD8\alpha^-$ stimulent la production d'IL-4, IL-5 et IL-10 (Maldonado-Lopez *et al.*, 1999; Pulendran *et al.*, 1999).

- *Optimiser la réponse des lymphocytes T et B*. Durant la réponse immunitaire, les lymphocytes subissent une phase d'expansion clonale, de contraction puis de mémoire. Un vaccin efficace serait celui qui stimulerait la phase d'expansion, réduirait la phase de contraction et stabiliserait les lymphocytes mémoire générés (Kaech *et al.*, 2002), tout en évitant l'induction d'une réponse excessive qui mènerait au dévelopement de pathologies autoimmunitaires. Ici aussi le choix

[1] **Adjuvant** : composé qui augmente et/ou module l'immunogénicité d'un antigène (Leroux-Roels, 2010). Les sels d'aluminium sont parmi les adjuvants les plus utilisés et les plus efficaces mais leur mode d'action est encore sujet à débat à l'heure actuelle.

d'un adjuvant est primordial car il pourrait influencer la phase d'expansion clonale et le maintien des lymphocytes mémoires en favorisant la survie des DCs, l'expression de signaux de costimulation, la sécrétion de cytokines pro-infammatoires, etc.

3.3.2 Compréhension du monde microbien

La recherche en vaccination est rarement couronnée de succès si elle ne prend pas en compte l'étude du pathogène concerné. En effet, le cycle infectieux du microorganisme envahisseur et ses mécanismes d'échappement à la réponse immunitaire doivent être connus pour mieux cibler les mécanismes effecteurs qui doivent être induits par la vaccination.

Exemple : la réponse de mémoire protectrice contre *Listeria monocytogenes*

L'exemple cité ici reprend les recherches menées par l'équipe de G. Lauvau sur *Listeria monocytogenes*. Elles apportent des informations essentielles sur les mécanismes effecteurs induits par *Listeria* et qui confèrent une protection efficace lors d'une seconde infection. Il apparaît également dans ces recherches que la présence de cellules mémoire ne coïncide pas toujours avec la protection (Ahmed & Gray, 1996; Zinkernagel *et al.*, 1996; Zinkernagel, 2012). Une étude approfondie des mécanismes protecteurs s'avère donc essentielle dans les différents modèles de recherche vaccinale.

Listeria monocytogenes est une bactérie intracellulaire facultative à Gram positif. Une fois internalisée par les cellules phagocytaires, elle est confinée dans un phagosome dont elle s'échappe pour atteindre le cytosol de la cellule infectée, ceci grâce à la production d'une toxine capable de former des pores dans la membrane, la listeriolysine O (LLO) (Gaillard *et al.*, 1986).

Listeria est détectée par les TLR2 (Torres *et al.*, 2004) et TLR5 (Hayashi *et al.*, 2001), mais également par des PRRs cytosoliques comme les NLRs et NALP3 (Warren *et al.*, 2008). La réplication de *Listeria* dans le cytosol induit des cascades de signalisation qui stimulent rapidement la maturation des cellules dendritiques. Celles-ci activent les lymphocytes T $CD8^+$ à se différencier en lymphocytes T mémoires (Jung *et al.*, 2002). Lors d'une seconde

infection, ces lymphocytes T mémoires se différencient en cellules effectrices produisant de l'IFN-γ et du TNF-α et présentant une activité cytolytique (Muraille *et al.*, 2007). Ces fonctions effectrices ne sont toutefois pas suffisantes pour conférer une protection efficace lors d'une ré-infection. En effet, pour être pleinement fonctionnels et protecteurs, les lymphocytes T $CD8^+$ mémoires doivent sécréter une molécule pro-inflammatoire, CCL3, qui active les cellules phagocytaires mononucléées (MPCs) à produire du TNF-α (Narni-Mancinelli *et al.*, 2007). À son tour le TNF-α stimule la production de dérivés toxiques de l'oxygène (ROS, reactive oxygen species) par les MPCs et les neutrophiles, ce qui mène à l'élimination de *Listeria*. La production de ROS nécessite la sécrétion de CCL3 par les lymphocytes T $CD8^+$ mémoires (Narni-Mancinelli *et al.*, 2007; Narni-Mancinelli *et al.*, 2011). Ces études préliminaires confirment la nécessité de deux niveaux de régulation dans le déroulement d'une réponse secondaire protectrice : (a) *une phase dépendante de l'antigène*, dans laquelle les lymphocytes T $CD8^+$ mémoires sont ré-activés et contrôlent l'activation du système immunitaire inné (Narni-Mancinelli *et al.*, 2011), et (b) *une phase indépendante de l'antigène*, dans laquelle les cellules phagocytaires mononucléées coordonnent l'immunité innée et stimulent les activités bactéricides des cellules effectrices (Narni-Mancinelli *et al.*, 2007).

Toutefois, comme suggéré dans la dernière étude de Lauvau (Soudja *et al.*, 2012), les mécanismes qui contrôlent la ré-activation des lymphocytes T $CD8^+$ mémoires peuvent également être indépendants de l'antigène. Les auteurs décrivent que la ré-activation de ces cellules est initialement orchestrée par une population particulière de monocytes ($LY6C^+CCR2^+$) générant un contexte inflammatoire par la sécrétion d'IL-18 et d'IL-15. Ces cytokines induisent rapidement l'activation des lymphocytes T $CD8^+$ mémoires mais également des cellules NK (Soudja *et al.*, 2012).

D'autre part, les recherches menées sur le cycle infectieux de *Listeria* ont pu mettre en évidence que la réplication de la bactérie dans le cytosol des cellules infectées (Hamilton *et al.*, 2006; Muraille *et al.*, 2005; von Koenig *et al.*, 1982) associée à une quantité suffisante de ligands de PRRs dans le cytosol étaient des prérequis nécessaires pour l'induction de lymphocytes T $CD8^+$ mémoires et protecteurs *in vivo* ($CCL3^+$ IFN-γ^+ TNF-α^+ et cytolytiques) (Muraille *et al.*, 2007; Rahmoun *et al.*, 2011). En effet, il a été démontré que le système de sécrétion secA2 est directement impliqué dans la génération de lymphocytes T $CD8^+$ mémoires protecteurs (Muraille *et al.*, 2007; Rahmoun *et al.*, 2011). L'utilisation de

différents mutants de ce système de sécrétion a pu mettre en évidence qu'une ou plusieurs des protéines sécrétées dans le cytosol par ce système étai(en)t nécessaire(s) à l'activation optimale des DCs, et par là même à la mise en place d'une immunité protectrice de longue durée (Rahmoun *et al.*, 2011). L'identification de la ou les protéine(s) impliquée(s) dans ce processus et par la suite le NLR et la voie de signalisation induite par la reconnaissance de son ligand seront d'un grand intérêt pour la recherche vaccinale. Ces recherches illustrent combien il est important de connaître le cycle infectieux des pathogènes en vaccination.

4 La brucellose

La brucellose est une maladie infectieuse causée par une α-protéobactérie à Gram négatif du genre *Brucella*. Bien que décrite pour la première fois au XIX° siècle par D. Bruce, des traces plus anciennes d'infections chez l'homme ont pu être mises en évidence dans des ossements égyptiens datant de l'an 750 avant J.-C. ainsi que sur les sites d'Herculaneum et Pompei détruits par une éruption volcanique en 79 après J.-C. (Capasso, 2002). Aussi connue sous le nom de fièvre de Malte ou fièvre ondulante, la brucellose est donc une maladie ancienne dont l'impact sanitaire et économique reste à ce jour un facteur préoccupant (Seleem *et al.*, 2010).

4.1 Épidémiologie

Avec plus de 500 000 nouveaux cas reportés chaque année chez l'homme, l'incidence élevée de la brucellose dans les pays en voie de développement classe cette maladie parmi les cinq zoonoses bactériennes les plus communes au monde[1] (Pappas *et al.*, 2006b). La distribution géographique de la brucellose est en constante évolution, mais les zones les plus touchées sont le Moyen-Orient avec plus de 70 cas par 100 000 habitants (contre moins de 1/100 000 en GB, USA et Australie (Cutler *et al.*, 2005)), le bassin méditerranéen (Portugal, Espagne, Italie, Grèce, Turquie), l'Amérique latine, l'Asie et l'Afrique (Godfroid *et al.*, 2005; Pappas *et al.*, 2006b).

Les pertes économiques associées à cette maladie sont liées d'une part à une diminution de la productivité dans les élevages (avortements, diminution de la production laitière, etc.) et d'autre part à l'incidence sur la santé publique (coûts des traitements, perte de productivité). Par exemple, en Amérique Latine, les pertes annuelles liées à la brucellose sont estimées à environ 600 millions de dollars (Seleem *et al.*, 2010). Toutefois, à cause de son issue non fatale, de ses symptômes cliniques peu reconnaissables, de sa quasi éradication dans le nord de l'Europe et en Amérique du Nord ainsi que de sa re-distribution

[1] Selon l'OMS, toute maladie ou infection qui se transmet d'un animal vertébré à un humain, et vice-versa, est classée parmi les zoonoses. Plus de 200 zoonoses ont déjà été décrites à ce jour. Les zoonoses bactériennes les plus communes sont la salmonellose, la campylobactériose, l'anthrax, la brucellose, l'infection par *Escherichia coli* VéroToxigéniques (VTEC), la leptospirose, la peste, la fièvre Q, la shigellose et la tularémie.

dans les pays en voie de développement (Pappas, 2010), l'intérêt pour la brucellose décroît en Occident.

4.2 Transmission

Ce n'est qu'en 1905 que la brucellose a été définie comme zoonose (Wyatt, 2005) car, à l'origine, les scientifiques pensaient que les bactéries résidaient dans le sol et étaient inhalées par l'homme (Vassallo, 1996). L'homme est généralement infecté par l'ingestion de produits laitiers non pasteurisés ou par l'inhalation de bactéries lors des mises bas ou avortements dans les troupeaux infectés. La transmission d'homme à homme a rarement été rapportée (Meltzer *et al.*, 2010). L'éradication de la brucellose chez les animaux domestiques apparaît dès lors comme la seule façon de diminuer l'incidence de la maladie chez l'homme. De nombreux programmes de vaccination ciblant les troupeaux d'élevage ont permis de diminuer l'incidence de la brucellose humaine, voir de l'éradiquer dans une dizaine de pays dont la Belgique et le Luxembourg (Pappas *et al.*, 2006b). Toutefois de nouveaux foyers infectieux sont régulièrement mis à jour chez les animaux domestiques mais également dans la faune sauvage (Gregoire *et al.*, 2012; Zheludkov & Tsirel'son, 2010), indiquant une ré-émergence continuelle de cette maladie (Godfroid *et al.*, 2005; Pappas, 2010; Seleem *et al.*, 2010).

4.3 Pathologie

4.3.1 Brucellose humaine

« The disease rarely kills anybody, but it often makes a patient wish he were dead » (TIME magazine 1943, before the discovery of antibiotic treatments)

La brucellose humaine comprend deux phases, l'une aigue, l'autre chronique. La phase aigue se caractérise par des symptômes tels qu'une fièvre ondulante, des sueurs nocturnes et une fatigue anormale (asthénie). Une analyse du sang en période de fièvre peut révéler une bactériémie, qui est décrite comme un évènement précoce et transitoire de la maladie, précédant l'infection des macrophages (Humphreys & Moore, 1941; Pappas &

Papadimitriou, 2007). Utile au diagnostic différentiel, la détection de *Brucella* dans le sang n'est toutefois pas une procédure aisée en biologie clinique (Yagupsky, 1999). La présence de bactéries dans le sang serait pourtant synonyme du développement de foyers secondaires d'infection, liés à des complications cliniques, et serait associée à un risque plus élevé de rechutes (Pappas & Papadimitriou, 2007; Solera *et al.*, 1998).

En absence de traitements, la phase chronique s'établit avec la formation de foyers infectieux ostéo-articulaires (66% des cas), neurologiques, hépatiques, génitaux et cardiaques (Colmenero *et al.*, 1996; Young, 1995). De nombreuses complications peuvent survenir dont la plus grave est l'endocardite qui survient dans 2% des cas et qui est à l'origine de 80% des décès causés par la brucellose (Reguera *et al.*, 2003).

4.3.2 Brucellose animale

Chez l'hôte naturel, la brucellose est souvent asymptomatique, avec peu de signes cliniques notables, mais les animaux infectés peuvent toutefois disséminer des bactéries via leurs sécrétions (Capparelli *et al.*, 2009). Chez la femelle en gestation, le signe clinique le plus fréquent est l'avortement (au dernier trimestre de gestation), qui mène généralement à une contamination des autres membres du troupeau. L'avortement est dû à l'invasion et la réplication des bactéries au sein des cellules du trophoblaste, induisant une nécrose des membranes et conduisant à l'issue fatale de la gestation (Anderson *et al.*, 1986). Chez le mâle, une invasion des organes reproducteurs est souvent observée et conduit à une stérilité partielle ou totale (Enright, 1990; Moreno & Moriyon, 2006).

4.4 Traitements et vaccination

Chez l'homme, aucune prophylaxie vaccinale n'est disponible à ce jour (Perkins *et al.*, 2010). Lors d'une contamination, l'OMS recommande l'utilisation d'une double antibiothérapie composée de doxycycline et de rifampicine durant une période de 6 semaines (Ariza *et al.*, 2007). Le risque de rechute n'est pas négligeable, c'est pourquoi de nombreuses études visent à améliorer l'antibiothérapie (Ariza *et al.*, 2007).

Chez l'animal, plusieurs vaccins vivants atténués sont utilisés à l'heure actuelle (**table 5**). La souche vaccinale *B. melitensis* Rev.1 est utilisée depuis plus de 40 ans chez les petits

ruminants et protège 70 à 85 % des animaux contre une réinfection par une souche virulente de *B. melitensis*, mais également de *B. ovis* (Blasco, 1997; Garin-Bastuji *et al.*, 1998). La souche S19 de *B. abortus* est utilisée chez les bovins et la souche *B. suis* S2 est surtout utilisée chez les moutons et les chèvres en Asie, dans les vastes prairies semi-arides où le vaccin est administré par voie orale via l'eau des puits (Xin, 1986).

Les souches Rev.1, S19 et S2 présentent toutefois une virulence résiduelle (Alton *et al.*, 1967) et peuvent causer l'avortement chez les animaux en gestation. Elles sont également infectieuses pour l'homme. De plus, ces vaccins sont à l'origine de problèmes d'interférence lors des tests sérologiques, empêchant la distinction entre un animal vacciné ou infecté. En effet, tout comme les souches virulentes, ils induisent la production d'anticorps dirigés contre la chaîne O du lipopolysaccharide (LPS) de *Brucella*, ceux-là même qui sont recherchés pour le diagnostic de la brucellose.

Pour pallier cet inconvénient, un autre vaccin vivant atténué est utilisé aux USA en remplacement de la souche S19, il s'agit de la souche rugueuse RB51 de *B. abortus*, faible productrice de la chaîne O du LPS (Schurig *et al.*, 1991). Malgré son approbation aux USA, l'efficacité de cette souche chez l'hôte naturel a été mise en doute par plusieurs études (Jimenez de Bagues *et al.*, 1995; Kreeger *et al.*, 2002; Olsen *et al.*, 2006). De plus on lui reproche sa résistance à la rifampicine, l'un des antibiotiques utilisé lors du traitement thérapeutique chez l'homme (Moriyon *et al.*, 2004).

Table 5. Souches vaccinales contre la brucellose animale (d'après (Moreno & Moriyon, 2006)).

Souches vaccinales	Caractéristiques
B. melitensis Revision (Rev.) 1	- protectrice contre les souches virulente de *B. melitensis* et *B. ovis* (Blasco, 1997; Garin-Bastuji et al., 1998) - utilisée chez les petits ruminants (chèvres, moutons) - isolée dans les années 1950 à partir de souches de *B. melitensis* devenues dépendantes de la streptomycine, mais qui, après réversion, ont perdu cette caractéristique et sont restées résistantes à cet antibiotique (Alton & Elberg, 1967). La ou les cause(s) de son atténuation demeure(nt) inconnue(s), mais il semblerait que celle-ci soit liée à l'altération de certaines propriétés de la membrane externe.
B. abortus strain (S)19	- protectrice contre les souches virulentes de *B. abortus* - utilisée chez les bovins - isolée dans les années 1920 dans du lait de vache laissé à température ambiante durant 1 an en laboratoire. Les raisons précises de son atténuation sont inconnues, mais cette souche présente clairement un trafic intracellulaire altéré (Elzer et al., 1994).
B. suis strain (S)2	- protectrice contre les souches virulentes de *B. suis* - utilisée chez les porcs, moutons, chèvres et bovins - isolée en Chine après des transferts en série en milieu de culture de la souche virulente B. suis 1. Elle présente le même niveau d'atténuation que la souche S19.
B. abortus RB51	- protectrice contre les souches virulentes de *B. abortus* - utilisée chez les bovins - isolée en 1991 (Schurig et al., 1991), il s'agit d'un mutant rugueux (dépourvu de la chaîne O du LPS) spontané de la souche virulente de *B. abortus* 2308 obtenu après des passages répétés *in vitro*. Son atténuation est notamment liée à la présence d'une séquence d'insertion (IS711) dans le gène wboA qui est impliqué dans la synthèse de la chaîne O (Vemulapalli et al., 2000). Il est résistant à la rifampicine.

4.5 Les différentes espèces de *Brucella*

Brucella est un coccobacille appartenant aux α-protéobactéries. Elle est phylogénétiquement proche de bactéries qui ont co-évolué avec leurs hôtes animaux ou végétaux, aussi bien dans une relation symbiotique (*Sinorhizobium meliloti*) que pathogène (*Bartonella spp., Rickettsia spp., Agrobacterium tumefaciens)* (Moreno & Moriyon, 2006). Tout comme d'autres bactéries à Gram négatif, la membrane externe de *Brucella* se caractérise par la présence de LPS. Selon la présence ou l'absence de la chaîne polysaccharidique O du LPS, on distingue les bactéries « lisses » ou « rugueuses », respectivement.

Les récentes découvertes de nouvelles espèces ont quelque peu modifié la classification du genre *Brucella*. Actuellement, on compte 10 espèces de *Brucella*, caractérisées par leur préférence d'hôte (**table 6**). Sept d'entre elles infectent les animaux terrestres (*B. melitensis, abortus, suis, ovis, canis, neotomae,* et *B. microti*), 2 infectent les mammifères marins (*B. ceti* et *B. pinnipedialis*) et la dernière, *B. inopinata*, découverte dans un implant mammaire, n'a pas encore de préférence d'hôte connue à ce jour (Scholz *et al.*, 2010). Deux nouvelles espèces ont récemment été isolées, l'une chez un patient atteint d'une maladie pulmonaire chronique (Tiller *et al.*, 2010) et l'autre chez des babouins qui présentaient une mortalité natale anormale (Schlabritz-Loutsevitch *et al.*, 2009). Enfin, en mai 2012, des bactéries isolées chez des grenouilles pourraient constituer une nouvelle espèce du genre *Brucella* (Eisenberg *et al.*, 2012). Les espèces les plus infectieuses pour l'homme sont *B. melitensis, B. abortus* et *B. suis*. On considère que 99% des infections humaines sont causées par *B. melitensis* (Wallach *et al.*, 1997).

Table 6. Préférence d'hôtes de *Brucella* (d'après (Pappas, 2010))

Espèces	Hôte
Anciennes espèces	
B. melitensis	Moutons, chèvres, chameaux
B. abortus	Bovins, buffles, élans, yacks, chameaux
B. suis	Cochons, sangliers, rennes, caribous, rongeurs
B. canis	Chiens
B. ovis	Moutons
B. neotomae	Rongeurs
Nouvelles espèces	
B. ceti	Marsouins, dauphins, baleines
B. pinnipedialis	Phoques
B. microti	Renards roux, campagnols communs
B. inopinata	Inconnu

4.6 *Brucella* et la cellule hôte

4.6.1 Brucella, une bactérie extracellulaire facultative

Brucella est considérée comme une bactérie intracellulaire facultative. La désignation « facultative » provient du fait qu'elle est capable de se multiplier dans un milieu de culture, sans requérir l'environnement cellulaire. Il a toutefois été proposé de considérer *Brucella* comme une bactérie « extracellulaire facultative » vu son adaptation exceptionnelle au milieu intracellulaire et son inaptitude à survivre durant une période prolongée en dehors de l'hôte (Moreno & Moriyon, 2002). Au sein de la cellule hôte, *Brucella* est confinée dans une vacuole dont elle dévie le trafic intracellulaire pour éviter la fusion phagolysosomale. Cette vacuole, qui acquiert durant son trafic des propriétés membranaires propres au réticulum endoplasmique (Celli *et al.*, 2003), constitue une niche de survie et de réplication favorable à *Brucella* (Celli, 2006; von Bargen *et al.*, 2012). Le caractère chronique de la brucellose proviendrait essentiellement de l'aptitude de *Brucella* à gagner furtivement sa niche de réplication intracellulaire (Martirosyan *et al.*, 2011).

4.6.2 Cellules hôtes

Brucella présente un tropisme pour les cellules du système réticulo-endothélial (macrophages, cellules réticulaires des organes hématopoiétiques et cellules endothéliales). *In vitro*, un grand nombre de types cellulaires ont été décrits comme pouvant être infectés par *Brucella*. Le consensus est toutefois de considérer que *Brucella* peut se répliquer au sein des cellules phagocytaires telles que les macrophages (Forestier *et al.*, 1999) et les cellules dendritiques (Billard *et al.*, 2005), et des cellules non-phagocytaires telles que les trophoblastes (Anderson *et al.*, 1986; Detilleux *et al.*, 1990), les cellules épithéliales (Pizarro-Cerda *et al.*, 1998) et les fibroblastes (Holland & Pickett, 1956).

In vivo il a récemment été démontré que *Brucella* infecte préférentiellement les macrophages et les cellules dendritiques (Archambaud *et al.*, 2010; Copin *et al.*, 2012), mais une autre étude suggère qu'une petite proportion de cellules infectées serait des lymphocytes B (Goenka *et al.*, 2012).

Lymphocytes B régulateurs (Bregs), une nouvelle niche d'infection ?

Goenka *et al* ont récemment démontré que*, in vivo*, les lymphocytes B représentaient 10% des cellules infectées par *B. abortus* 2308 (Goenka *et al.*, 2012). Malgré l'absence de réplication de *Brucella* au sein de ces lymphocytes, ces cellules pourraient constituer une niche d'infection contribuant à la chronicité de la maladie. En effet, d'une part les lymphocytes B activés présentent une longévité élevée et d'autre part ces cellules sont décrites comme étant des « Bregs » pouvant réguler la réponse immunitaire via leur production de TGF-β1. Les mêmes auteurs avaient récemment démontré le rôle immuno-régulateur des lymphocytes B lors d'une infection par *B. abortus* 2308 (Goenka *et al.*, 2011).

5 La réponse immunitaire contre *Brucella*

5.1 Modèle d'étude : la souris

5.1.1 Du cobaye à la souris

La brucellose a fait l'objet de nombreuses études dans plusieurs modèles expérimentaux animaux, incluant les embryons de poulet, les lapins, rats, hamsters, gerbilles, cobayes et souris (Garcia-Carillo, 1990; Moreno & Moriyon, 2006; Silva *et al.*, 2011). Pendant de nombreuses années, le cobaye a constitué le principal modèle d'intérêt car il présentait une susceptibilité élevée à l'infection et les symptômes développés par ces animaux étaient semblables à ceux observés chez l'homme (Moulton & Meyer, 1958). Cependant, depuis près de 20 ans, le modèle murin est préférentiellement utilisé, notamment pour des raisons économiques, pour la facilité d'élevage ainsi que pour les nombreux outils expérimentaux développés chez la souris. De plus, le développement de souches génétiquement modifiées et la connaissance approfondie que l'on dispose de leur système immunitaire en font un modèle très pratique. Toutefois, l'interprétation des résultats obtenus chez des souris génétiquement modifiées requiert une certaine prudence car d'autres fonctions que celle initialement visée par la manipulation génétique peuvent être affectées (Goldszmid *et al.*, 2007; Kim *et al.*, 2008; Locksley *et al.*, 1993).

La souris contrôle relativement bien l'infection, sans toutefois éviter l'établissement d'une infection chronique (Cheers, 1984). Elle présente néanmoins peu de symptômes propres à la brucellose humaine, ce qui impose certaines précautions quant aux comparaisons avec la maladie observée chez l'homme.

5.1.2 Les souris, des BALB/c aux C57BL/6

Certaines souches de souris sont plus résistantes à l'infection par *Brucella* que d'autres. Parmi les souris dites susceptibles, on compte les souches DBA2, C3H/He et BALB/c, tandis que les souches C57BL/10 et C57BL/6 sont comparativement plus résistantes. Dans ce travail, nous avons utilisé les souris BALB/c et C57BL/6. La susceptibilité des souris BALB/c se manifeste notamment par une charge bactérienne dans la rate et le foie 10 fois

plus élevée que celle observée chez les souris C57BL/6. Elle est associée à une splénomégalie plus importante et une production moindre d'IFN-γ (Copin *et al.*, 2007; Murphy *et al.*, 2001b), la cytokine clé de la réponse Th1 qui est développée contre *Brucella*.

Plusieurs hypothèses ont été avancées pour expliquer la susceptibilité des souris BALB/c. Parmi celles-ci, on notera le caractère naturellement biaisé de leur réponse immunitaire qui serait une réponse pro-Th2 (Hemmers & Mowen, 2009), inapte à contrôler l'infection par une bactérie intracellulaire telle que *Brucella*. Cette particularité avait été démontrée dans le modèle *Leishmania*, notamment par l'utilisation de souris BALB/c rendues génétiquement déficientes pour la réponse de type Th2 (Radwanska *et al.*, 2007; Stamm *et al.*, 1998). Chez ces animaux, une meilleure réponse Th1 était observée et corrélait avec un meilleur contrôle de l'infection parasitaire. Cependant, dans ce modèle, l'activation d'une réponse Th2 avait pu être clairement mise en évidence chez les souris BALB/c infectées (Locksley *et al.*, 1987; Locksley *et al.*, 1991).

Lors de l'infection de souris BALB/c par *B. abortus* 2308, bien que la neutralisation de l'IL-4 par des anticorps monoclonaux diminue la charge bactérienne, aucune réponse de type Th2 n'a pu être clairement identifiée (Fernandes *et al.*, 1996). Dès lors, le biais de réponse immunitaire observé chez les souris BALB/c infectées par le parasite *Leishmania* ne serait pas applicable lors d'une infection par *Brucella*.

En 2006, il a été suggéré que les souris BALB/c infectées par *B. abortus* 2308 étaient capables de produire une réponse Th1, mais que celle-ci étaient amoindrie suite à une diminution transitoire de l'expression du récepteur à l'IL-12 (IL-12Rβ2) (Sathiyaseelan *et al.*, 2006). Dans d'autres modèles, cette diminution a toutefois été attribuée à une production précoce d'IL-4 (Guler *et al.*, 1997; Launois *et al.*, 1997).

Par ailleurs, Murphy *et al* ont mis en évidence, lors d'une restimulation *in vitro,* l'absence de production d'IFN-γ à un point précis de la cinétique d'infection par *B. abortus* 2308, à savoir 3 semaines (Murphy *et al.*, 2001b). Le même groupe de recherche a montré quelques années plus tard que l'administration d'IL-12 recombinant (IL-12r) restaurait cette production d'IFN-γ chez les souris BALB/c infectées (Sathiyaseelan *et al.*, 2006). Sachant que l'IL-12Rβ2 requière l'IL-12 et l'IFN-γ pour son expression et sa maintenance (Smeltz *et al.*, 2002), l'administration d'IL-12r assurerait le maintien de cette expression et, par là même, le phénotype Th1 de la réponse immunitaire.

5.1.3 Modes d'infection expérimentaux

Loin d'être le mode d'infection le plus proche de la contamination naturelle, la voie intra-péritonéale (i.p.) est néanmoins la méthode d'infection expérimentale la plus couramment utilisée. Elle induit une rapide distribution systémique de *Brucella* dans l'organisme et assure une infection persistante des organes tels que la rate et le foie (Stevens *et al.*, 1994). Techniquement simple à maîtriser, la voie i.p. garantit une grande sécurité aux chercheurs. De plus ce mode d'infection permet l'utilisation de plus grands volumes d'inoculum et est donc moins sujet aux erreurs d'injection.

La cavité péritonéale, et après ?

La cavité péritonéale contient par définition les viscères et est délimitée par une membrane séreuse, le péritoine. Sous le péritoine, on retrouve un tissu riche en vaisseaux sanguins et lymphatiques. Cette propriété fait du péritoine un lieu d'échanges important et assure le drainage des substances présentes dans la cavité péritonéale. Plus particulièrement, l'existence de stomates au sein du péritoine recouvrant le diaphragme fournit une route directe entre la cavité péritonéale et les vaisseaux lymphatiques. Ces vaisseaux lymphatiques se déversent ensuite dans le flux sanguin pour finalement atteindre les ganglions lymphatiques médiastinaux (Abu-Hijleh *et al.*, 1995).
Ce trajet avait déjà été mis en évidence par une précédente étude en modèle murin (Marco *et al.*, 1992) où les auteurs avaient injecté par voie i.p. de l'encre de chine ou la bactérie *Listeria monocytogenes*. Ils avaient alors démontré que le drainage lymphatique de la cavité péritonéale était principalement réalisé via les ganglions lymphatiques médiastinaux avant une dissémination systémique dans l'organisme. L'étude n'excluait toutefois pas la possibilité que *L. monocytogenes* circule librement dans la lymphe sans être nécessairement phagocytée par des macrophages péritonéaux. Cela implique que ces bactéries pourraient atteindre facilement la circulation sanguine, ce qui expliquerait leur présence rapide dans le foie et la rate (10 minutes après injection). Il est à noter que *B. melitensis* est également présente dans la rate et le foie 10 minutes après injection i.p. (Copin *et al.*, 2012).
Outre cette fonction de résorption, la cavité péritonéale joue un rôle immunitaire non négligeable. En effet, elle contient des macrophages et des lymphocytes T et B (Composto *et al.*, 2010), notamment la classe de lymphocytes B-1 responsables de la production d'anticorps naturels (Boes, 2000).

La voie intra-veineuse (i.v., généralement dans la veine caudale) ou la voie sous-cutanée (s.c., dans le dos ou le coussinet plantaire) sont deux autres méthodes utilisées, chacune induisant une colonisation plus (i.v.) ou moins (s.c.) rapide des organes de l'animal (Pardon & Marly, 1976; Pardon & Marly, 1978).

Enfin, la voie intra-nasale (i.n.) apparaît comme la plus représentative de l'infection humaine par aérosol. Procédure risquée pour l'expérimentateur, elle apporte toutefois des données cruciales sur l'infection des poumons, des ganglions para-thymiques et le déroulement de la réponse immunitaire induite (Archambaud *et al.*, 2010; Mense *et al.*, 2001).

5.1.4 Déroulement de l'infection par voie intrapéritonéale dans le modèle murin

Après injection d'une dose de $4x10^4$ CFU de *Brucella* par voie i.p., les organes du système réticulo-endothélial tels que la rate et le foie, sont rapidement infectés (**figure 12**). Toutefois, l'infection ne persiste à des taux mesurables que dans la rate, qui est dès lors l'organe préférentiel pour suivre l'évolution de la charge bactérienne. Selon le profil de réplication de *Brucella* dans cet organe, on distingue généralement 4 phases (Copin *et al.*, 2007; Grillo *et al.*, 2012) :

- **Phase de colonisation** (les 2 premiers jours), caractérisée par une réponse pro-inflammatoire très faible, en comparaison à ce qui peut être observé chez d'autres pathogènes tels que *Salmonella* (Barquero-Calvo *et al.*, 2007)
- **Phase aigüe** (jusqu'au $20^{ème}$ jour), qui correspond au pic d'infection (atteint vers le $5^{ème}$ jour dans la rate (Copin *et al.*, 2007)), avec pour conséquences une splénomégalie, l'apparition de granulomes (Copin *et al.*, 2012; Hort *et al.*, 2003), corrélée avec entre autres avec la détection de cellules IFN-γ^+ et $iNOS^+$. Durant la première semaine d'infection, l'IFN-γ est produit par les cellules NK, les lymphocytes T $CD4^+$ et T $CD8^+$, mais seuls les lymphocytes T $CD4^+$ maintiennent cette production par la suite (Copin *et al.*, 2007). Les principales cellules productrices d'iNOS sont des cellules dendritiques inflammatoires (Copin *et al.*, 2007). Parallèlement les lymphocytes B commencent à produire des anticorps

spécifiques de *Brucella*, majoritairement des IgM et un peu plus tardivement des IgG3 et IgG2a (Elzer *et al.*, 1994; Goenka *et al.*, 2011).

- **Phase plateau** (jusqu'à la 8ème ou 11ème semaine d'infection), caractérisée par un maintien de la charge bactérienne. Durant cette période, on notera toutefois une nette diminution de l'inflammation, associée à une réduction de la splénomégalie.
- **Phase chronique,** caractérisée par un déclin lent de la charge bactérienne (peut se prolonger au-delà de la 36ème semaine d'infection). De manière consistante avec la brucellose humaine, la persistance de l'infection chez les souris se caractérise par une résurgence continue des bactéries dans plusieurs organes, dont la rate (**figure 13**). Malgré l'apparente absence de réponse immunitaire durant cette période, les cellules de la rate maintiennent leur capacité de réponse lors d'une stimulation, ce qui démontre la mise en place d'une mémoire immunologique de longue durée (Kahl-McDonagh & Ficht, 2006; Stevens *et al.*, 1994).

Le profil et la durée des différentes phases de l'infection ainsi que la splénomégalie sont dépendantes de la dose d'infection, des souris et des souches bactériennes utilisées (Grillo *et al.*, 2012). Ainsi l'utilisation d'une dose sub-létale d'infection (> $2x10^8$ CFU/souris) induit une saturation de la rate à un point tel qu'aucun pic de CFU n'est observable et que près de 50% des animaux décèdent dans les 2 jours suivant l'infection (Barquero-Calvo *et al.*, 2007). La dose optimale d'infection par voie i.p. se situe entre 10^4 et $5x10^6$ CFU/souris (Grillo *et al.*, 2012).

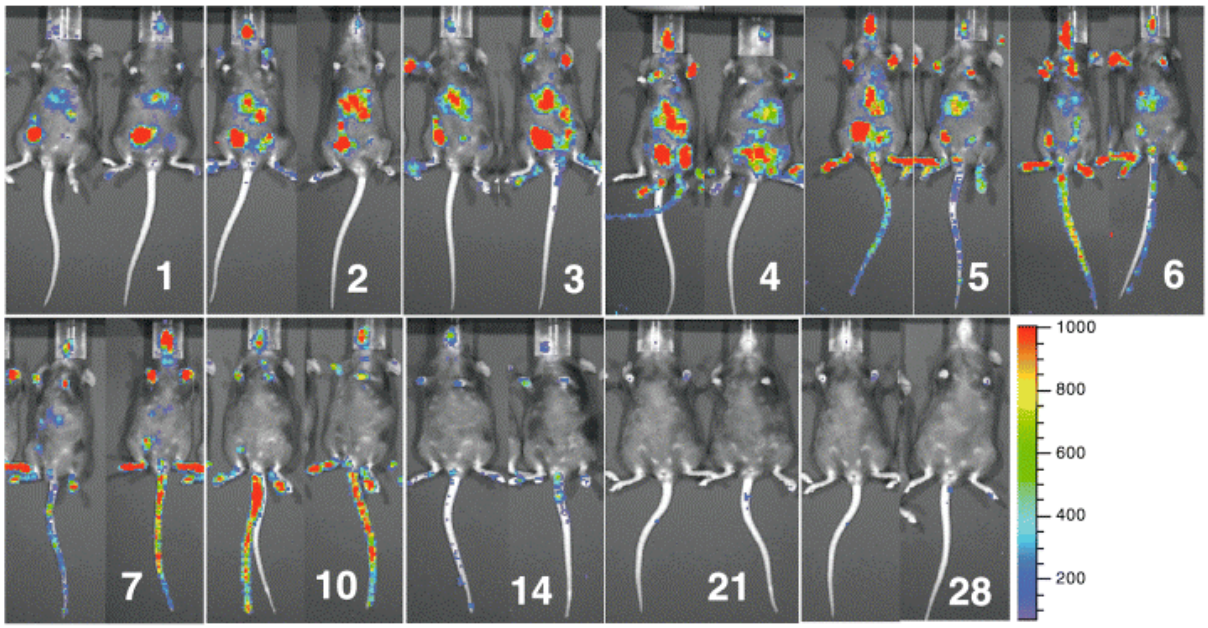


Figure 12. Déroulement de l'infection chez les souris C57BL/6 après injection i.p. de la souche bioluminescente *B. melitensis* GR023 (1x10^7 CFU) (Rajashekara *et al.*, 2005). Les chiffres en bas des images indiquent les jours post-infection. L'échelle de couleurs représente le nombre approximatif de photons. Après injection, une localisation systémique de *B. melitensis* est observée, touchant particulièrement la rate et le foie, mais également les régions submandibulaires et génitales. Au fil du temps, l'infection persiste principalement au niveau des extrémités (queue, pattes), témoignant la préférence de ce pathogène pour les zones articulaires et de basse température.

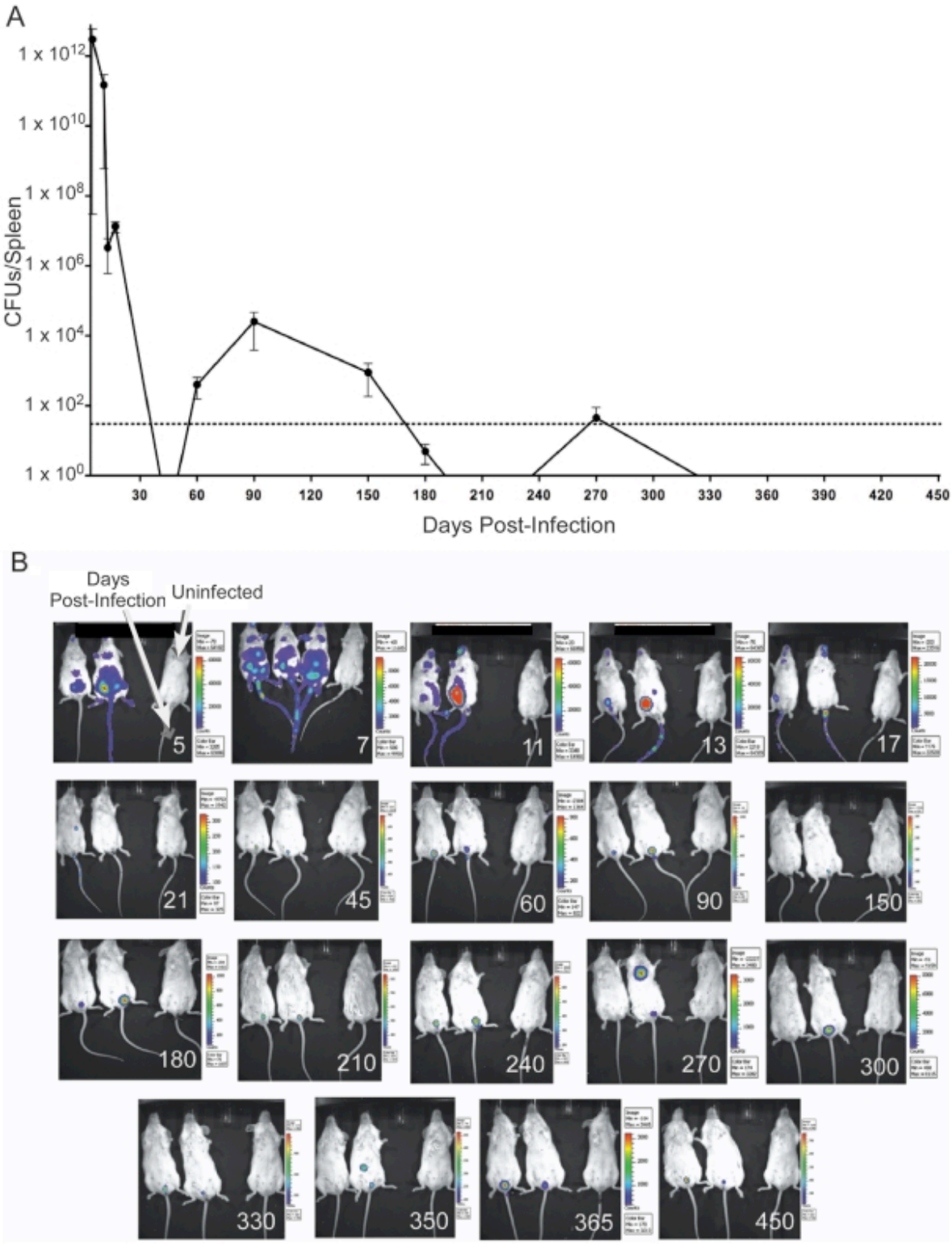


Figure 13. Persistance de l'infection chez les souris BALB/c après injection i.p. de la souche bioluminescente *B. melitensis* GR023 (1x10^7 CFU) (Durward *et al.*, 2012)**. A**, CFUs par rate. **B**, Monitoring de l'infection par bioluminescence. Les chiffres en bas des images indiquent les jours post-infection. L'échelle de couleurs représente le nombre approximatif de photons. L'infection chronique de *B. melitensis* se caractérise par une valeur de CFUs qui varie périodiquement au cours du temps, passant au-dessus ou en-dessous du niveau de détection. La résurgence de l'infection se traduit par l'apparition de « hotspots » au niveau des zones uro-génitales mais également à d'autres localisations.

5.2 Réponse immunitaire innée

Le SI est capable de contrôler l'infection par *Brucella*, mais son incapacité à éliminer cette bactérie est liée aux nombreuses stratégies qu'elle met en place pour échapper aux différents mécanismes effecteurs du SI inné. En comparaison avec d'autres bactéries à Gram négatif (*Salmonella*, *Escherichia coli*, etc.), *Brucella* est donc souvent décrite comme une bactérie furtive (Barquero-Calvo *et al.*, 2007; Gorvel, 2008; Letesson *et al.*, 2002; Martirosyan *et al.*, 2011), ce qui témoigne de son adaptation à l'environnement intracellulaire et de ses propriétés particulièrement peu immuno-stimulantes (**figure 14**).

5.2.1 La détection de Brucella : TLRs et MyD88

La reconnaissance de *Brucella* par le système immunitaire serait principalement dépendante du Toll Like Receptor (TLR)-9 (Copin *et al.*, 2007; Huang *et al.*, 2005; Macedo *et al.*, 2008), dont la déficience en modèle murin augmente la susceptibilité à l'infection et se traduit par une réduction de la production d'IL-12 par les macrophages et les DCs (Macedo *et al.*, 2008) et une réduction de la fréquence de cellules IFN-γ^+ et iNOS$^+$ (Copin *et al.*, 2007). Une action conjointe du TLR4 et TLR9 a également été proposée, car les souris TLR4$^{-/-}$ présentent elles aussi une légère susceptibilité à l'infection et une moindre fréquence de cellules IFN-γ^+ et iNOS$^+$ (Copin *et al.*, 2007) (voir **Appendix - 1**).

Reconnu par le TLR4, le lipopolysaccharide (LPS) est un constituant majeur de la membrane externe chez les bactéries à Gram négatif. Sa détection stimule les neutrophiles, les monocytes, les macrophages et d'autres types cellulaires à exprimer des molécules d'adhésion et à produire une variété de cytokines pro-inflammatoires (Morrison & Ryan, 1987). Le LPS de *Brucella* fait défaut à cette description, car sa structure particulière (**figure 15**) lui confère une faible endotoxicité (Barquero-Calvo *et al.*, 2007; Conde-Alvarez *et al.*, 2012; Lapaque *et al.*, 2006; Moreno *et al.*, 1981; Rasool *et al.*, 1992) et permet à *Brucella* de résister au complément (Eisenschenk *et al.*, 1999) et aux peptides cationiques (Freer *et al.*, 1996; Martinez de Tejada *et al.*, 1995). Les résultats concernant l'implication du TLR4 dans la détection de *Brucella* font donc encore actuellement l'objet de controverses (Barquero-Calvo *et al.*, 2007; Campos *et al.*, 2004; Conde-Alvarez *et al.*, 2012; Copin *et al.*, 2007; Pei *et al.*, 2012; Weiss *et al.*, 2005).

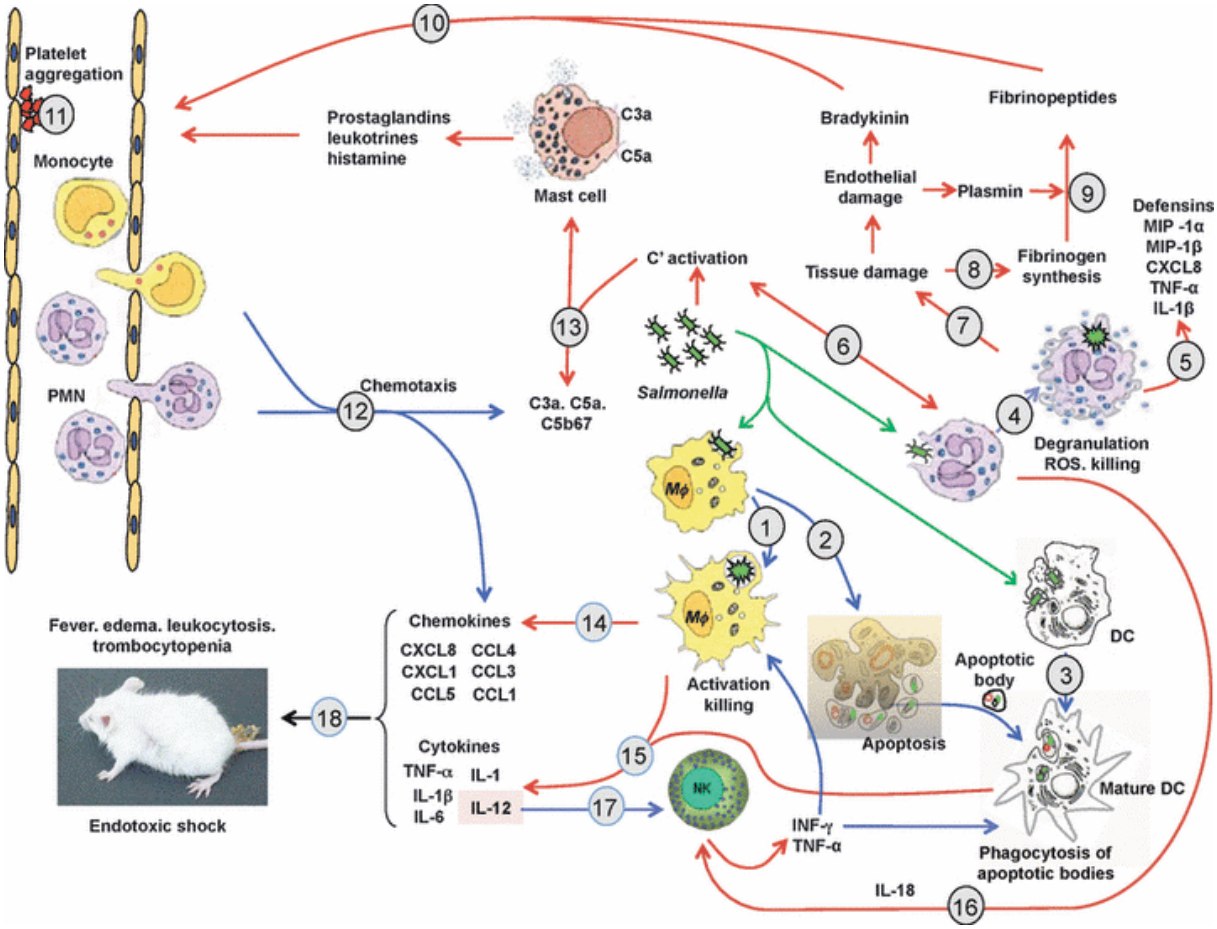


Figure 14. *Brucella*, une bactérie particulièrement peu pro-inflammatoire (d'après (Martirosyan *et al.*, 2011)). Chez la souris, les mécanismes pro-inflammatoires induits dès les premières heures suivant l'infection par *Salmonella* (flèches) ne sont pas activés par *Brucella* (ronds gris). Après phagocytose, *Brucella* n'induit pas l'activation des macrophages (Mϕ) (1) et protège ces cellules de l'apoptose (2). Les cellules dendritiques (DCs), qui ont également internalisé *Brucella*, ne subissent pas de processus de maturation (3). Les leucocytes polymorphonucléaires (PMNs) ne libèrent pas leurs granules cytoplasmiques (4), et par conséquent, les médiateurs pro-inflammatoires qu'ils contiennent (5). *Brucella* n'active pas la cascade du complément, que cela soit de manière directe ou indirecte, via les granulocytes activés (6). Peu de dommages tissulaires sont donc engendrés (7). L'absence d'activation des leucocytes et du complément compromet la synthèse des protéines pro-inflammatoires du foie, comme le fibrinogène (8), et la production de fibrinopeptides (9) qui stimulent la perméabilisation des vaisseaux sanguins (10). Par conséquent, l'infection par *Brucella* n'induit pas de changements significatifs de la formule sanguine et les coagulopathies sont rares durant la période d'incubation (11). L'infection par *Brucella* n'induit qu'un faible recrutement de cellules pro-inflammatoires sur le site d'infection, ceci est sans doute lié à l'absence de facteurs chimioattractants tels que les chimiokines produites par les cellules (12) et les composants du complément (13). Parmi les caractéristiques remarquables de la brucellose, on peut citer la faible quantité de chimiokines et cytokines produites par les Mϕs et les DCs infectés (14, 15), la faible quantité de médiateurs pro-inflammatoires sécrétés par les PMNs (16), ainsi que l'implication limitée des cellules Natural Killer (NK) (17). La conséquence finale de ce faible niveau d'activation du SI inné est l'absence des symptômes typiques du choc endotoxique observé lors d'infections par d'autres bactéries à Gram négatif, comme *Salmonella* (18), ce qui est en accord avec les observations cliniques chez l'homme et l'hôte naturel.

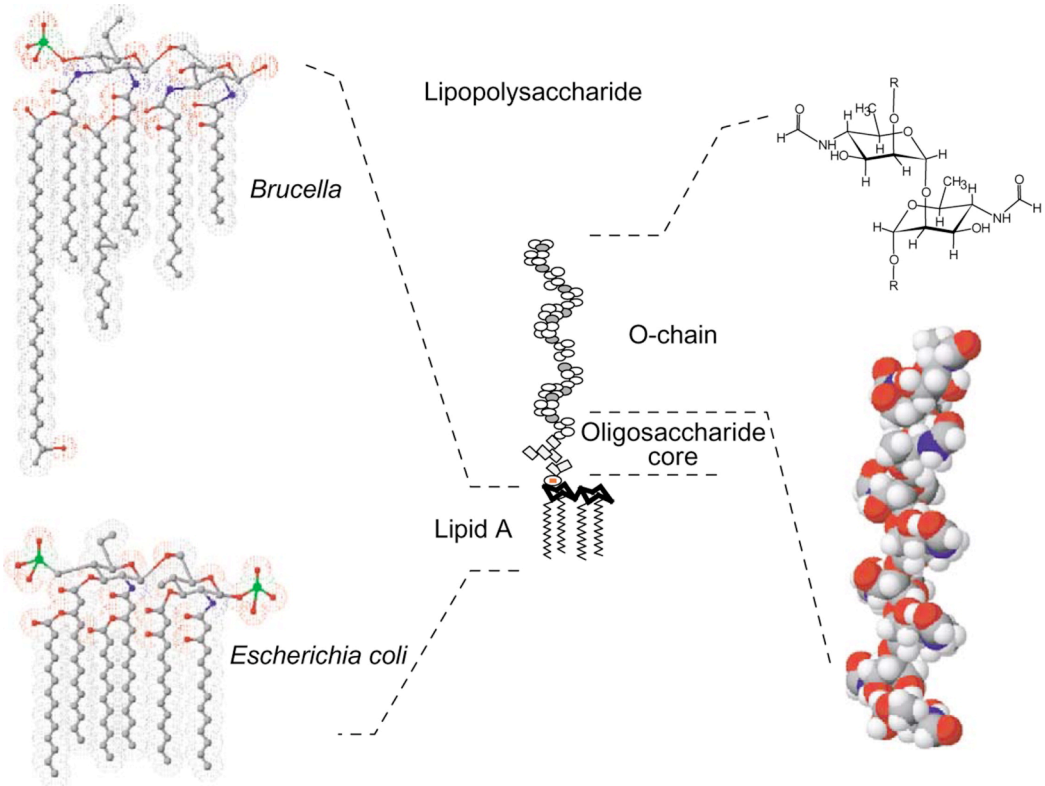


Figure 15. Le LPS de *Brucella*, une couverture anti-feu (d'après (Lapaque *et al.*, 2005)). Le LPS de *Brucella* est constitué d'une chaîne O polysaccharidique (qui confère le phénotype « lisse » de la souche, par opposition aux souches « rugueuses » dépourvues de chaîne O), d'un corps (oligosaccharide) et d'un lipide A, ce dernier étant considéré comme le composant endotoxique par excellence chez les autres bactéries à Gram négatif. Le faible potentiel immuno-stimulant du LPS provient (i) d'une chaîne O plus longue qui masque les groupements anioniques du lipide A (cibles des peptides cationiques) et bloque l'accès au complément, (ii) d'un core d'oligosaccharide branché (Conde-Alvarez *et al.*, 2012) et (iii) d'un lipide A présentant un nombre réduit de groupements phosphates (Martinez de Tejada *et al.*, 1995) et caractérisé par un squelette de diaminoglucose (au lieu de glucosamine) et par des chaînes acylées plus longues (C18-C19, C28) que ce qui est observé chez *Escherichia coli*.

Tout comme le LPS, la flagelline de *Brucella* est modifiée et échappe à la détection par le TLR5 (Andersen-Nissen *et al.*, 2005; Lapaque *et al.*, 2009; Terwagne *et al.*, 2012). Cependant une récente étude a démontré que la flagelline est détectée par d'autres PRRs présents dans le cytosol et que cette détection est essentielle pour limiter la persistance de *Brucella* en modèle murin (Terwagne *et al.*, 2012).

L'utilisation de *Brucella* tuée par la chaleur (Heat-Killed, HK) a mis en évidence d'autres voies de détection, via des PAMPs sans doute dévoilés par le traitement à la chaleur.

Giambartolomei *et al* a ainsi démontré que des formes lipidées des Omp (Outer Membrane Protein) de *B. abortus* 2308, purifiées après expression en *E. coli*, induisent la production de médiateurs pro-inflammatoires et sont détectées par le TLR2 (Giambartolomei *et al.*, 2004). Une autre étude a également démontré que *B. abortus* HK (HKBA) induit la production de TNF-α via une voie dépendante du TLR2 tandis que la production d'IL-12p40 dépendrait quant à elle du TLR9 (Huang *et al.*, 2003).

Enfin, *Brucella* pourrait être détectée par d'autres récepteurs intracellulaires, encore inconnus à ce jour. En effet, une étude a démontré que la réplication de *Brucella abortus* en macrophages murins induit la production des protéines Irgm3 et Irga6[1], via une voie dépendante de MyD88 mais indépendante des TLR2, 4, 5 et 9 (Lapaque *et al.*, 2009). Il est à noter que l'implication de ces protéines dans la persistance de *B. abortus* 2308 *in vivo* a récemment été démontrée (Ritchie *et al.*, 2012).

Le rôle crucial de la molécule adaptatrice MyD88 participant à la cascade de signalisation des TLRs a été démontré à maintes reprises (voir **Appendix - 1**) (Copin *et al.*, 2007; Copin *et al.*, 2012; Huang *et al.*, 2003; Macedo *et al.*, 2008; Pei *et al.*, 2012; Weiss *et al.*, 2005). Chez les souris C57BL/6 déficientes pour cette protéine, on note une susceptibilité élevée accompagnée d'une altération de la production d'IFN-γ et d'iNOS (Copin *et al.*, 2007). D'autres études ont également démontré un rôle de MyD88 dans la maturation des DCs ainsi que dans l'induction d'IL-12 et de TNF-α lors d'une stimulation par du HKBA (Huang *et al.*, 2003; Macedo *et al.*, 2008). La susceptibilité des souris MyD88$^{-/-}$ semble donc liée à la maturation compromise des DCs mais également à l'absence d'IL-12 car l'administration d'IL-12r à ces animaux améliore quelque peu leur résistance à l'infection (Macedo *et al.*, 2008). La réponse Th1 contre *Brucella* semble donc régulée par la voie de signalisation dépendante de MyD88.

[1] Protéines appartenant à la famille des IRG (Immune-Related GTPases), il s'agit de médiateurs anti-microbiens induits par l'IFN-γ et impliqués dans la résistance envers les pathogènes intracellulaires. Après la phagocytose du pathogène, ces protéines sont recrutées au niveau des membranes du phagosome. Elles stimulent la maturation du phagosome et accélèrent sa fusion avec le lysosome, contribuant de la sorte au contrôle de la multiplication bactérienne (Taylor *et al.*, 2004).

5.2.2 L'activation des DCs – manipulation du système

Avec *Coxiella burnetii* (Shannon *et al.*, 2005) et *Francisella tularensis* (Ben Nasr *et al.*, 2006), *Brucella* fait partie des rares bactéries capables d'infecter et de proliférer au sein des cellules dendritiques sans induire leur maturation (Billard *et al.*, 2005; Salcedo *et al.*, 2008). Tout comme *Coxiella*, *Brucella* utiliserait cette capacité à son avantage afin d'échapper à la réponse immunitaire (Shannon *et al.*, 2005). Les études de Billard *et al* ont en effet démontré *in vitro* que les cellules dendritiques humaines infectées par *B. suis* sont bloquées dans leur processus de maturation et de présentation des antigènes. La production d'IL-12 et de TNF-α est également inhibée chez ces cellules, ce qui affecte drastiquement l'activation des lymphocytes T (Billard *et al.*, 2007b). Cette immunomodulation serait réalisée par l'intermédiaire de la chaîne O du LPS de *Brucella* (Billard *et al.*, 2007a) et de l'Omp 25 (Billard *et al.*, 2007b). Toutefois une récente étude réalisée dans notre laboratoire ((Copin *et al.*, 2012), voir **Appendix-3**) et menée *in vivo* en modèle murin nuance les résultats de Billard *et al*. Il a en effet été démontré que, dès les 12 premières heures suivant l'infection par une haute dose de *B. melitensis*, les DCs représentent 10 à 30% des cellules infectées dans la rate. De plus l'infection de la rate induit une massive relocalisation et une maturation des DCs. Toutefois, en accord avec les études de Billard *et al*, ce processus de maturation ne semble pas concerner les DCs infectées. Il serait initié par l'inflammation dépendante de la voie MyD88 et ne nécessite pas de phénomène infectieux puisqu'on l'observe aussi chez les souris inoculées avec *B. melitensis* HK (Copin *et al.*, 2012).

D'autres études ont montré que *Brucella* pouvait interférer avec la maturation des DCs via la production d'une protéine contenant un domaine TIR (Toll/interleukin-1 receptor) et nommée TcpB ou Btp1 (Salcedo *et al.*, 2008). Les protéines à domaine TIR jouent un rôle essentiel dans l'induction de la réponse immunitaire innée, car elles sont des composants importants dans les voies de signalisation intracellulaires dépendantes des TLRs et menant à l'activation de NF-κB. Lorsqu'elle est exprimée en cellules eucaryotes, TcpB induit la dégradation de MAL/TIRAP (MyD88-adapter-like/TIR domain-containing adaptor protein), bloquant de la sorte l'expression du gène NF-κB induite par la voie de signalisation des TLR2 et TLR4 (Chaudhary *et al.*, 2012; Radhakrishnan *et al.*, 2009; Sengupta *et al.*, 2010). Une récente étude a démontré *in vitro* que la protéine TcpB pouvait également altérer la réponse

adaptative lors d'une infection par *B. melitensis,* en inhibant les fonctions cytotoxiques des lymphocytes T $CD8^+$ (Durward *et al.*, 2012).

5.2.3 La réponse innée effectrice anti-Brucella - les armes du SI

IFN-γ et iNOS

La réponse protectrice anti-*Brucella* est principalement dépendante de l'IFN-γ (Baldwin & Parent, 2002; Murphy *et al.*, 2001b; Stevens *et al.*, 1992). Seule l'absence de cette cytokine est fatale pour les souris infectées, tant C57BL/6 que BALB/c (voir **Appendix-1**) (Baldwin & Parent, 2002; Murphy *et al.*, 2001b). La déficience d'aucun autre effecteur immunitaire, ni de la voie TLR/MyD88/IL-12 n'a jusqu'ici eu un tel impact sur la survie des animaux infectés. L'administration d'IFN-γ recombinant aux souris BALB/c augmente leur résistance à l'infection par *B. abortus* 2308 (Stevens *et al.*, 1992), tandis que la neutralisation de cette cytokine augmente drastiquement leur susceptibilité (Fernandes & Baldwin, 1995; Fernandes *et al.*, 1996; Zhan & Cheers, 1993) (voir **Appendix-1**). L'IFN-γ est produit dès la première semaine suivant l'infection. À 5 jours post-infection, cette production est assurée par les cellules NK, les lymphocytes T $CD4^+$ et T $CD8^+$. Plus tardivement, seuls les lymphocytes T $CD4^+$ conservent la capacité de produire l'IFN-γ (Copin *et al.*, 2007). Le rôle des lymphocytes T dans le contrôle de l'infection sera abordé plus loin dans ce manuscript.

Bien que les cellules NK soient activées et produisent de l'IFN-γ, elles ne semblent pas avoir de rôle majeur dans le contrôle initial de l'infection par *B. abortus* 2308 en modèle murin (Fernandes *et al.*, 1995). Une récente étude a toutefois suggéré un rôle de ces cellules dans la régulation des lymphocytes B (Gao *et al.*, 2011). Les auteurs ont rapporté que, dans le cadre d'une immunisation par *B. abortus* HK (HKBA), la déplétion des cellules NK affectait drastiquement la production d'IgG2c. Ils proposent que cette régulation puisse impliquer un contact direct entre les cellules NK et les lymphocytes B. Enfin, contrairement à ce qui est observé en modèle murin, l'action cytotoxique des cellules NK a pu être mise en évidence *in vitro* sur des macrophages humains infectés par *B. suis* (Dornand *et al.*, 2004).

L'une des principales fonctions de l'IFN-γ serait d'activer les mécanismes bactéricides des cellules phagocytaires (Jiang & Baldwin, 1993; MacMicking *et al.*, 1997). En 2002, une

étude a démontré que les souris gp91phox$^{-/-}$, déficientes pour la NADPH oxydase produisant les dérivés toxiques de l'oxygène, et les souris iNOS/NOS2$^{-/-}$ étaient susceptibles à l'infection par *B. abortus*, mais que l'absence d'oxyde nitrique (NO) affectait plus sévèrement la résistance des souris (Ko *et al.*, 2002b). En 2007, notre laboratoire a mis en évidence que la production de NO dépendait directement de la présence d'IFN-γ. Cette production de NO est majoritairement réalisée par des cellules dendritiques inflammatoires (CD11b^{+}CD11c^{+}LY-6C^{+}MHCII^{+}) également nommées TNF iNOS producing-DCs (Tip-DCs) (Copin *et al.*, 2007).

Granulomes

Parmi les armes du système immunitaire utilisées contre *Brucella*, la formation de granulomes apparaît comme un point essentiel de défense (Copin *et al.*, 2012; Enright *et al.*, 1990; Hort *et al.*, 2003; Jimenez de Bagues *et al.*, 2011; Spink, 1956). Conséquences d'une réponse Th1 et souvent décrits lors d'infections chroniques telles que celle induite par *Mycobacterium tuberculosis* (Ramakrishnan, 2012), les granulomes sont des structures cellulaires et fibreuses très organisées, composées principalement de monocytes activés, eux-mêmes entourés de lymphocytes T et de granulocytes. Leur formation est initiée par les cellules dendritiques (Kang *et al.*, 2008) qui induisent le recrutement des autres cellules. La fonction principale du granulome serait de contenir et d'isoler l'infection. Néanmoins, il a été observé dans plusieurs modèles que le granulome pouvait également être exploité par le pathogène pour assurer sa dissémination (Davis & Ramakrishnan, 2009).

Notre laboratoire a récemment étudié *in situ* la formation des granulomes dans la rate de souris infectées par *B. melitensis* ((Copin *et al.*, 2012), voir **Appendix-3**). Les granulomes sont observés dans la pulpe rouge dès le 5ème jour d'infection et sont composés de monocytes matures activés (CD11b^{+} CD11c^{-} F4/80^{+} MHCII^{+}) et de DCs inflammatoires (CD11b^{+} CD11c^{+} F4/80^{-} MHCII^{+} iNOS^{+}), entourés de lymphocytes T (CD90.2^{+}) et de granulocytes (CD11b^{+} Ly-6G^{+}). Leur présence est dépendante de la réponse Th1 car l'absence de MyD88, d'IL-12 ou d'IFN-γ altère fortement la formation et la composition de ces granulomes et est associée à une susceptibilité élevée à l'infection. Toutefois, leur formation se produit chez les souris RAG$^{-/-}$, dépourvues de lymphocytes T et B, sans doute grâce au maintien transitoire de la production d'IFN-γ par les cellules NK. Ces granulomes sont cependant moins organisés et moins denses (Copin *et al.*, 2012).

TNF-α et IL-12

Le TNF-α et l'IL-12 sont deux cytokines clés de la réponse immunitaire anti-*Brucella* dont le rôle est étroitement lié à celui de l'IFN-γ.

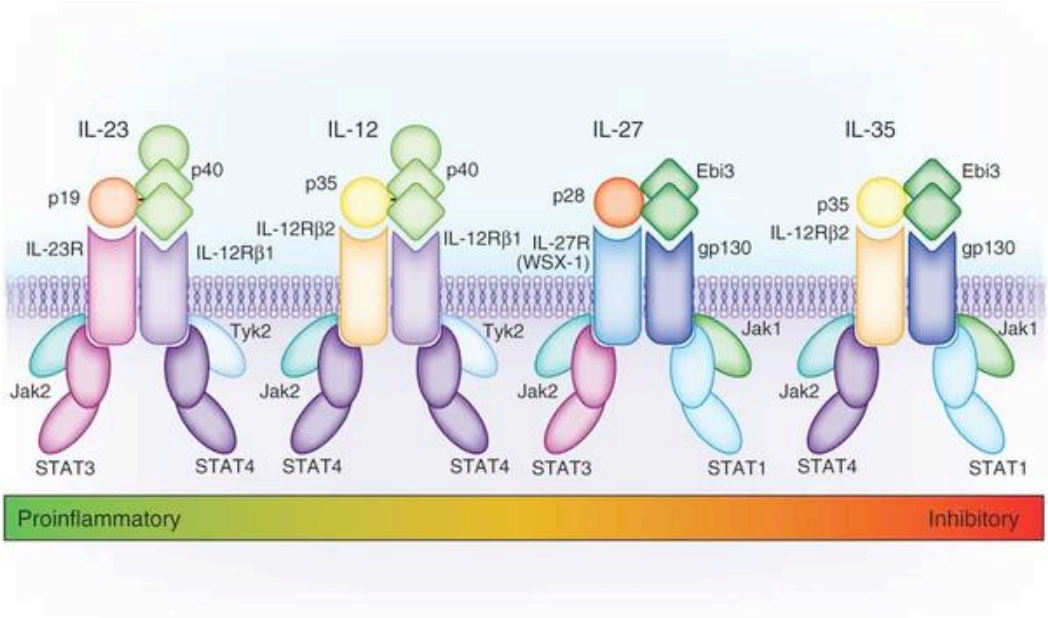
L'implication du TNF-α a été démontrée par des études *in vitro* (Jiang *et al.*, 1993) mais aussi *in vivo*, notamment par l'utilisation de souris $TNFR^{-/-}$ (voir **Appendix-1**) (Zhan & Cheers, 1998). L'absence du récepteur au TNF-α compromet la résistance des souris et affecte la production d'IL-12 et de NO par les macrophages, ce qui confirme l'implication du TNF-α dans l'activation des activités bactéricides des macrophages (Jiang *et al.*, 1993). Toutefois la production d'IFN-γ chez ces souris reste inchangée (Zhan & Cheers, 1998). Deux autres études menées *in vivo* et basées sur la neutralisation du TNF-α par des anticorps monoclonaux ont également révélé une exacerbation de l'infection chez les animaux traités (Murphy *et al.*, 2001a; Zhan *et al.*, 1996).

L'IL-12, dont la production serait partiellement sous le contrôle du TNF-α (Zhan & Cheers, 1998), apparaît elle aussi comme un facteur essentiel au contrôle de l'infection par *Brucella*. La neutralisation de l'IL-12 par des anticorps monoclonaux conduit à une exacerbation de l'infection par *B. abortus* S19 pendant plus de 6 semaines, accompagnée par une profonde altération de la production d'IFN-γ et de NO (Zhan *et al.*, 1995; Zhan *et al.*, 1996). La formation de granulomes dans le foie de ces animaux est également compromise (Zhan *et al.*, 1996). Les souris déficientes pour l'IL-12p40 sont incapables de contrôler l'infection par *B. abortus* 2308. Chez ces animaux, la charge bactérienne demeure élevée pendant au moins 4 semaines (voir **Appendix-1**) (Ko *et al.*, 2002b). Enfin, l'administration d'IL-12 recombinant aux souris BALB/c infectées par *B. abortus* 2308 permet une augmentation de leur résistance, et restitue la production d'IFN-γ lors d'une stimulation *in vitro* durant la période du hiatus (3 semaines post infection) décrit par Sathyaseelan *et al*. (Sathiyaseelan *et al.*, 2006).

*L'IL-12, une famille de cytokines impliquées dans la régulation des réponses des lymphocytes T (**figure 16**)*

L'IL-12 est un hétérodimère formé par une sous-unité p35 et une sous-unité p40. Cette cytokine était autrefois étroitement liée à la réponse de type Th1 (Magram *et al.*, 1996). Plusieurs cytokines hétérodimériques ont depuis lors été identifiées et, de par leur homologie de structure avec l'IL-12, ont été regroupées pour former la famille de l'IL-12. Ces cytokines exercent toutefois des fonctions distinctes.
La sous-unité p40 de l'IL-12 peut s'associer non seulement à l'IL-12p35 pour former l'IL-12p70 (réponse Th1), mais également à une autre molécule, nommée p19, pour former l'IL-23 (Oppmann *et al.*, 2000). L'IL-23 est impliquée dans la réponse Th17 et induit les mêmes voies de signalisation intracellulaire (Jak, STAT) que l'IL-12 mais différents complexes de liaison à l'ADN sont formés (Parham *et al.*, 2002), ce qui explique que les activités induites par les deux cytokines peuvent parfois paraître redondantes mais elles demeurent toutefois différentes.
Une autre cytokine, l'IL-27, est formée par l'association de EBI3 (Epstein-Barr virus-induced gene 3), une sous-unité structurellement proche de la sous-unité p40, avec la protéine p28. L'IL-27 initie la différenciation des lymphocytes Th1 et limite le développement des réponses Th2, Th17 et iTreg (Pflanz *et al.*, 2002).
La sous-unité p35 de l'IL-12 est également constitutive de l'IL-35, une cytokine récemment décrite qui supprime la prolifération des lymphocytes T et induit la différenciation des lymphocytes T naïfs en lymphocytes T regs producteurs d'IL-35 (iTr35) (Collison *et al.*, 2007).

Figure 16. Structure des cytokines de la famille de l'IL-12 et de leurs récepteurs (Vignali & Kuchroo, 2012).



5.3 Réponse immunitaire adaptative

La réponse adaptative joue un rôle essentiel dans le contrôle de l'infection par *Brucella*. Les études de transfert passifs d'anticorps ou de lymphocytes menées fin des années 1980 en modèle murin ont démontré que tant la réponse humorale que la réponse cellulaire exerçaient un rôle protecteur contre la brucellose (Araya *et al.*, 1989). Plus récemment, l'utilisation de souris $RAG^{-/-}$ dépourvues de lymphocytes B et T, a confirmé cette hypothèse (Copin *et al.*, 2012; Izadjoo *et al.*, 2000). La susceptibilité élevée observée chez ces souris lors d'une infection par *B. melitensis* par voie i.n. (Izadjoo *et al.*, 2000) ou par voie i.p. (Copin *et al.*, 2012) ne conduit cependant pas à une issue fatale, suggérant que des phénomènes compensatoires sont mis en place. La survie de ces souris pourrait partiellement s'expliquer par la présence de granulomes, dont la mise en place a pu être réalisée grâce à la production précoce d'IFN-γ par les cellules NK ((Copin *et al.*, 2012), voir **Appendix-3**).

5.3.1 Lymphocytes T

5.3.1.1 Lymphocytes T $CD4^+$ ou T $CD8^+$, histoire d'une controverse

De nombreuses études ont tenté de déterminer quelle(s) population(s) de lymphocytes T ($CD4^+$ ou $CD8^+$) est/sont protectrice(s) lors d'une infection par *Brucella*. Ce point est crucial pour la compréhension du cycle infectieux de *Brucella* et l'établissement de nouveaux vaccins protecteurs, notamment via l'identification du/des antigène(s) et voie(s) de présentation antigénique(s) impliqués dans une réponse immunitaire protectrice.

Dans le cas de la brucellose, l'implication des lymphocytes T $CD4^+$ et/ou $CD8^+$ est un sujet controversé, alimenté par la publication de nombreux résultats conflictuels.

En 1989, des études de transferts passifs ont démontré que les lymphocytes T $CD4^+$ et $CD8^+$ avaient tous deux un effet protecteur lors d'une infection par *B. abortus* S19 (Araya *et al.*, 1989). Depuis lors, les études menées sur les souris $MHC\text{-}II^{-/-}$ ont mis en évidence que la présence des lymphocytes T $CD4^+$ était essentielle (Goenka *et al.*, 2011) ou dispensable (Oliveira & Splitter, 1995) au contrôle de l'infection (voir **Appendix-1**). D'autres études

menées *in vitro* ont mis en évidence que le LPS de *Brucella* inhibait la présentation des antigènes en MHC-II, ce qui pourrait altérer la réponse des lymphocytes T $CD4^+$ (Barrionuevo *et al.*, 2008; Forestier *et al.*, 2000).

L'implication des lymphocytes T $CD8^+$ dans la résistance à l'infection a été démontrée par l'utilisation d'anticorps monoclonaux neutralisants anti-CD8α (Mielke, 1991; Murphy *et al.*, 2001a; Pavlov *et al.*, 1982) et de souris C57BL/6 β2-microglobuline$^{-/-}$ (Oliveira & Splitter, 1995). À l'opposé, les lymphocytes T $CD8^+$ ont récemment été associés à la persistance de *Brucella*. En effet, bien que légèrement susceptibles en début d'infection (1 semaine), les souris β2-microglobuline$^{-/-}$ éliminent plus rapidement les bactéries durant la phase plateau de l'infection (Goenka *et al.*, 2011; Murphy *et al.*, 2001b). Un résultat similaire a été observé chez les souris C57BL/6 perforine$^{-/-}$ (Murphy *et al.*, 2001b) (voir **Appendix - 1**). De plus, la récente étude de Durward *et al* (Durward *et al.*, 2012) a mis en évidence que les fonctions cytotoxiques des lymphocytes T $CD8^+$ étaient inhibées par la protéine TcpB de *B. melitensis*, réduisant ainsi la qualité de réponse de ces cellules sur le long terme.

La fonction des lymphocytes T $CD8^+$ pourrait être, dans un premier temps, de lyser les cellules infectées par *Brucella* afin de rendre les bactéries accessibles aux cellules phagocytaires activées, mais par la suite, le rôle de ces lymphocytes pourrait être limité, voire défavorable, de par leurs potentielles fonctions immuno-régulatrices (Baldwin & Goenka, 2006; Myers *et al.*, 2005).

Il est à noter que l'activation des lymphocytes T $CD8^+$ n'est pas la seule fonction effectrice affectée par la déficience en β2-microglobuline. En effet, cette protéine est également requise pour la formation de la molécule CD1d, qui permet la reconnaissance des antigènes lipidiques par les lymphocytes T natural killer (NKT). D'autres études ont également indiqué que l'absence de la β2-microglobuline se traduit par un catabolisme accéléré des IgG, affectant de la sorte la réponse anticorps (Christianson *et al.*, 1997; Kim *et al.*, 2008). Concrètement, les souris déficientes en β2-microglobuline sont déficientes en lymphocytes T $CD8^+$ et NKT, et présentent des taux circulants très faibles d'IgG. L'interprétation de leur susceptibilité à l'infection par *Brucella* est donc fort complexe.

5.3.1.2 Lymphocytes T et production d'IFN-γ

La production d'IFN-γ est souvent corrélée à la résistance à l'infection par *Brucella*, notamment suite aux études comparatives menées chez les souris BALB/c et C57BL/6 qui présentent des degrés de résistance différents associés à des fréquences différentes de cellules IFN-γ^+ (Copin *et al.*, 2007; Fernandes *et al.*, 1996; Murphy *et al.*, 2001b). L'effet protecteur observé lors des transferts de lymphocytes T serait en partie dépendant de l'IFN-γ car l'augmentation de résistance observée chez les souris receveuses est amoindrie par un traitement avec des anticorps monoclonaux anti-IFN-γ (Zhan & Cheers, 1993). Dès lors, le rôle des lymphocytes T dans la protection pourrait être de produire de l'IFN-γ.

Tant les lymphocytes T $CD4^+$ que les $CD8^+$ produisent de l'IFN-γ *in vitro* et *in vivo* (Baldwin & Goenka, 2006; Copin *et al.*, 2007; Fernandes *et al.*, 1996; Zhan *et al.*, 1996). Toutefois, Copin *et al* ont montré que les lymphocytes T $CD4^+$ constituaient la principale population produisant cette cytokine dès le 10ème jour d'infection par *B. melitensis* et au delà (74% des cellules IFN-γ^+ sont $CD4^+$). À 5 jours post infection, la fréquence de cellules IFN-γ^+ parmi les lymphocytes T $CD4^+$ et $CD8^+$ est comparable, cependant seuls les lymphocytes T $CD4^+$ voient cette fréquence augmenter durant l'infection (Copin *et al.*, 2007).

La récente étude de Goenka *et al,* menée sur des souris C57BL/6 infectées par *B. abortus* 2308, a confirmé l'importance des lymphocytes T $CD4^+$ par plusieurs observations (Goenka *et al.*, 2011) :

- l'intensité de fluorescence moyenne (mfi) d'IFN-γ est significativement plus importante chez les lymphocytes T $CD4^+$ que chez les $CD8^+$,
- les cellules spléniques prélevées chez les souris β2-microglobuline$^{-/-}$ infectées produisent la même quantité d'IFN-γ que celles prélevées chez des souris WT lors d'une restimulation *in vitro*, tandis que celles prélevées chez des souris Aβ$^{-/-}$ (MHC-II$^{-/-}$) sont incapables d'assurer ce niveau de production,
- l'absence de l'une ou l'autre population de lymphocytes T affecte différemment la résolution de l'infection : à 10 semaines post-infection, les souris β2-microglobuline$^{-/-}$ ont 90% de CFU en moins que les souris WT ou Aβ$^{-/-}$, tandis que les souris Aβ$^{-/-}$ contrôlent moins efficacement l'infection et présentent des symptômes pathologiques exacerbés (émaciement, léthargie).

5.3.1.3 Réponses des lymphocytes T non conventionels

Les lymphocytes T régulateurs $CD4^+CD25^+$ sont généralement considérés comme protecteurs car ils préservent l'organisme hôte des effets néfastes d'une réponse immunitaire exacerbée, mais leur implication potentielle dans les infections chroniques est cependant à prendre en considération (Taylor *et al.*, 2005). Une seule étude a démontré, par l'utilisation d'anticorps neutralisants anti-CD25, l'effet défavorable des lymphocytes T régulateurs sur le contrôle de l'infection par *B. abortus*, soulevant ainsi la possibilité de leur implication dans la persistance de *Brucella* (Pasquali *et al.*, 2010).

Les lymphocytes Tγδ peuvent jouer un rôle essentiel dans le contrôle des infections bactériennes, et il a été proposé que les souris T $\gamma\delta^{-/-}$ présentaient une réponse altérée aux bactériémies causées par les bactéries à Gram négatif comme *Klebsiella pneumoniae* (Moore *et al.*, 2000). Dans le cas de la brucellose humaine, un recrutement des lymphocytes Tγδ est observé dans le sang des patients infectés et ces cellules présentent une activité cytolytique envers les cellules infectées par *Brucella in vitro* (Bertotto *et al.*, 1993; Ottones *et al.*, 2000). Récemment, une étude a démontré un rôle protecteur de ces cellules en modèle murin et bovin (Skyberg *et al.*, 2011). Bien que ces cellules prolifèrent et produisent de l'IL-17 et une faible quantité d'IFN-γ suite à l'infection par *B. abortus*, leur implication apparaît très limitée dans le temps (Skyberg *et al.*, 2011).

L'implication des **lymphocytes iNKT** (invariant Natural Killer T cells) dans le contrôle de la brucellose humaine a également été rapportée (Bessoles *et al.*, 2009). Ces lymphocytes T reconnaissent les glycolipides présentés par le complexe CD1d et peuvent exprimer l'un ou l'autre co-récepteur CD4 ou CD8. L'activité anti-*Brucella* de ces cellules impliquerait principalement des mécanismes cytotoxiques contact-dépendants tels que l'induction de la voie Fas et la libération de granules lytiques (Bessoles *et al.*, 2009).

5.3.1.4 Sous classes de réponse immunitaire

Brucella est une bactérie intracellulaire et son infection induit, en accord avec le modèle Th1/Th2/Th17, une réponse dominante de type Th1. Les études comparatives menées en modèle murin avec les souris C57BL/6 et BALB/c ont alimenté un réel débat quant à une implication potentielle de la réponse Th2 chez les souris BALB/c (voir

paragraphe 5.1.2 ci-dessus). À ce jour, les éléments qui semblent affecter le plus drastiquement le contrôle de l'infection par *Brucella* sont étroitement liés à la réponse Th1 (voir **Appendix - 1**) :

- les souris IFN-$\gamma^{-/-}$ C57BL/6 et BALB/c ne survivent pas à l'infection par *B. abortus* 2308 au-delà de 6,5 et 10,5 semaines, respectivement (Brandao *et al.*, 2012; Murphy *et al.*, 2001b)
- les souris C57BL/6 IRF-$1^{-/-}$ (Interferon Regulatory Factor-1), déficientes pour la production d'IFN-γ, d'IL-12p40 et de TNF-α, l'induction d'iNOS, l'activation des lymphocytes T $CD8^+$ et des cellules NK, ne survivent pas à l'infection par *B. abortus* 2308 au-delà de 2 semaines. L'administration d'IL-12 recombinant chez ces animaux permet toutefois de prolonger modérément leur survie, suggérant un mécanisme de protection dépendant de l'IL-12 mais indépendant de l'IFN-γ (Ko *et al.*, 2002b)
- les souris IL-12p$40^{-/-}$ et ICSB$P^{-/-}$ (IFN Consensus Sequence Binding Protein, déficientes pour l'induction des ROI, IL-12p40 et IRF2) infectées par *B. abortus* 2308 présentent une charge bactérienne élevée qui se maintient, sans toutefois causer la mort des animaux (Ko *et al.*, 2002b)
- la voie de signalisation dépendante de MyD88, dont l'absence affecte la résistance des souris mais également la production d'IFN-γ et d'iNOS (Copin *et al.*, 2007), apparaît cruciale au contrôle de l'infection par *Brucella* (Copin *et al.*, 2007; Copin *et al.*, 2012; Huang *et al.*, 2003; Macedo *et al.*, 2008; Weiss *et al.*, 2005)

La réponse de type Th17 n'a été étudiée que très récemment dans le cadre d'une infection par *Brucella*, aucune donnée n'existait à ce sujet lorsque nous avons commencé nos études sur les réponses T helper. En 2012, Giambartolomei *et al* ont démontré en modèle murin que les lymphocytes T producteurs d'IL-17 étaient impliqués dans l'ostéoclastogénèse, un processus pathologique à l'origine des complications ostéo-articulaires décrites dans la brucellose humaine (Colmenero *et al.*, 1996; Giambartolomei *et al.*, 2012). Toutefois, l'absence d'IL-17Rα (Skyberg *et al.*, 2011) ou la neutralisation de l'IL-17A (Pasquevich *et al.*, 2011) ne semblent pas affecter le contrôle de l'infection par *B. abortus* 2308.

5.3.2 Lymphocytes B

5.3.2.1 Lymphocytes B : armés contre Brucella

La réponse humorale induite par *Brucella* en modèle murin est caractéristique de celle induite par des antigènes T-indépendants (Betts *et al.*, 1993; Kurtz & Berman, 1986). La production d'IgM spécifiques de la chaîne O du LPS prédomine durant les 2 premières semaines suivant l'infection et, parallèlement, la fréquence d'IgG augmente progressivement au cours des 3 premières semaines. Au-delà, les IgG3 et, dans une moindre mesure, les IgG2a, représentent les isotypes les plus abondants, tandis que les IgG1 et IgG2b sont présents en faibles quantités (**figure 17**) (Elzer *et al.*, 1994; Goenka *et al.*, 2011). Ce profil de réponse anticorps est typique d'une réponse Th1 et, comme l'ont montré Finkelman *et al*, est dépendant de l'IFN-γ (Finkelman *et al.*, 1988).

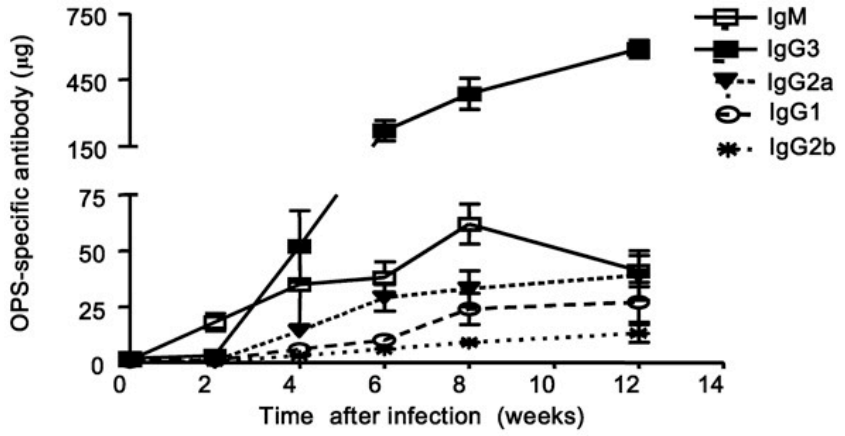


Figure 17. Production d'anticorps spécifiques du polysaccharide O (OPS) après infection de souris C57BL/6 avec *Brucella abortus* S2308 (5x10^4 CFU) (d'après (Goenka *et al.*, 2011)). n=5 ; les données sont représentées en tant que la moyenne +/- SE de 5 expériences.

Le principal mécanisme de protection lié à la présence d'anticorps spécifiques de *Brucella* serait l'opsonisation (Elzer *et al.*, 1994; Eze *et al.*, 2000; Winter *et al.*, 1989). Les IgM et IgG3 sont considérées comme des opsonines qui, une fois fixées à la surface des bactéries, augmentent leur phagocytose ainsi que l'activité bactéricide de la cellule phagocytaire. Ceci

serait réalisé par l'intermédiaire du récepteur Fc (FcR) (Elzer *et al.*, 1994; Enright *et al.*, 1990; Nimmerjahn & Ravetch, 2008). L'activation du complément via les anticorps n'apparaît pas comme un mécanisme protecteur essentiel contre la brucellose. En effet, plusieurs études ont montré que *Brucella* était résistante au complément (Eisenschenk *et al.*, 1999; Fernandez-Prada *et al.*, 2001).

L'implication des anticorps a notamment été démontrée par des études de transferts passifs de sérum immun ou d'anticorps monoclonaux spécifiques de *Brucella* (**Table 7**). L'efficacité de ces transferts était fortement dépendante de la souche de *Brucella* utilisée pour l'immunisation (Araya & Winter, 1990; Elzer *et al.*, 1994), du moment de prélèvement du sérum immun (Araya *et al.*, 1989) ou du type d'anticorps monoclonaux choisi (Elzer *et al.*, 1994; Montaraz *et al.*, 1986; Winter *et al.*, 1989).

Table 7. Impact du transfert passif d'anticorps dans la protection lors d'une infection par la souche virulente (S2308) ou atténuée (S19) de *Brucella abortus*, en modèle murin (d'après (Baldwin & Goenka, 2006)). OPS, Polysaccharide-O du LPS de *Brucella* ; p.i., post-infection

Souche de souris	Souche de *B. abortus*	Anticorps transférés	Résultats	Références
BALB/c	S2308	Anticorps monoclonaux spécifiques de l'OPS (IgG3, IgG2a)	Effet protecteur à 1 et 4 semaines p.i.	(Montaraz et al., 1986)
	S19	Sérum immun de souris donneuses infectées par S19	Protection significative lorsque les donneuses sont infectées depuis > 3 semaines	(Araya et al., 1989)
	S19 ou S2308	Sérum immun de souris donneuses infectées par S19 ou S2308	Le sérum immun provenant de donneuses infectées par S19 ou S2308 confère une meilleure protection contre S19 que contre S2308 (1 semaine p.i.).	(Araya & Winter, 1990)
	S19 ou S2308	Anticorps monoclonaux spécifiques de l'OPS (IgM, IgG3)	Les IgM confèrent une meilleure protection contre S2308, tandis que les IgG3 confèrent une meilleure protection contre S19.	(Elzer et al., 1994)

CD1	S2308	Anticorps monoclonaux spécifiques de l'OPS (IgG2a, IgG1)	Les IgG2a empêchent l'établissement d'une infection lors d'un challenge réalisé avec une basse dose, tandis que les IgG1 n'ont pas d'effet.	(Winter et al., 1989)

5.3.2.2 Lymphocytes B : victimes d'une manipulation insidieuse

Les récentes études menées sur des souris déficientes pour les lymphocytes B, dénommées Igh6$^{-/-}$, MuMT$^{-/-}$ ou Jh$^{-/-}$ selon les auteurs et/ou la portion du gène visée, ont suggéré un rôle inattendu des lymphocytes B (Goenka *et al.*, 2011; Goenka *et al.*, 2012; Rolan & Tsolis, 2007; Rolan *et al.*, 2009) (voir **Appendix - 1**).

Dans les études de Rolan *et al*, il apparaît que les souris Igh6$^{-/-}$ contrôlent plus efficacement l'infection par *Brucella abortus* 2308 que les souris WT, du moins durant la phase plateau de l'infection (3 semaines post infection). Toutefois ces souris sont moins aptes à éliminer le mutant non virulent du système de sécrétion de type 4 (T4SS) de *Brucella*, à savoir VirB (Rolan & Tsolis, 2007). Les mêmes auteurs suggèrent que l'absence d'anticorps chez les souris Igh6$^{-/-}$ infectées par le mutant virB favoriserait sa localisation extracellulaire et empêcherait donc son élimination par les cellules phagocytaires (Rolan *et al.*, 2009). Ils proposent également que les souris Igh6$^{-/-}$ présentent un défaut de développement des cellules dendritiques (Moulin *et al.*, 2000; Rolan *et al.*, 2009).

Plus récemment, Goenka *et al* ont suggéré que les lymphocytes B pouvaient contribuer au caractère chronique de l'infection par *Brucella*, notamment en fournissant une niche d'infection intracellulaire (Goenka *et al.*, 2012) pourvue de fonctions immunosuppressives (Goenka *et al.*, 2011). Il s'agirait dès lors d'une nouvelle stratégie élaborée par *Brucella* pour survivre et persister au sein de l'hôte.

Le caractère « Bregs » des lymphocytes B infectés par *B. abortus* 2308 a été proposé suite à l'observation que les souris C57BL/6 MuMT$^{-/-}$ présentent une meilleure résistance à l'infection corrélée à une augmentation de la fréquence de lymphocytes T IFN-γ^{+} et à une diminution de la fréquence de cellules IL-10^{+}. Ce résultat n'est pas lié à l'absence d'anticorps

car le transfert de sérum immun chez les souris MuMT$^{-/-}$ ne modifie pas le phénotype observé (Goenka *et al.*, 2011).

Il avait déjà été démontré en 2006 que la proline racémase PrpA de *B. abortus* 2308 agissait comme un agent mitogène T-indépendant des lymphocytes B et stimulait leur production d'IL-10. PrpA a été décrit comme un élément essentiel à la virulence de *Brucella* car, via l'induction d'IL-10, il provoque une suppression transitoire de la réponse immunitaire qui permettrait l'établissement d'une infection chronique (Spera *et al.*, 2006).

L'impact négatif de l'IL-10 sur la réponse IFN-γ a déjà été décrit à plusieurs reprises (Fernandes & Baldwin, 1995; Fernandes *et al.*, 1996). L'IL-10 est une cytokine anti-inflammatoire produite par les macrophages activés, mais également par les lymphocytes T et B (Gillan *et al.*, 2005; Mizoguchi & Bhan, 2006; Moore *et al.*, 2001). Déterminante dans le maintien d'un équilibre entre pathologie et protection, son impact négatif sur la résistance aux infections bactériennes ou parasitaires a déjà été rapporté dans d'autres modèles (Bermudez & Champsi, 1993; Silva *et al.*, 1992).

Goenka *et al* mentionnent également que les mécanismes d'immunosuppression diffèrent selon la souche de souris, C57BL/6 ou BALB/c. Bien que les souris BALB/c déficientes pour les lymphocytes B (Jh$^{-/-}$) contrôlent elles aussi plus efficacement l'infection que les BALB/c WT, l'effet immunosuppresseur exercé par les lymphocytes B serait réalisé par l'intermédiaire du TGF-β, une autre cytokine anti-inflammatoire produite par les macrophages et les lymphocytes B, et qui diminue la réponse Th1 (Mizoguchi & Bhan, 2006; Parekh *et al.*, 2003). Chez les souris BALB/c Jh$^{-/-}$, bien qu'aucune augmentation de la fréquence de lymphocytes T IFN-γ^{+} ne soit observée, l'IFN-γ apparaît comme un élément déterminant pour l'élimination de *B. abortus.* En effet, les souris double knock-out (KO) Jh$^{-/-}$ IFN-γ$^{-/-}$ sont incapables de contrôler l'infection comme observé chez les simples KO Jh$^{-/-}$ et présentent une charge bactérienne comparable à celle des BALB/c WT. De manière surprenante, ces doubles KO succombent à l'infection en 5 semaines, alors que les simples KO IFN-γ$^{-/-}$ survivent jusqu'à 10,5 semaines, ce qui indique que les lymphocytes B limiteraient la progression de l'infection chez les souris IFN-γ$^{-/-}$ (Goenka *et al.*, 2011).

6 Vaccination contre la brucellose

6.1 Problématique de la vaccination contre la brucellose

Les principaux vaccins utilisés actuellement en élevage sont les souches *B. melitensis* Rev.1 chez les petits ruminants et *B. abortus* S19 chez les bovins (voir aussi **table 5**). L'absence de vaccin pour l'homme et les problèmes de virulence résiduelle et d'interférence avec les tests diagnostiques sont à l'origine d'un intérêt croissant pour ce domaine de recherche. Toutefois, tandis que de nouveaux candidats vaccinaux sont publiés régulièrement, peu d'études s'intéressent à l'identification des effecteurs immunitaires essentiels à la protection des animaux vaccinés. Les études de transferts menées par Winter (Araya *et al.*, 1989; Araya & Winter, 1990; Winter *et al.*, 1989) ont démontré que tant la réponse cellulaire que la réponse humorale ont une action protectrice contre la brucellose. En partie sur base de ces 2 critères, un amoncellement de données a été publié sur de nouveaux vaccins potentiels, parfois à tort, comme le suggère Grillo dans sa dernière revue.

« ***[…] practically all live strains (no matter whether they are partially or fully attenuated) or even killed Brucella provide significant levels of protection. Moreover, immunization with Brucella LPS, outer membrane proteins, bacterial extracts or even with phylogenetically related bacteria (e.g. Ochrobactrum) may reduce infection with respect to unvaccinated mice.*** » (Grillo *et al.*, 2012)

6.2 Vaccin vivant, tué ou inactivé ?

Quels que soient les modèles d'infection, il est généralement reconnu que les vaccins vivants confèrent une meilleure protection que les vaccins tués (Kawamura *et al.*, 1992; Mitsuyama *et al.*, 1990; Montaraz & Winter, 1986; Scott *et al.*, 1990; Tsukada *et al.*, 1991). Dans les études de vaccination menées sur *Brucella*, l'utilisation de *B. abortus* tuée à la chaleur (HKBA) comme un adjuvant stimulant la mise en place d'une réponse immunitaire protectrice Th1 est couramment rapportée (Finkelman *et al.*, 1988; Street *et al.*, 1990). Toutefois certaines études ont tempéré le rôle des vaccins non-vivants dans l'induction

d'une réponse Th1 protectrice, suggérant même qu'une telle immunisation pouvait mener à un profil de réponse typiquement Th2.

6.2.1 Extraits bactériens, faibles inducteurs d'une réponse Th1...

Les études de Zhan *et al* (Zhan *et al.*, 1993; Zhan *et al.*, 1995) ont comparé la résistance à l'infection et la production de cytokines chez des souris ayant reçu des lymphocytes T $CD4^+$ de donneuses infectées par la souche atténuée *B. abortus* S19 ou immunisées avec des protéines solubles de *Brucella*. Ils ont démontré que seuls les lymphocytes T $CD4^+$ issus de souris infectées étaient capables de transférer la résistance, tandis que ceux issus de souris immunisées ne le pouvaient pas. Le faible niveau de protection conféré par les cellules provenant des souris immunisées était comparable à celui conféré par des souris naïves. Lors de ces études, ils ont pu mettre en évidence une réelle dichotomie dans les profils de cytokines produites avec les 2 protocoles de transferts. Les souris ayant reçu des lymphocytes T $CD4^+$ de donneuses infectées présentaient une fréquence élevée de précurseurs de lymphocytes T $CD4^+$ IFN-γ^+ et peu de précurseurs de lymphocytes T $CD4^+$ IL-4^+. Le profil inverse, caractérisé par une production d'IL-4, était observé chez les souris ayant reçu des lymphocytes T $CD4^+$ de donneuses immunisées.

En conclusion, en comparaison avec la réponse induite par une souche vivante, les antigènes solubles de *Brucella* non seulement induisent une réponse trop faible, mais cette réponse serait de plus inappropriée et non protectrice (Zhan *et al.*, 1995).

6.2.2 ... Brucella HK, bon inducteur d'une réponse Th1

Huang *et al* ont démontré que le HKBA pouvait induire une réponse Th1 (Huang *et al.*, 1999) et que le constituant qui induisait plus particulièrement cette réponse était l'ADN, via une stimulation du TLR9 (Huang *et al.*, 2005). Ils ont observé que plusieurs cytokines étaient produites suite à une stimulation par le HKBA : l'IL-12p40, l'IFN-α et l'IFN-γ (Huang *et al.*, 2005) ainsi que le TNF-α (Huang *et al.*, 2003). Ceci s'oppose aux études de Zhan *et al* qui avaient démontré que la production de TNF-α était fortement réduite chez les souris immunisées par du HKBA *in vitro* (Zhan *et al.*, 1995) et *in vivo* (Zhan *et al.*, 1993). Il est à noter que dans les études de Huang *et al*, le contrôle utilisé par les auteurs était le PBS et

non la souche vivante de *B. abortus*. Contrairement aux autres cytokines dont la production dépend du TLR9, le TNF-α serait induit suite à une stimulation du TLR2 (Huang *et al.*, 2003). Une étude plus récente a mis en évidence que, suite à une stimulation par du HKBA, le TLR2 exerçait une action régulatrice sur le TLR9, dont dépend la mise en place d'une réponse IL-12 appropriée (Zhang *et al.*, 2012). Huang *et al* ont aussi démontré que l'IL-12 produit en réponse au HKBA provenait majoritairement des DCs $CD8^-$ (Huang *et al.*, 2001) qui, contrairement aux DCs $CD8^+$, seraient plutôt impliquées dans l'induction d'une réponse Th2 (Maldonado-Lopez *et al.*, 1999; Pulendran *et al.*, 1999).

6.2.3 Brucella irradiée aux rayons gamma, une solution ?

La souche vaccinale *B. abortus* RB51 irradiée a été utilisée à plusieurs reprises (Magnani *et al.*, 2009; Sanakkayala *et al.*, 2005). En 2005, Sanakkayala *et al.* ont démontré que cette souche, bien qu'incapable de se répliquer, conservait sa capacité à stimuler une réponse immunitaire protectrice de type Th1, comme en témoignent la présence d'IgG2a et la production d'IL-12 et d'IFN-γ (Sanakkayala *et al.*, 2005).

Toutefois en 2010, Surendran *et al*. ont comparé *in vitro* la maturation et la sécrétion de cytokines par les DCs suite à une stimulation par la souche vaccinale rugueuse *B. abortus* RB51 ou lisse 2308, après un traitement aux rayons γ ou à la chaleur (HK) (Surendran *et al.*, 2010). Les auteurs ont mis en évidence que malgré une induction de la maturation des DCs par les 2 souches, qu'elles soient vivantes, irradiées ou inactivées, seules les souches vivantes stimulaient la sécrétion de cytokines par ces cellules (Surendran *et al.*, 2010).

6.3 Vers un meilleur vaccin, pour l'homme et l'animal ?

Outre les recherches menées sur l'effet protecteur des souches de *Brucella* tuées ou inactivées, de nombreuses études se concentrent également sur la création de vaccins sous-unitaires, ciblant spécifiquement l'activation des effecteurs nécessaires à la mise en place d'une immunité protectrice. Le succès d'un tel vaccin dépend fortement de la voie de présentation antigénique stimulée et de l'adjuvant choisi. Ainsi, selon l'antigène utilisé, les études vaccinales désignent comme protecteurs les lymphocytes T $CD4^+$ (Cassataro *et al.*, 2005a; Oliveira *et al.*, 1996) ou $CD8^+$ (Cassataro *et al.*, 2005b; Durward *et al.*, 2010; Yu *et al.*,

2007) ou encore elles proposent une coopération de ces deux populations (Araya *et al.*, 1989; He *et al.*, 2001; Munoz-Montesino *et al.*, 2004; Pasquevich *et al.*, 2009). Il règne donc une certaine confusion sur la question de savoir quelle population de lymphocytes doit être activée par le candidat vaccinal pour induire une protection efficace.

L'absence de règles précises pour l'évaluation d'un vaccin protecteur engendre dès lors la publication d'études où les données présentées sont insuffisantes pour conclure qu'il s'agit d'un candidat potentiel. Par exemple, l'étude de Verma (Verma *et al.*, 2012) propose un vaccin induisant une réponse pro-Th2. Une étude de Leclerq (Leclerq *et al.*, 2002) démontre que malgré la mise en place d'une réponse Th1, attestée par la production d'IFN-γ et d'IgG2a, aucune protection n'est conférée par le candidat vaccinal. Peu d'études ont tenté de caractériser la réponse immunitaire secondaire dans le cadre d'une immunisation par la souche virulente de *Brucella*. Par défaut, les auteurs d'études sur des candidats vaccinaux utilisent donc comme référence ce qui a été démontré en réponse primaire à l'infection, tels la présence de cellules productrices d'IFN-γ et la mise en place d'une réponse humorale de type Th1 (IgG2a principalement) (Arenas-Gamboa *et al.*, 2011; Gonzalez *et al.*, 2008; Kahl-McDonagh & Ficht, 2006; Kahl-McDonagh *et al.*, 2007; Pasquevich *et al.*, 2009). Or il a déjà été démontré dans plusieurs modèles que les effecteurs requis pour une réponse immunitaire secondaire protectrice n'étaient pas toujours les mêmes que ceux qui sont mobilisés lors d'une réponse primaire (Khader *et al.*, 2007; Lin *et al.*, 2009; Nakayama *et al.*, 2010; Narni-Mancinelli *et al.*, 2011; Schito *et al.*, 1998; Seo *et al.*, 2010; Soudja *et al.*, 2012).
Il est donc urgent d'améliorer notre compréhension de la réponse immunitaire développée à l'encontre de *Brucella* et d'identifier les effecteurs nécessaires à la mise en place d'une réponse de mémoire protectrice, ceci afin de définir des balises concrètes pour l'évaluation des candidats vaccins.

Objectives

Bacteria of the genus *Brucella* are facultative intracellular Gram-negative coccobacilli that infect humans and animals. By modulating both immune responses and the physiology of the host cells, they are able to survive lifelong in the infected host. Described as one of the most common bacterial zoonotic disease worldwide, brucellosis still presents challenges in vaccination since a great majority of commercially available animal vaccines are based on live attenuated strains of *Brucella* that cause disease in humans and there is currently no available vaccine for humans. Rare studies have tried to characterize the nature of the protective immune response induced by vaccination and thus identify protective immune markers for the development of a rational strategy to select candidate vaccines. Moreover several parts of the immune response elicited by *Brucella* remain controversial or unsolved.

Therefore, this work aimed to

(i) identify the lymphocyte subsets, signaling pathways and the nature of immune response (Th1, Th2, Th17) involved in the control of a *B. melitensis* infection. For this purpose, we compared wild type mice with a large panel of C57BL/6 and BALB/c mice rendered genetically deficient for key elements of immune response.

(ii) investigate more deeply the poorly described bacteremia caused by *B. melitensis*. We monitored the bacteremia in mice for several days after primary infection and try to identify the localization of *B. melitensis* in blood cells using imagestream analysis, confocal and electronic microscopy.

(iii) evaluate the contributions of humoral response, T cell subsets and signaling pathways in the development of a protective immune memory response against a secondary infection by *B. melitensis*. To this end we compared the protection status and the elicited memory immune response induced by live and heat killed bacteria in wild-type and genetically-deficient mice.

Results

1 Part I : Crucial role of gamma interferon-producing $CD4^+$ Th1 cells but dispensable function of $CD8^+$ T cell, B cell, Th2 and Th17 responses in the control of *Brucella melitensis* infection in mice

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Adaptive immune responses are critical for providing the memory function that is the key player in vaccination. As an intracellular bacterium, *Brucella* is known to elicit a Th1 immune response. Th1-polarized T cells produce IFN-γ, which activates the bactericidal functions in macrophages to hamper the intracellular survival of *Brucella*. However, despite numerous studies, the relative importance of $CD4^+$ and/or $CD8^+$ T cells in *Brucella* immunity remains controversial. Adoptive transfers with $CD4^+$, $CD8^+$, and whole T-cell populations suggest that both T-cell populations are important in controlling brucellosis (Araya *et al.*, 1989). However, experiments with genetically deficient mice for either $CD4^+$ (MHC-$II^{-/-}$) or $CD8^+$ (β2-$microglobulin^{-/-}$) T cells or using depleting monoclonal antibodies provided discordant results, creating confusion (Goenka *et al.*, 2011; Murphy *et al.*, 2001a; Oliveira & Splitter, 1995; Pavlov *et al.*, 1982).

At the beginning of our work in 2007, the significance of humoral immunity in murine brucellosis was commonly accepted since many serum passive-transfer experiments could reduce bacterial infection (Araya *et al.*, 1989; Montaraz *et al.*, 1986). Two recent works have challenged the positive role of B cells in the control of *Brucella* infection by suggesting a regulatory role for B cells that hamper the resolution of the infection (Goenka *et al.*, 2011; Goenka *et al.*, 2012).

The paradigm of T helper immune responses has evolved to include at least three T cell subsets: Th1, Th2 and Th17. To our knowledge no study has attempted to compare the involvement of those three T cell subsets in the control of *Brucella* infection. Data in this field are often originated from studies comparing the following two strains of mice: C57BL/6 mice that manage to control *Brucella* infection through an efficient Th1 response ("resistant mice") and BALB/c mice, whose susceptibility appear to be linked to a Th2 bias.

Therefore the first part of this thesis aimed to evaluate the respective contribution of B cells and Th1, Th2, Th17 immune responses during the course of *B. melitensis* infection.
To this end, we compared the bacterial load and the immune response elicited by *B. melitensis* infection in wild-type mice and a large panel of C57BL/6 or BALB/c mice rendered genetically deficient for key elements of immune response[1]. All mice were bred in the same animal facilities to avoid discrepancies resulting from environmental or dietary differences. Indeed recent evidence (Gill & Finlay, 2011) suggests that the immune response can be strongly modify by these parameters.

Our results confirm the central role of MHC-II-dependent antigen presentation to $CD4^+$ T cells and the IFN-γ-mediated Th1 response in the control of *B. melitensis* chronic infection, whereas the absence of B cells, MHC-I-dependent antigen presentation and Th2 and Th17 responses appear to have no important positive or negative impact.

[1] Additional results on genetically deficient mice tested during this thesis can be found in the Appendix section (chapter 2). Because they have not been always replicated or display variability, these results are only shown for guidance and will not been discussed here.

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Crucial Role of Gamma Interferon-Producing CD4$^+$ Th1 Cells but Dispensable Function of CD8$^+$ T Cell, B Cell, Th2, and Th17 Responses in the Control of *Brucella melitensis* Infection in Mice

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***Brucella* spp. are facultative intracellular bacterial pathogens responsible for brucellosis, a worldwide zoonosis that causes abortion in domestic animals and chronic febrile disease associated with serious complications in humans. There is currently no approved vaccine against human brucellosis, and antibiotic therapy is long and costly. Development of a safe protective vaccine requires a better understanding of the roles played by components of adaptive immunity in the control of *Brucella* infection. The importance of lymphocyte subsets in the control of *Brucella* growth has been investigated separately by various research groups and remains unclear or controversial. Here, we used a large panel of genetically deficient mice to compare the importance of B cells, transporter associated with antigen processing (TAP-1), and major histocompatibility complex class II-dependent pathways of antigen presentation as well as T helper 1 (Th1), Th2, and Th17-mediated responses on the immune control of *Brucella melitensis* 16 M infection. We clearly confirmed the key function played by gamma interferon (IFN-γ)-producing Th1 CD4$^+$ T cells in the control of *B. melitensis* infection, whereas IFN-γ-producing CD8$^+$ T cells or B cell-mediated humoral immunity plays only a modest role in the clearance of bacteria during primary infection. In the presence of a Th1 response, Th2 or Th17 responses do not really develop or play a positive or negative role during the course of *B. melitensis* infection. On the whole, these results could improve our ability to develop protective vaccines or therapeutic treatments against brucellosis.**

Brucella (alphaproteobacteria) spp. are facultative intracellular Gram-negative coccobacilli that infect humans as well as domestic mammals (cattle, sheep, swine, camels, etc.) and wild mammals (deer, bison, etc.). Animal infection leads to abortion and infertility with huge economic costs. Human brucellosis is an ancient disease mainly transmitted through ingestion, inhalation, or contact with conjunctiva or skin lesions. It is characterized by undulant fever that if left untreated can result in chronic and sometimes lifelong disease, with serious clinical manifestations (12, 15, 23, 56). Human brucellosis remains a significant public health concern, with more than 500,000 new human cases reported annually (56). There is currently no available vaccine against human brucellosis, and all commercially available animal vaccines are based on live attenuated strains of *Brucella* (*Brucella melitensis* Rev.1, *B. abortus* S19, *B. abortus* RB51) (20, 54). Despite their effectiveness, these live vaccines have disadvantages, such as being infectious and causing disease in humans, interfering with diagnosis, resulting in abortions when administered to pregnant animals, and bearing antibiotic resistance (54). Thus, the ideal safe vaccine would be a nonreplicating subunit vaccine that specifically targets the critical aspects of the immune response necessary to induce immunity. The success of a subunit vaccine is strongly dependent on the choice of appropriate antigen-presenting pathways and a suitable adjuvant. Therefore, a better understanding of the role played by various lymphocyte subsets during infection and the identification of T helper cytokine profiles associated with protective and counterprotective immune response will be very useful for the development of a safe protective subunit vaccine.

Immune responses to *Brucella* spp. have been studied mainly in mouse models (4, 25). There has been much evidence demonstrating that the gamma interferon (IFN-γ)-mediated T helper 1 (Th1) immune response is crucial for the control of *Brucella* infection. Supplementing infected mice with recombinant IFN-γ (66) results in better clearance of *B. abortus* in the spleen, and neutralizing endogenous IFN-γ by administering monoclonal antibodies (MAbs) results in decreased control of bacterial growth (18, 19, 74). *Brucella*-infected MyD88-deficient (MyD88$^{-/-}$) (10) and interleukin-12 p40 (IL-12p40)-deficient (IL-12p40$^{-/-}$) (37) C57BL/6 mice display reduced production of IFN-γ and 10- to 100-fold higher bacterial counts in the spleen. Finally, IFN-γ-de-

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ficient (IFN-$\gamma^{-/-}$) and interferon responsive factor 1 (IRF-1)-deficient (IRF-$1^{-/-}$) C57BL/6 mice are the rare strains that succumb to *B. abortus* (6, 37, 52) or *B. melitensis* (2) infection. In contrast, Th2 responses are frequently considered detrimental during *Brucella* infection. Indeed, BALB/c mice, which present a well-known Th2 bias in numerous infectious models (27, 42), display a reduced ability to control *Brucella* infection (10, 19, 52). Moreover, neutralization of IL-4 by MAb can reduce bacterial counts in some models (19). Up to now, studies of the function of Th17 responses in immunity to *Brucella* organisms have been scarce. Nevertheless, Th17 responses have been shown to contribute to host defense against several intracellular microorganisms, such as *Mycobacterium tuberculosis* (14, 33, 68).

Adaptive immunity is clearly implicated in the control of chronic *Brucella* infection (29). However, the relative roles of B cells, $CD4^+$ cells, and $CD8^+$ T cells in protection remain controversial. B cells have been found to be involved in resistance to the attenuated *B. abortus virB* mutant (61) and susceptibility to virulent *B. abortus* (24). Depending on the studies, major histocompatibility complex class II (MHC-II)-deficient (MHC-$II^{-/-}$) mice demonstrate that the presence of $CD4^+$ T cells appears to be important (24) or dispensable (55) in the control of infection. $CD8^+$ T cells have been shown to play a role in resistance to infection by depleting $CD8^+$ T cells with anti-CD8α monoclonal antibodies (48, 51, 58) and using β_2-microglobulin-deficient (β_2-microglobulin$^{-/-}$) mice (55). Both CD4 and CD8 T cells have been shown to be equally protective in adoptive transfer studies (1). These results are subject to multiple interpretations.

Using a large panel of gene-deficient mice, we evaluated the contributions of the humoral response and T cell subsets in protective immunity against *B. melitensis*, the most frequent cause of human brucellosis (12). To our knowledge, this study is the first to analyze the course of infection of virulent *Brucella* in transporter associated with antigen processing (TAP-1)-, IL-4-, IL-12p35-, IL-17α receptor (IL-17Rα)-, IL-21R-, and IL-22-deficient mice. Our results confirm the central role of MHC-II-dependent antigen presentation to $CD4^+$ T cells and the IFN-γ-mediated Th1 response in the control of *B. melitensis* infection, whereas the absence of B cells, MHC-I-dependent antigen presentation, and Th2 and Th17 responses appears to have no important positive or negative impact.

MATERIALS AND METHODS

Ethics statement. The animal handling and procedures of this study 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 (Royal Decree on the Protection of Experimental Animals of 6 April 2010, published on 14 May 2010). The complete protocol was reviewed and approved by the Animal Welfare Committee of the Facultés Universitaires Notre-Dame de la Paix (FUNDP; Namur, Belgium) (permit number 05-558).

Mice and reagents. MyD88$^{-/-}$ C57BL/6 mice (31) were obtained from S. Akira (Osaka University, Osaka, Japan). TAP-1-deficient (TAP-$1^{-/-}$) C57BL/6 mice (70) and MHC-$II^{-/-}$ C57BL/6 mice (13) were obtained from Jörg Reimann (University of Ulm, Ulm, Germany). IL-12p35$^{-/-}$ C57BL/6 mice (47) and IL-12p40$^{-/-}$ C57BL/6 mice (44) were obtained from B. Ryffel (University of Orleans, Orleans, France). IL-$21R^{-/-}$ C57BL/6 mice (39) were obtained from F. Andris (Université Libre de Bruxelles, Brussels, Belgium). IL-17Rα$^{-/-}$ C57BL/6 mice (53) and IL-$22^{-/-}$ C57BL/6 mice (38) were obtained from L. Dumoutier (Ludwig Institute for Cancer Research, Brussels Branch, Brussels, Belgium). Recombinant activating gene (RAG)-deficient (RAG$1^{-/-}$) C57BL/6 mice (49) were obtained from S. Goriely (Université Libre de Bruxelles, Brussels, Belgium). RAG2γc$^{-/-}$ C57BL/6 mice (7) were obtained from Michel Y. Braun (Université Libre de Bruxelles, Brussels, Belgium). CD28$^{-/-}$ and CD40$^{-/-}$ C57BL/6 mice (30, 64) and B cell-deficient (MuMT$^{-/-}$) C57BL/6 mice (36) were purchased from The Jackson Laboratory (Bar Harbor, ME). IL-12p40$^{-/-}$ BALB/c mice (44) were obtained from V. Flamand (Université Libre de Bruxelles, Brussels, Belgium). Wild-type C57BL/6 mice and BALB/c mice were purchased from Harlan (Bicester, United Kingdom) and were used as controls. All wild-type and deficient mice used in this study were bred in the animal facility of the Gosselies Campus of the Free University of Brussels (ULB; Brussels, Belgium).

B. melitensis strain 16 M (biotype 1, ATCC 23456) was initially isolated from an infected goat and grown in a biosafety level III laboratory facility. The culture was grown overnight with shaking at 37°C in 2YT medium (Luria-Bertani broth with a double quantity of yeast extract) to stationary phase and was washed twice in phosphate-buffered saline (PBS) by centrifugation at 3,500 × *g* for 10 min before use for mouse inoculation as previously described (10).

Mouse infection. Mice were injected intraperitoneally (i.p.) with the indicated dose of *B. melitensis* in 500 μl of PBS. Control animals were injected with the same volume of PBS. The infectious doses were validated by plating serial dilutions of inocula. At selected time intervals, mice were sacrificed by cervical dislocation. Immediately after the mice were killed, the spleen was collected for bacterial count and flow cytometry analyses.

Bacterial count. Spleens were recovered in PBS–0.1% Triton X-100 (Sigma). We performed successive serial dilutions in PBS to obtain the most accurate bacterial count and plated them onto 2YT medium plates. The numbers of CFU were counted after 4 days of culture at 37°C.

RNA purification and real-time RT-PCR. RNA was extracted from total spleen tissue. Spleen samples were collected and immediately frozen and stored at −80°C until processing. RNA was then extracted from frozen tissue with TriPure isolation reagent (Roche) according to the instructions of the manufacturer. RNA samples were treated with DNase (Fermentas). Reverse transcription (RT) and real-time PCRs were carried out using LightCycler 480 RNA master hydrolysis probes (one-step procedure) on a LightCycler 480 real-time PCR system (Roche Diagnostics). Primer sequences are described in Table S1 in the supplemental material.

Cytofluorometric analysis. As previously described (11), spleens were harvested, cut in very small pieces, and incubated with a cocktail of DNase I fraction IX (100 μg/ml; Sigma-Aldrich Chimie SARL, Lyon, France) and 1.6 mg/ml of collagenase (400 Mandl units/ml) at 37°C for 30 min. After washing, spleen cells were filtered and first incubated in saturating doses of purified 2.4G2 (anti-mouse Fc receptor; ATCC) in 200 μl PBS–0.2% bovine serum albumin–0.02% NaN_3 (fluorescence-activated cell sorter [FACS] buffer) for 20 min on ice to prevent antibody binding to Fc receptor. A total of 3×10^6 to 5×10^6 cells were stained on ice with various fluorescent MAb combinations in FACS buffer and further collected on a FACSCalibur cytofluorometer (Becton Dickinson, BD). We purchased the following MAbs from BD Biosciences: fluorescein isothiocyanate (FITC)-coupled 145-2C11 (anti-CD3ε), FITC-coupled H57-597 (anti-TCRβ), phycoerythrin (PE)-coupled RM4-5 (anti-CD4), and PK136 (anti-NK1.1). The cells were analyzed on a FACSCalibur cytofluorometer. Cells were gated according to size and scatter to eliminate dead cells and debris from the analysis.

Intracellular cytokine staining. Spleen cells were incubated for 4 h in RPMI 1640–5% fetal calf serum with 1 μl/ml Golgi Plug (BD Pharmingen) at 37°C in 5% CO_2. The cells were washed with FACS buffer and stained for cell surface markers before fixation in PBS–1% paraformaldehyde (PFA) for 15 to 20 min on ice. These cells were then permeabilized for 30 min using a saponin-based buffer (10× Perm/Wash [BD Pharmingen] in FACS buffer) and stained with allophycocyanin (APC)-coupled XMG1.2 (anti-IFN-γ; BD Biosciences). After final fixation in PBS–1% PFA, cells were analyzed on a FACScalibur cytofluorometer. No signal was detectable with control isotypes.

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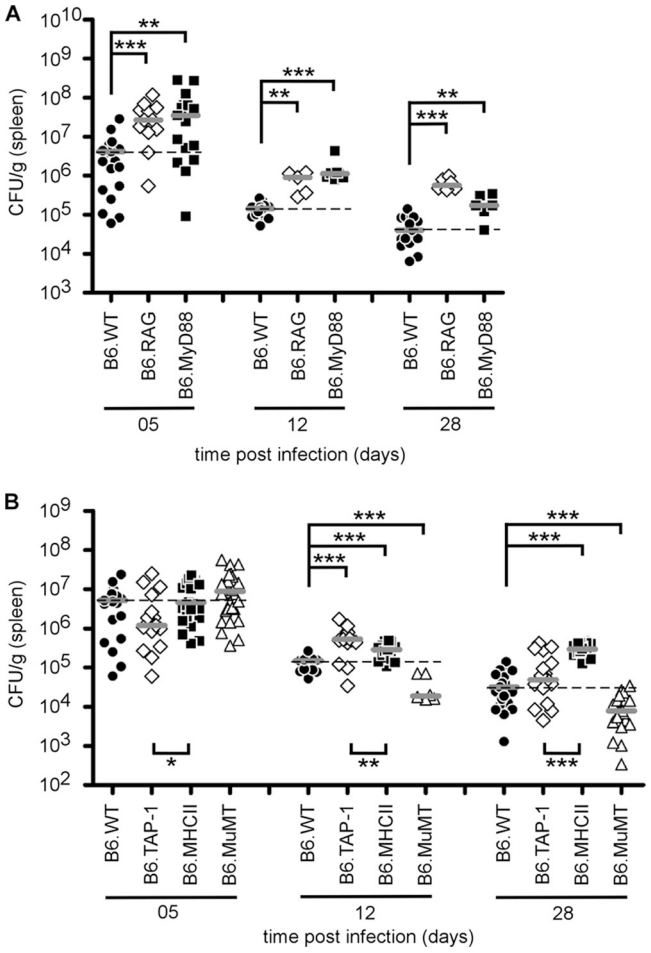

FIG 1 Course of *B. melitensis* infection in the spleen of C57BL/6 (B6) versus RAG$^{-/-}$, MyD88$^{-/-}$, TAP-1$^{-/-}$, MHC-II$^{-/-}$, and MuMT$^{-/-}$ mice. Wild-type (WT) and deficient mice were injected i.p. with 4×10^4 CFU of *B. melitensis* and sacrificed at the indicated times. The data represent the number of CFU per gram of spleen. Gray bars represent the medians. These results are representative of two independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Statistical analysis. We used a Mann-Whitney or Wilcoxon-Mann-Whitney test provided by the GraphPad Prism program 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. *P* values of <0.05 were considered to represent a significant difference.

RESULTS

Major role of CD4$^+$ T cells in control of the plateau phase of chronic *B. melitensis* infection. The precise role of different components of adaptive immunity in the control of *Brucella* growth is quite controversial (1, 3, 6, 24, 48, 51, 52, 55, 58, 60, 61). Here, we compared the course of *B. melitensis* infection in the spleen of various C57BL/6 mice rendered genetically deficient for key elements of adaptive immune response: RAG (T and B cells), MHC-II (CD4$^+$ T cells), TAP-1 (CD8$^+$ T cells), and MuMT (B cells). As an internal control, we used MyD88$^{-/-}$ mice that present well-established susceptibility to *B. melitensis* infection (10, 71). Wild-type and deficient mice were infected i.p. with 4×10^4 CFU of *B. melitensis* (Fig. 1A and B). As expected, RAG deficiency was correlated with an increased bacterial load in the spleen (Fig. 1A). Comparative analysis of TAP-1$^{-/-}$, MHC-II$^{-/-}$, and MuMT$^{-/-}$ mice showed a clear unique implication of CD4$^+$ T cells in the late control of *B. melitensis* infection, as demonstrated by the fact that at 28 days postinfection (p.i.), only MHC-II$^{-/-}$ mice were unable to control the infection. In contrast, at 28 days p.i., TAP-1$^{-/-}$ mice displayed an unchanged CFU count in the spleen, and as previously reported by Goenka et al. (24), MuMT$^{-/-}$ mice exhibited a reduced bacterial load compared with wild-type mice.

IFN-γ is well-known as a key cytokine that regulates resistance to *Brucella* infection (10, 19, 52), as mice deficient for IFN-γ do not survive infection (6, 52). In the spleen, early IFN-γ production (at 5 days) derives equally from NK cells, CD4$^+$ T cells, and CD8$^+$ T cells, while later sustained IFN-γ production (at 12 days) is mainly dependent on CD4$^+$ T cells (10). Using flow cytometry analyses, we investigated the impact of different targeted genetic deficiencies on the frequency of IFN-γ-producing cells in the spleen. It is important to note that the cells were never stimulated *in vitro* before intracellular cytokine staining. Thus, the IFN-γ detected in these cells derived only from natural *in vivo* activation during the course of infection.

At 5 days p.i., a highly significant increase of IFN-γ-positive (IFN-γ$^+$) cells was observed in RAG$^{-/-}$ and MuMT$^{-/-}$ mice compared with wild-type mice (Fig. 2A). In RAG$^{-/-}$ mice, this surprisingly elevated frequency of IFN-γ$^+$ cells was mainly due to NK cells (Fig. 2B; see Fig. S1 in the supplemental material). This was confirmed by the complete absence of IFN-γ in the spleens of RAGγc$^{-/-}$ mice deficient for NK cells (see Fig. S1 in the supplemental material). As previously described by Goenka et al. (24), MuMT$^{-/-}$ mice displayed a higher frequency of IFN-γ-producing T cells, associated with better control of the infection (Fig. 2A). Despite a severe deficiency in CD4$^+$ T or CD8$^+$ T cells (see Fig. S2 in the supplemental material), the total frequency of IFN-γ-producing cells was not reduced either in MHC-II$^{-/-}$ mice or in TAP-1$^{-/-}$ mice. Similar results, due to transitory compensations, have been reported by Das (16) after injection of anti-CD3 antibodies.

At 12 days p.i., only RAG$^{-/-}$ mice displayed a strongly diminished frequency of IFN-γ-producing cells, since NK cells were no longer able to assume IFN-γ production (Fig. 2A). Interestingly, at 12 days p.i., MHC-II$^{-/-}$ and TAP-1$^{-/-}$ mice displayed a similar frequency of IFN-γ-producing cells, but at 28 days p.i., only MHC-II$^{-/-}$ mice failed to control *Brucella* infection (Fig. 1B). As expected, IFN-γ production was taken over by CD8$^+$ T cells in MHC-II$^{-/-}$ mice (Fig. 2B and C). Note that splenomegaly and the number of spleen cells (data not shown) were similar in both strains of infected mice. This strongly suggests that resistance to *Brucella* not only is restricted to IFN-γ production but also requires functional CD4$^+$ T cells.

BALB/c mice do not present a Th2 bias during the course of *Brucella melitensis* infection. Since the discovery of the Th1 and Th2 paradigm, resistance to *Brucella* has been described to be associated with a Th1 response and susceptibility has been described to be associated with a Th2 response, notably, because BALB/c mice display reduced control of infection compared with that displayed by C57BL/6 mice (4, 19). BALB/c mice are well-known for their Th2 bias in several infection models, such as the *Leishmania major* infection model (41–43). The recent discovery of the Th17 response and its implication in the control of infection

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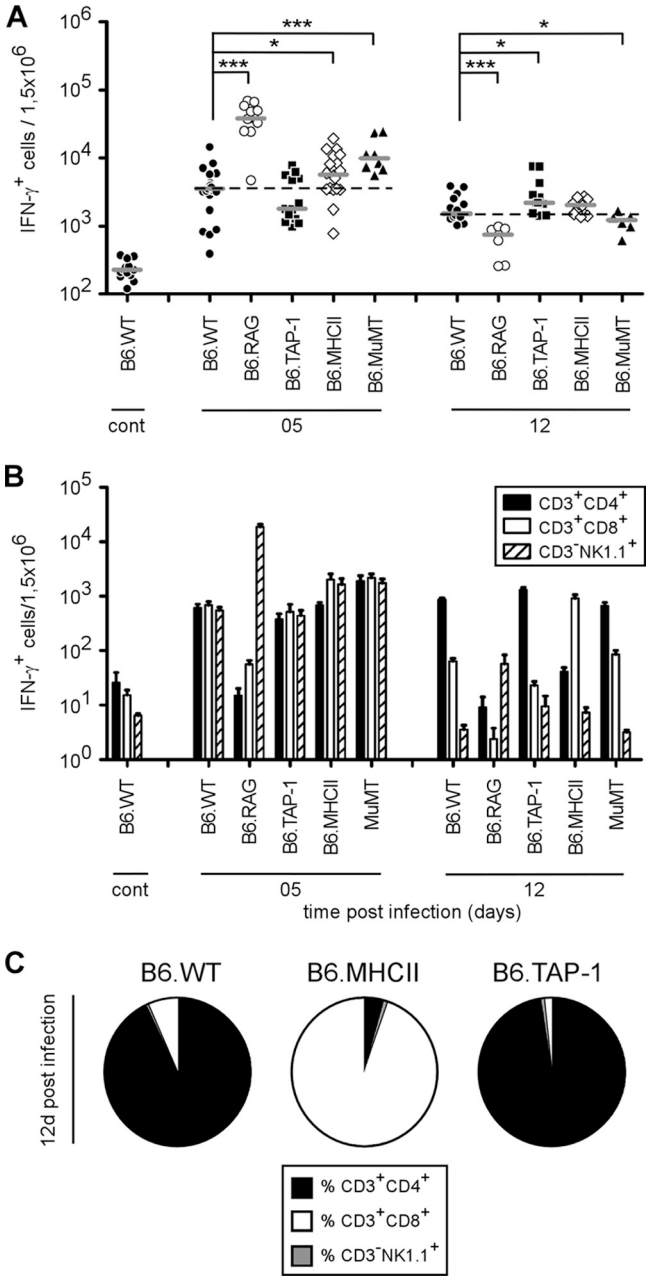

FIG 2 Flow cytometry analysis of IFN-γ-producing spleen cells following *B. melitensis* infection. Wild-type, RAG$^{-/-}$, TAP-1$^{-/-}$, MHC-II$^{-/-}$, and MuMT$^{-/-}$ C57BL/6 mice were injected i.p. with PBS (as a control) or 4×10^4 CFU of *B. melitensis* and sacrificed at the indicated times. Spleen cells were harvested and analyzed by flow cytometry. Cells were gated according to size and scatter to exclude dead cells and debris from the analysis. Spleen cells from individual mice were first analyzed for forward size scatter (FSC) versus IFN-γ production and then for cell surface markers. The frequency of IFN-γ-positive cells in uninfected deficient mice was similar to the one observed in uninfected wild-type mice. (A) Number of IFN-γ-positive cells per 1.5×10^6 spleen cells acquired. Each piece of data represents the value obtained from an individual spleen, and the data are representative of two independent experiments. Gray bars represent the medians. cont, control; *, $P < 0.05$; ***, $P < 0.001$. (B) IFN-γ-positive cells in each group were then analyzed for CD3, CD4, CD8, and NK1.1 expression. The data represent the mean ± SEM of groups described in panel A. (C) The percentages of IFN-γ-positive cells 12 days p.i. are represented in a sectoral diagram according to cell surface markers.

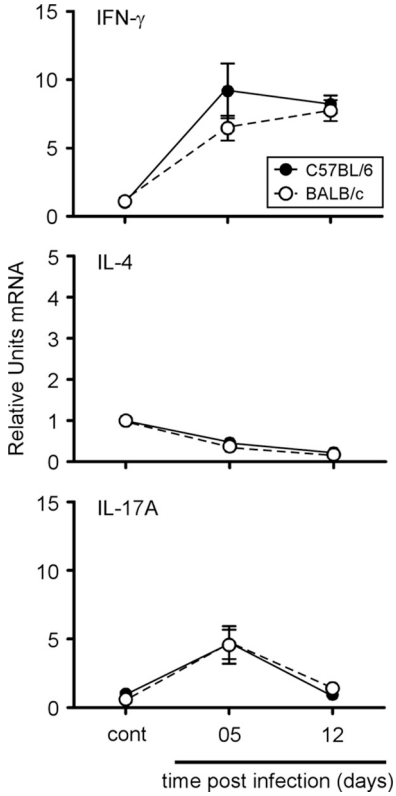

FIG 3 Kinetics of IFN-γ, IL-4, and IL-17A gene expression in spleen cells from C57BL/6 or BALB/c mice during the course of *B. melitensis* infection. C57BL/6 and BALB/c mice were injected i.p. with PBS (control) or 4×10^4 CFU *B. melitensis*. After the indicated times, spleen cells were collected and total RNA was extracted and analyzed by real-time PCR. mRNA levels were normalized using β-actin mRNA as the reference and compared with those obtained under uninfected conditions (control). The data are expressed as the mean ± SEM of 16 animals per time point pooled from two independent experiments.

by several intracellular bacteria have sparked debate on the role of each subclass of Th response during the course of *Brucella* infection.

In order to identify the response profile dominating *Brucella* infection in C57BL/6 and BALB/c mice, we compared the expression of IFN-γ, IL-4, and IL-17A by quantitative real-time PCR in spleen at 5 and 12 days p.i. (Fig. 3). As for the flow cytometry analysis, this test was performed on the entire organ without *in vitro* stimulation or purification of the spleen cells. As expected, IFN-γ mRNA was indeed detected in C57BL/6 and BALB/c mice and peaked at 5 days p.i. Both IL-4 and IL-17A mRNA expression remained weakly detectable during infection, and only IL-17A expression displayed a small and short upregulation at 5 days p.i. No statistically significant differences were observed between C57BL/6 and BALB/c mice. Thus, as determined by quantitative RT-PCR, BALB/c mice do not seem to present a Th2 bias following *Brucella* infection.

Ablation of the Th2 or Th17 response does not affect the control of *Brucella melitensis* infection in C57BL/6 mice. In order to functionally compare the impact of each subclass of Th response, we compared the susceptibility of C57BL/6 mice genetically deficient for IL-12p35 (Th1), IL-12p40 (Th1 and Th17), IL-4 (Th2), and IL-17Rα, IL-21R, and IL-22 (Th17). Mice were infected, and bacterial counts in the spleen were examined at 5, 12, and 28 days

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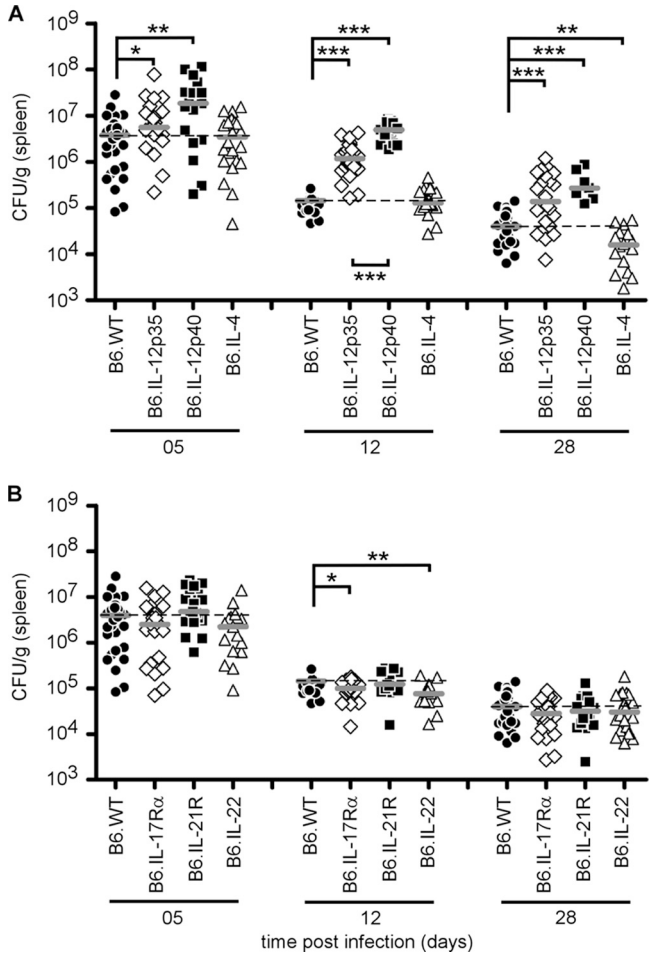

FIG 4 Course of *B. melitensis* infection in the spleen of wild-type and Th response-deficient C57BL/6 mice. Wild-type, IL-12p35$^{-/-}$ (Th1), IL-12p40$^{-/-}$ (Th1 and Th17), IL-4$^{-/-}$ (Th2), IL-17Rα$^{-/-}$, IL-21R$^{-/-}$, and IL-22$^{-/-}$ (Th17) C57BL/6 mice were injected i.p. with 4×10^4 CFU of *B. melitensis* and sacrificed at the indicated times. The data represent the number of CFU per gram of spleen. Gray bars represent the medians. These results are representative of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

p.i. (Fig. 4A and B). Only IL-12p35$^{-/-}$ and IL-12p40$^{-/-}$ mice displayed significantly higher bacterial counts than wild-type mice at each time point after infection, confirming the crucial role of the Th1 response in the control of *Brucella* infection. Surprisingly, IL-4 deficiency only slightly but significantly improved the resistance of C57BL/6 mice at 28 days p.i. (Fig. 4A). Mice deficient for elements (IL-17Rα, IL-21R, IL-22) of the Th17 response controlled the infection like wild-type mice, suggesting no major positive role for the Th17 response (Fig. 4B).

Flow cytometry analyses of the frequency of IFN-γ$^+$ cells in the spleen during the course of *B. melitensis* infection confirm the close relationship existing between resistance to infection and IFN-γ production by CD4$^+$ T cells (Fig. 5A to D). As expected, IL-12p35 and IL-12p40 deficiency strongly decreased the frequency of IFN-γ$^+$ cells at 5 and 12 days p.i. compared with that in wild-type mice (Fig. 5A and B). IL-4, IL-17Rα, IL-21R, and IL-22 deficiency (Fig. 5C and D) affected this frequency very moderately, suggesting that the Th2 and Th17 responses have only minor effects on the Th1 immune response against *B. melitensis* infection in C57BL/6 mice.

In order to better identify the signal required to induce IFN-γ production during *Brucella* infection, we compared the frequency of IFN-γ-producing cells in the spleen of CD28$^{-/-}$, CD40$^{-/-}$, MyD88$^{-/-}$, and IL-12p35$^{-/-}$ C57BL/6 infected mice. MyD88$^{-/-}$ and IL-12p35$^{-/-}$ mice were used as internal controls. The results (see Fig. S3 in the supplemental material) showed that deficiency of both CD28 and CD40 has only a minor impact on the frequency of IFN-γ-producing cells in the spleen, suggesting that the MyD88 and IL-12 pathways remain the main tested pathways regulating IFN-γ production during *Brucella* infection.

Ablation of the Th2 response does not improve the resistance of BALB/c mice to *Brucella melitensis* infection. We then compared the susceptibility of wild-type, IL-12p40$^{-/-}$ (Th1 and Th17), and IL-4R$^{-/-}$ (Th2) BALB/c mice. As expected, IL-12p40 deficiency drastically reduced the resistance of BALB/c mice (Fig. 6), demonstrating that the Th1 response, even in this strain, is the main protective response. Surprisingly, IL-4R deficiency did not enhance the resistance of BALB/c mice at 5 and 12 days p.i. (Fig. 6). Only a very modest improvement of CFU counts in the spleen was observable at 28 days p.i.

Analysis of IFN-γ-producing cell frequency by flow cytometry (Fig. 7A and B) showed a strong defect of IFN-γ production in IL-12p40$^{-/-}$ mice. In contrast to C57BL/6 mice, IL-4R deficiency in BALB/c mice was associated with an enhanced Th1 response, as shown by the 2-fold higher number of IFN-γ$^+$ cells at 5 days p.i. However, this higher frequency was not maintained until 12 days (Fig. 7A and B) and was not associated with better control of the infection at the same time point (Fig. 6).

DISCUSSION

Brucellae are exquisitely well adapted to their mammalian hosts. They disseminate in all tissues and escape many immune system components through stealthy intracellular growth (46). However, this silent infection leads to a chronic febrile disease with potentially serious complications over the long term (12, 15, 23, 56). Empirical research has failed to develop a safe protective vaccine for humans (54, 59). Killed vaccines are not protective, and the live attenuated vaccines used in animals are too virulent for humans. One solution could be subunit vaccination candidates including *Brucella* antigens associated with adjuvants that mimic patterns of pathogenesis (POPs) in order to select appropriate immune effector mechanisms. POPs are defined as a combination of signals including the production of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) under specific infection conditions (69). Characterization of *Brucella*-specific POPs requires good knowledge of its *in vivo* infectious strategy, which, unfortunately, is still largely enigmatic. Using the same mouse model, we recently characterized (11) macrophages and dendritic cells as the major infected cells in the spleen and liver and identified granuloma formation as a main trait of resistance to *Brucella* infection *in situ*. Strikingly, the construction of granuloma structures during the early phase of chronic infection appeared to be largely independent of adaptive immunity, as the same structures are observed in RAG$^{-/-}$ mice. In contrast, they are strictly dependent on MyD88/IL-12p35/IFN-γ-mediated pathways (11). This observation could explain why IFN-γ (6, 52) or IRF-1 (37) deficiency, but not RAG deficiency

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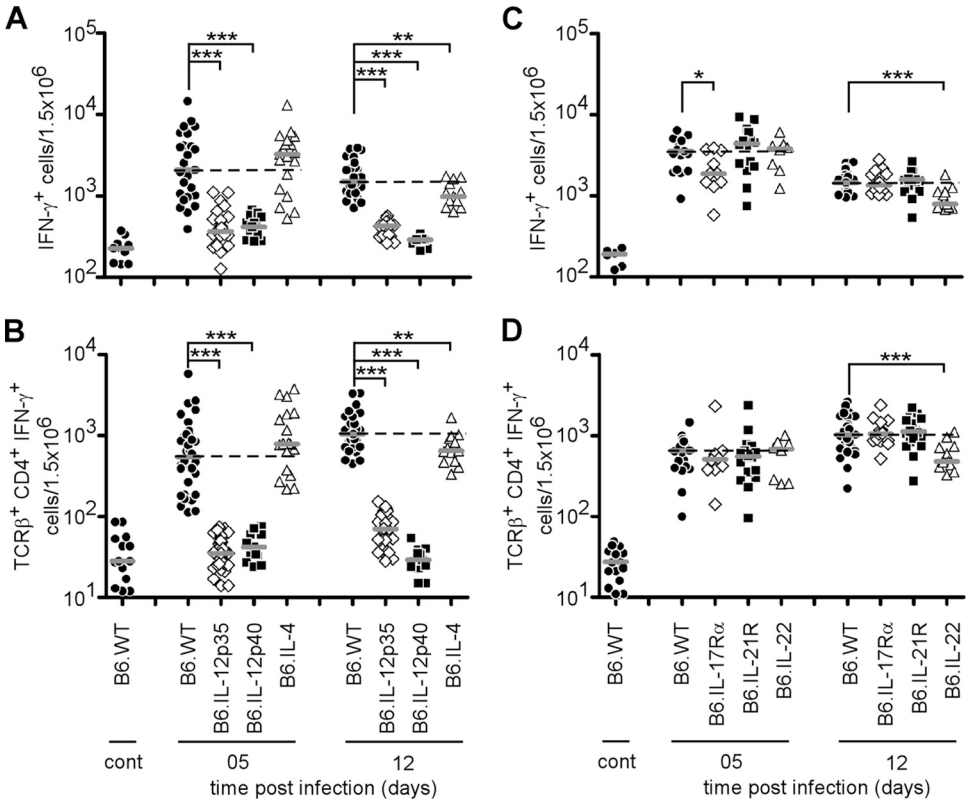

FIG 5 Flow cytometry analysis of IFN-γ-producing spleen cells following *B. melitensis* infection. Wild-type, IL-12p35$^{-/-}$, IL-12p40$^{-/-}$, IL-4$^{-/-}$, IL-17Rα$^{-/-}$, IL-21R$^{-/-}$, and IL-22$^{-/-}$ C57BL/6 mice were injected i.p. with PBS (control) or 4×10^4 CFU of *B. melitensis* and sacrificed at the indicated times. Spleen cells were collected and analyzed by flow cytometry. Cells were gated according to size and scatter to exclude dead cells and debris from analysis. Spleen cells from individual mice were first analyzed for forward size scatter versus IFN-γ production and then for cell surface markers. The frequency of IFN-γ-positive cells in uninfected deficient mice was similar to the one observed in uninfected wild-type mice. (A, C) Number of IFN-γ-positive cells per 1.5×10^6 spleen cells acquired. (B, D) IFN-γ-positive cells were then analyzed for T cell receptor β (TCRβ) and CD4 expression. The data represent the number of IFN-γ$^+$, T cell receptor β-positive, and CD4$^+$ cells from the groups described in panels A and B. Each piece of data represents the value obtained from an individual spleen. These data are representative of two independent experiments. Gray bars represent the medians. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

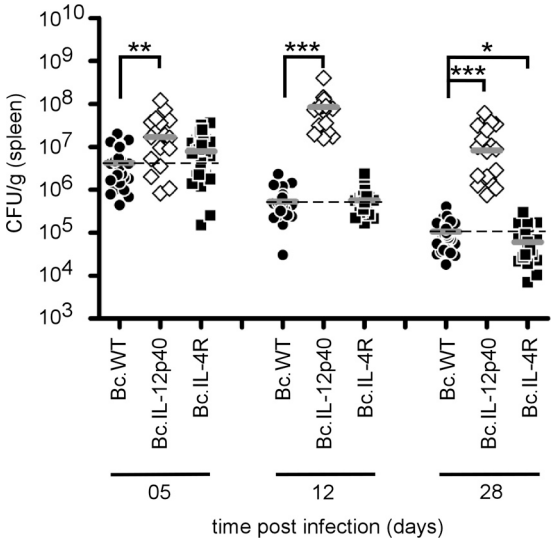

FIG 6 Course of *B. melitensis* infection in the spleen of wild-type and Th response-deficient BALB/c mice. Wild-type, IL-12p40$^{-/-}$ (Th1 and Th17), and IL-4R$^{-/-}$ (Th2) BALB/c mice were injected i.p. with 4×10^4 CFU of *B. melitensis* and euthanized at the indicated times. The data represent the number of CFU per gram of spleen. Gray bars represent the medians. These results are representative of two independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

(29), is fatal to *Brucella*-infected mice. Indeed, in RAG$^{-/-}$ mice, NK cells produce high counts of IFN-γ, as shown in this work. Though adaptive immunity components are not crucial to survival of *Brucella* infection, they do play a key role in successful vaccination. Depending on the studies considered, their roles appear to be very controversial (3).

We first attempted to determine if the control of *B. melitensis* infection involves a humoral immune response (circulating antibodies and B cells). Several groups have previously analyzed this question, with contradictory results. Natural antibodies have been reported to favor *Brucella* control by facilitating its elimination by phagocytosis (61). Many serum transfer experiments have demonstrated a positive role of antibodies in protecting mice against *Brucella* infection (1, 50, 72). However, as previously reported by Goenka et al. (24), we observed here that MuMT$^{-/-}$ mice cleared the infection from the spleen more rapidly than wild-type mice and displayed a higher frequency of IFN-γ-producing T cells at the peak of infection. Enhanced IFN-γ production could be due to a regulatory role of B cells, as hypothesized by Goenka et al. (24). On the whole, these results strongly suggest that natural or induced humoral immunity does not contribute positively to the control of primary *Brucella* infection, without excluding a possible protective role for specific antibodies in the secondary immune response.

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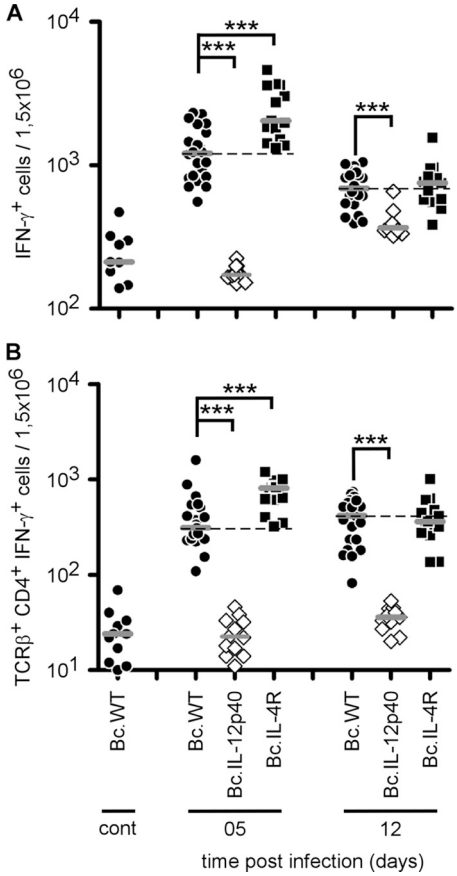

FIG 7 Flow cytometry analysis of IFN-γ-producing cells following *B. melitensis* infection. Wild-type, IL-12p40$^{-/-}$, and IL-4R$^{-/-}$ BALB/c mice were injected i.p. with PBS (control) or 4×10^4 CFU of *B. melitensis* and sacrificed at the indicated times. Spleen cells were collected and analyzed by flow cytometry. Cells were gated according to size and scatter to exclude dead cells and debris from analysis. Spleen cells from individual mice were first analyzed for forward size scatter versus IFN-γ production and then for cell surface markers. The frequency of IFN-γ-positive cells in uninfected deficient mice was similar to the one observed in uninfected wild-type mice. (A) Number of IFN-γ-positive cells per 1.5×10^6 spleen cells acquired. (B) IFN-γ-positive cells were analyzed for T cell receptor β (TCRβ) and CD4 expression. The data represent the number of IFN-γ$^+$, T cell receptor β-positive, and CD4$^+$ cells from the groups described in panel A. Each piece of data represents the value obtained from an individual spleen, and the data are representative of two independent experiments. Gray bars represent the medians. ***, $P < 0.001$.

The precise roles of CD4$^+$ and CD8$^+$ T cells during *Brucella* infection remain particularly unclear (reviewed in reference 3). Indeed, a positive role for CD8$^+$ T cells in protection has been shown by adoptive transfer (1), depletion with anti-CD8α monoclonal antibodies (48, 51, 58), and infection of β_2-microglobulin$^{-/-}$ mice (52, 55). In contrast, a negative role for CD8$^+$ T cells has also been reported in studies (24, 52) showing that β_2-microglobulin$^{-/-}$ mice cleared the infection better than their wild-type counterparts. On the other hand, the importance of CD4$^+$ T cells has also been demonstrated by adoptive transfer (1) and, recently, by infecting MHC-II$^{-/-}$ mice (24). Finally, a study using mixed infection with *B. abortus* 2308 and the *virB* mutant showed no impact of β_2-microglobulin deficiency on the control of wild-type bacterial infection and a greater ability to control this infection for CD4$^{-/-}$ mice (60). These contradictory results can be explained by multiple parameters, such as the mouse and *Brucella* strains and the experimental design used. For example, studies showing a central role for CD8$^+$ T cells and no implication of CD4$^+$ T cells in β_2-microglobulin$^{-/-}$ and MHC-II$^{-/-}$ mice used *Brucella abortus* S19 (55), which is an attenuated strain of *Brucella abortus* known to be more susceptible to rapid intraphagocyte destruction after opsonization than a virulent strain (17). In depletion experiments, results must be interpreted cautiously, as inaccurate data can be generated by the administration procedure or a lack of specificity of the antibodies used. For example, the anti-CD8α antibodies used to deplete CD8$^+$ T cells also target both CD8α-positive dendritic cells and a particular subset of γδ T cells (8). Discrepancies can also result from the choice of inadequately or poorly characterized deficient mice. Indeed β_2-microglobulin$^{-/-}$ mice are now known to lack CD8$^+$ T cells but also CD1d-restricted NK T cell responses and circulating IgG antibodies (35), findings which render interpretation of the results in the context of infection very hazardous. Moreover, comparison of studies using experimental animals performed in different laboratories has raised new concerns since environment and diet as well as immune deficiencies (22) have been demonstrated to shape the gut microbiota of mice and strongly modify the immune response. However, the impact of immune deficiencies appears to be strongly reduced under homeostatic conditions (67). In agreement, we initially worked with mice coming from different animal facilities, and we observed significant discrepancies following infection, even between wild-type mice that originated from the same supplier. As a consequence, the present study compared wild-type mice with a large panel of deficient mice that were bred in the same animal facilities for a minimum of 1 year and that thus displayed similar hygienic status. These factors and conditions therefore bring more consistent results on the role of multiple components of adaptive immunity.

Here, we chose to compare infection in TAP-1$^{-/-}$ and MHC-II$^{-/-}$ C57BL/6 mice to evaluate the contribution of CD8$^+$ and CD4$^+$ T cell functions, respectively. Both types of deficient mice were able to control bacterial growth at the peak of infection. Although transient susceptibility was observed in both groups of mice by 12 days p.i., TAP-1$^{-/-}$ mice were able to control the infection at 28 days p.i. In contrast, MHC-II$^{-/-}$ mice displayed a 10-fold increased bacterial load at 28 days p.i., suggesting a protective role for MHC-II-dependent CD4$^+$ T cells during the chronic phase of infection. IFN-γ was produced by both CD4$^+$ and CD8$^+$ T cells at the peak of infection (5 days p.i.), but only CD4$^+$ T cells retained the ability to produce high levels of IFN-γ during the plateau phase of infection in wild-type mice (10; this study). It is very interesting to note that the frequency of IFN-γ-producing cells was maintained at similar levels during the plateau phase of infection in both MHC-II$^{-/-}$ and TAP-1$^{-/-}$ mice. In MHC-II$^{-/-}$ mice, IFN-γ production was restricted to CD8$^+$ T cells, showing that these cells were able to sustain IFN-γ production for more than 5 days. However, these cells did not provide protection since MHC-II$^{-/-}$ mice displayed enhanced CFU counts in the spleen compared with TAP-1$^{-/-}$ and wild-type mice. While several studies (4, 19, 52) support a correlation between IFN-γ production and protection, our data demonstrate that IFN-γ production must be associated with activated CD4$^+$ T cells to be protective, suggesting that CD4$^+$ T cells provide other undetermined additional protective cofactors.

The protective immune response against *Brucella* has historically been associated with the Th1 response, notably, because BALB/c mice, which display a well-known Th2 bias in several in-

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fection models (42, 43), show a reduced ability to control *Brucella* infection compared with C57BL/6 mice (4, 19). It has been hypothesized (19) that production of IL-4 in BALB/c mice reduces the production of IFN-γ and adversely affects the protective immune response to *Brucella*. Our data showed that infected C57BL/6 and BALB/c mice displayed similar weak expression of IL-4 mRNA in the spleen. Along the same lines, in BALB/c and C57BL/6 mice, the absence of IL-4 protein detectable by enzyme-linked immunosorbent assay following *in vitro* restimulation has been reported previously (19). Using IL-$4^{-/-}$ C57BL/6 and IL-$4R^{-/-}$ BALB/c mice, we demonstrate that IL-4 does not adversely affect host defense against *Brucella* infection. Indeed, IL-4 deficiency in C57BL/6 mice did not drastically enhance IFN-γ production. However, in IL-$4R^{-/-}$ BALB/c mice, a 2-fold increase in IFN-γ production was measurable at the peak of infection. This difference could be a consequence of the lack of responsiveness to both IL-4 and IL-13 in IL-$4R^{-/-}$ mice (32). Thus, we conclude that the low levels of IL-4 produced during *Brucella* infection in wild-type C57BL/6 and BALB/c mice do not affect the ability to mount a protective response. These observations support the idea, previously suggested by others (62), that the susceptibility of BALB/c mice to *Brucella* infection is not due to the development of a Th2 response but is due to an intrinsically lower ability to mount a Th1 response.

The recently characterized Th17 response has been shown to contribute to host defense against several extracellular pathogens, such as *Klebsiella pneumoniae* (26, 73), *Citrobacter rodentium* (45), and *Candida albicans* (28), as well as against intracellular microorganisms, such as *Listeria monocytogenes*, *Salmonella enterica*, and *Mycobacterium tuberculosis* (14, 33, 68). The role of Th17 during *Brucella* infection has not been extensively explored. Two studies have reported that IL-17A neutralization (57) or the absence of IL-17Rα (65) did not at all influence the subsistence and growth of *Brucella* at 1 month and 1 week postinfection, respectively. However, another recent study has demonstrated a role for IL-17-producing T cells in osteoclastogenesis during murine brucellosis (21). The Th17 response implicates the coordinated production of IL-17, IL-21, and IL-22 by $CD4^+$ T cells following IL-23 secretion by antigen-presenting cells (for a review, see reference 5). In this study, we compared the ability of wild-type, IL-$12p35^{-/-}$, IL-$12p40^{-/-}$ (IL-$12/23^{-/-}$), IL-$17R\alpha^{-/-}$, IL-$21^{-/-}$, and IL-$22^{-/-}$ mice to control *Brucella* infection. Only IL-$12p35^{-/-}$ and IL-$12p40^{-/-}$ mice displayed susceptibility to *Brucella* infection, suggesting that components of the Th17 response are not essential to bacterial clearance. However, we observed significant transitory higher susceptibility in IL-$12p40^{-/-}$ mice than IL-$12p35^{-/-}$ mice. This difference suggests cooperation between the Th1 and Th17 responses during *Brucella* infection. A similar phenomenon during *Mycobacterium tuberculosis* infection has been reported previously (9, 34). When IL-12p70 is available, IL-23 is dispensable for protection. In the absence of IL-12p70, the lack of IL-23 enhanced susceptibility to *M. tuberculosis*. These compensations have also been described for *Salmonella enterica* (63) and *Toxoplasma gondii* (40).

In summary, our study demonstrates the key role played by IFN-γ-producing Th1 $CD4^+$ T cells in the control of *Brucella* infection and the negligible importance of $CD8^+$ T cells or humoral immunity in the clearance of bacteria during primary infection. We also showed that an IL-4-dependent Th2 response does not develop or play a negative role during the course of infection. However, the Th17 response could partially compensate for the lack of a Th1 response. These results could potentially improve our ability to develop a protective vaccine or therapeutic treatment against brucellosis. They suggest that favoring IL-12 production during *Brucella* vaccination, for example, by targeting $CD8^+$ dendritic cells, should lead to a better induction and persistence of IFN-γ-producing $CD4^+$ T cells and concomitantly allow better protection against *Brucella* infection.

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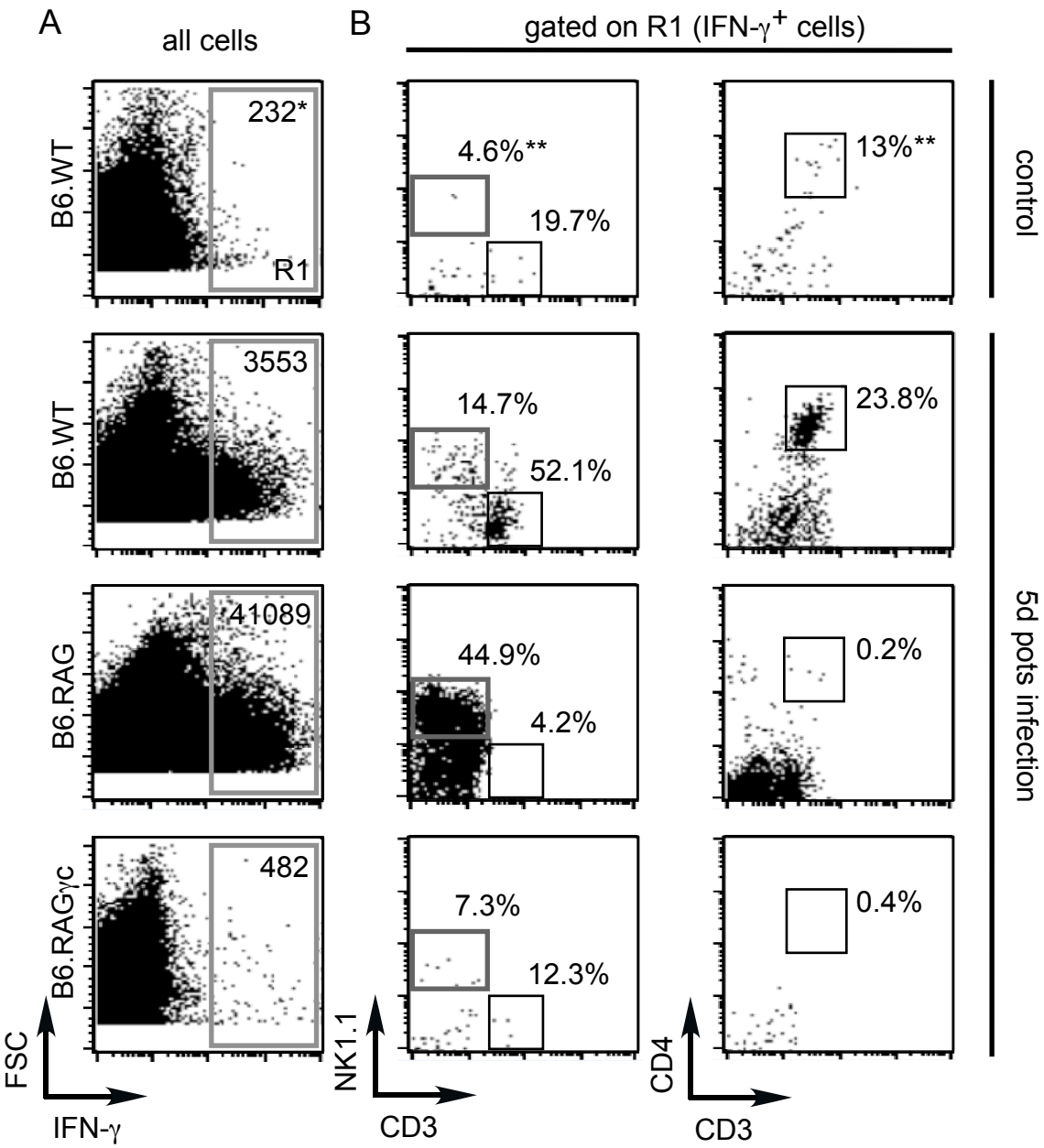
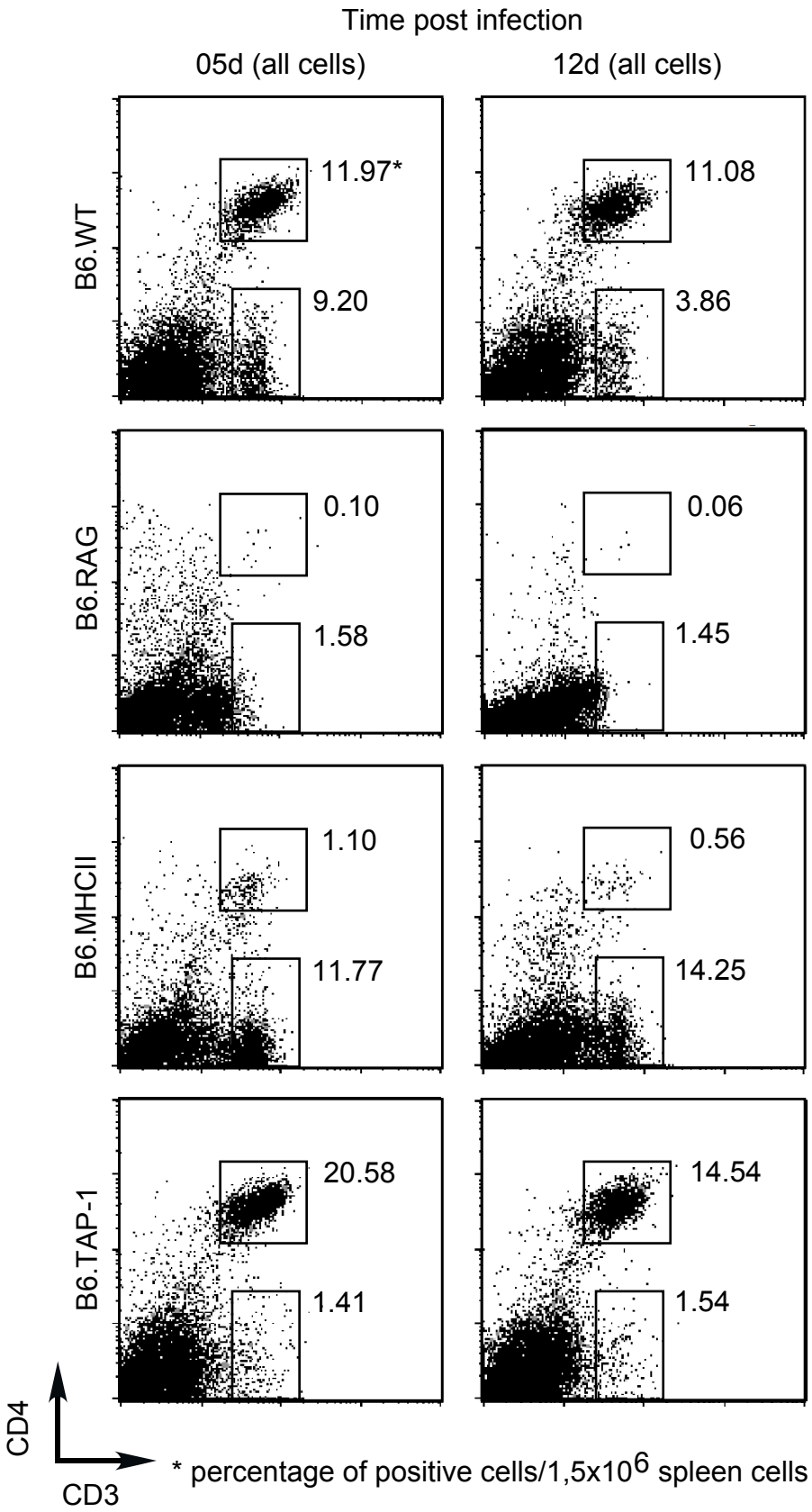
Supplemental material

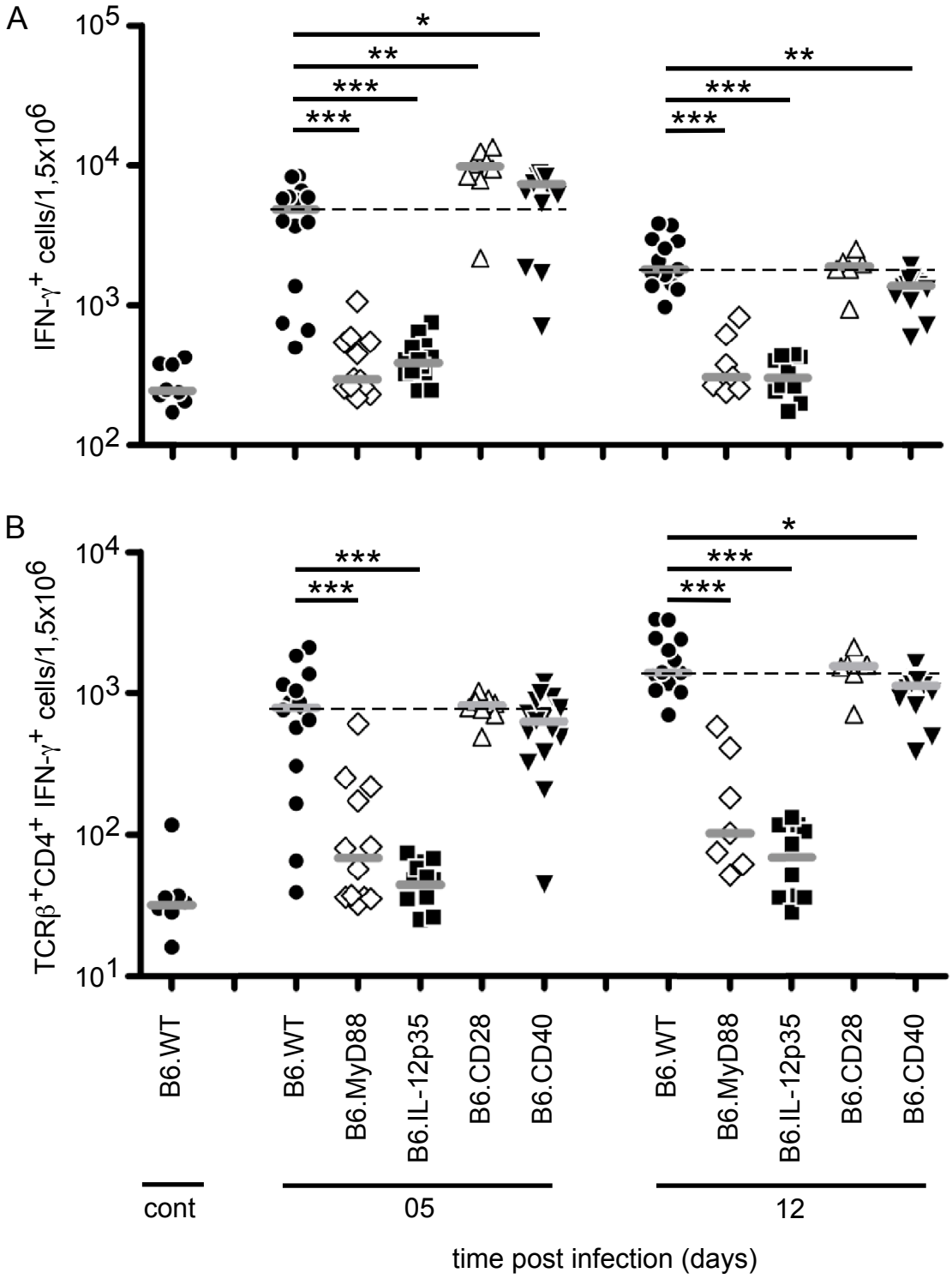


* number or ** percentage of positive cells/1,5x10^6 spleen cells

Supplementary Figure 1 (S1)



Supplementary Figure 2 (S2)



Supplementary Figure 3 (S3)

Figure S1. Flow cytometry analysis of IFN-γ-producing cells in wild-type versus RAG$^{-/-}$ and RAGγc$^{-/-}$ C57BL/6 mice infected by *B. melitensis*. Wild-type and deficient mice were injected i.p. with PBS (control) or 4x10^4 CFU of *B. melitensis*. Mice were sacrificed 5 days p.i. and spleen cells were collected and analyzed by flow cytometry. Cells were gated according to size and scatter to exclude dead cells and debris from analysis. Cells were first analyzed for Forward Size Scatter (FSC) versus IFN-γ production (A) and then IFN-γ positive cells were analyzed for CD3, NK1.1 and CD4 expression (B). In the selected quadrant, the number indicates the number (A) or the percentage of cells (B) per 1.5x10^6 spleen cells acquired and is representative of two independent experiments.

Figure S2. Flow cytometry characterization of splenic T cell populations from RAG$^{-/-}$, MHCII$^{-/-}$ and TAP-1$^{-/-}$ mice. Wild-type, RAG, MHCII and TAP-1 deficient C57BL/6 mice were injected i.p. with PBS (control) or 4x10^4 CFU of *B. melitensis*. Mice were sacrificed 5 and 12 days p.i. and spleen cells were collected and analyzed by flow cytometry. Cells were gated according to size and scatter to exclude dead cells and debris from analysis. Cells were analyzed for CD3 and CD4 expression. In the selected quadrant, numbers indicate the percentage of cells per 1.5x10^6 spleen cells acquired and are representative of two independent experiments.

Figure S3. CD28 and CD40 co-stimulatory molecules appear weakly implicated in the control of *B. melitensis* infection. Wild-type, IL-12p35, MyD88, CD28 and CD40 deficient C57BL/6 mice were injected i.p. with PBS (control) or 4x10^4 CFU of *B. melitensis* and sacrificed at the indicated time. A, Spleen cells were collected for flow cytometry analysis. Cells were gated according to size and scatter to exclude dead cells and debris from analysis. Spleen cells from individual mice were first analyzed for Forward Size Scatter (FSC) and

IFN-γ production. The data represent the number of IFN-γ positive cells per $1.5x10^6$ spleen cells acquired. Each piece of data represents the value obtained from an individual spleen and the data are representative of two independent experiments. B, IFN-γ positive cells were then analyzed for TCRβ and CD4 expression. The data represent the number of IFN-γ^+ TCRβ^+ $CD4^+$ cells from the groups described in B. Grey bars represent the median. Significant differences are denoted by an asterisk (*) for $p < 0.05$.

Supplemental table 1. Oligonucleotide sequences used for RT-PCR

	Oligonucleotides, 5'-3'
Murine β-actin	
Forward	TCC-TGA-GCG-CAA-GTA-CTC-TGT
Reverse	CTG-ATC-CAC-ATC-TGC-TGG-AAG
Probe	ATC-GGT-GGC-TCC-ATC-CTG-GC
Murine IFN-γ	
Forward	GGA-TGC-ATT-CAT-GAG-TAT-TGC
Reverse	GCT-TCC-TGA-GGC-TGG-ATT-C
Probe	TTT-GAG-GTC-AAC-AAC-CCA-CAG-GTC-CA
Murine IL-4	
Forward	AGA-TCA-TCG-GCA-TTT-TGA-ACG
Reverse	TTT-GGC-ACA-TCC-ATC-TCC-G
Probe	TCA-CAG-GAG-AAG-GGA-CGC-CAT-GC
Murine IL-17A	
Forward	GCT-CCA-GAA-GGC-CCT-CAG
Reverse	CTT-TCC-CTC-CGC-ATT-GAC-A
Probe	ACC-TCA-ACC-GTT-CCA-CGT-CAC-CCT-G

2 Part II : *Brucella melitensis* invades murine erythrocytes during infection

Marie-Alice Vitry, Delphine Hanot Mambres, Marjorie Vermeersch, Katrin Hack, Frédérique Lhommé, Jean-Marie Vanderwinden, Carl De Trez, David Pérez-Morga, Jean-Jacques Letesson and Eric Muraille

In preparation

Understanding immunological responses developed following infection is a major concern in the design of a successful vaccine. However the identification of the localization and the nature of infected cells is also a point to take into consideration. Previous study by our group has characterized the cell surface phenotype of infected cells in the spleen and the liver ((Copin *et al.*, 2012), see **Appendix-3**). This work also reported that *Brucella* was detectable in the spleen 10 minutes after i.p. inoculation, indicating that bacteria could transit freely by the blood to reach its targeted organs. However, the fate of *Brucella* from the entry point to the final destination in the host remains currently unknown.

In this context the second part of this thesis focus on the dissemination of *Brucella* through the blood (bacteremia), an early event happening just after *Brucella* infection and currently poorly described (Pappas & Papadimitriou, 2007). Bacteremia is one major characteristic of human brucellosis and is often associated with an increased risk of relapse. Moreover positive blood cultures are synonymous with secondary seeding and development of focal complications of the disease (Ariza *et al.*, 1995; Solera *et al.*, 1998).

In our study, we monitored the bacteremia in mice for several days after primary *B. melitensis* infection. The localization of *B. melitensis* in blood cells was investigated using imagestream analysis, confocal and electronic microscopy. We also analysed the impact of natural or induced antibody on the blood persistence of bacteria by using mice rendered genetically deficient[1].

Our results show that *B. melitensis* is able to persist several weeks in the blood of infected mice after i.p. inoculation. Bacteria were firstly localized extracellularly but

[1] Additional results on genetically deficient mice tested during this thesis can be found in the Appendix section (chapter 2). Because they have not been always replicated or display variability, these results are only shown for guidance and will not been discussed here.

surprisingly, after 24 hours of infection, were mainly found inside erythrocytes. To our knowledge, this particular niche of infection has never been described in brucellosis, although relatively common in other bacteria from the alpha-proteobacteria phylum such as *Anaplasma* and *Bartonella*. The finding that *B. melitensis* is able to infect erythrocytes and persist for a long time in the blood opens new areas of research in brucellosis. In particular, this phenomenon and old studies describing the presence of *Brucella* in blood-sucking arthropods (Gudoshnik, 1958; Ozsan, 1962; Wellmann, 1951; Wellmann, 1952; Zheludkov & Tsirel'son, 2010) strongly suggest a possible unexpected way of infection in the natural environment.

Manuscript

The following manuscript is still in preparation and is not submitted to a journal nor published. Therefore this is not a final version.

Brucella melitensis invades murine erythrocyte during infection

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Key words: *Brucella melitensis*, erythrocyte, infection

Running title: Erythrocyte infection by *Brucella melitensis*

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ABSTRACT

Brucella spp are facultative intracellular Gram-negative coccobacilli responsible for brucellosis, a worldwide zoonosis. Surprisingly, we observe that *Brucella melitensis* is able to persist several weeks in the blood of infected mice where it exhibits a brief extracellular phase. Imagestream analysis of blood cells showed that *Brucella* is mainly associated to erythrocytes. Examination by confocal laser scanning microscopy, as well as scanning and transmission electron microscopy, demonstrated that the localization of *B. melitensis* was intraerythrocytic. Bacteria did not seem to multiply in erythrocytes and were found free in the cytoplasm. Circulating Ig induced by vaccination impaired blood persistence following challenge. In conclusion, we demonstrate for the first time that *B. melitensis* is able to invade the erythrocytes *in vivo*. Erythrocyte colonization could protect the bacterial cells from the host's immune response and hamper antibiotic treatment. Our results should serve to the elaboration of novel strategies in treatment or prophylaxis of brucellosis.

INTRODUCTION

Brucella is a facultative intracellular α-Proteobacteria that causes abortion and infertility in mammals and leads to a debilitating febrile illness in humans. Although it is rarely fatal, human brucellosis can progress into a severe chronic disease without prolonged antibiotic treatment (Cutler *et al.*, 2005; Godfroid *et al.*, 2005). Despite significant progresses, the incidence of human brucellosis remains very high in endemic areas (Pappas *et al.*, 2006). As the complete eradication of *Brucella* would be unpractical due to its presence in a large range of wild mammals (Gregoire *et al.*, 2012; Zheludkov & Tsirel'son, 2010) and because antibiotic treatment is costly and patients frequently suffer from resurgence of the bacteria (Ariza *et al.*, 1995; Solera *et al.*, 1998), vaccination remains the only rational strategy to confer protection to populations living in endemic countries. Unfortunately, there is currently no available vaccine against human brucellosis as all commercially available animal vaccines are based on live attenuated strains of *Brucella* (Schurig *et al.*, 2002) that display several drawbacks and cause disease in humans. Progress in vaccination is impaired by our poor knowledge on *Brucella* life style *in vivo* and on the protective immune response induced by infection. In this context, we have recently demonstrated that dendritic cells and macrophage are the main infected cells in the spleen and liver during chronic *Brucella* infection (Copin *et al.*, 2012) and that IFN-γ producing $CD4^+$ T cell plays a crucial role in controlling infection (Vitry *et al.*, 2012). However, the mechanisms of early *Brucella* dissemination *in vivo* remain largely unknown. A prominent characteristic of brucellosis is the long incubation period. The current hypothesis is that this period, corresponding to a low activation of the innate immunity, 'opens an immunological window' and gives a chance to *Brucella* to spread throughout the organisms to establish a replication niche within phagocytic cells (Martirosyan *et al.*, 2011).

Bacteremia is one major characteristic of human brucellosis and is often associated with an increased risk of relapse. Moreover positive blood cultures are synonymous with secondary seeding and development of focal complications of the disease (Pappas & Papadimitriou, 2007; Solera *et al.*, 1998). Bacteremia in mice infected by intraperitoneal injection, the most frequent mode of experimental infection, has been reported in some studies (Braude, 1951; Rajashekara *et al.*, 2005). However, the mechanism of *Brucella* persistence in the blood has never been further investigated. In this study, we monitored the bacteremia in mice for several days after intraperitoneal inoculation of a *B. melitensis* 16M

strain constitutively expressing the mCherry fluorescent tracer. The location of *B. melitensis* in blood cells was analyzed using imagestream analysis, confocal and electronic microscopy. We also analyzed the impact of natural or induced antibody on the blood persistence of bacteria by using mice rendered genetically deficient.

Our results show that *B. melitensis* is able to persist at least two weeks in the blood of infected mice after intraperitoneal inoculation. Bacteria were first located extracellularly but surprisingly, after 24 hours of infection, were mainly found inside erythrocytes. To our knowledge, this particular niche of infection has never been described in brucellosis, although relatively common in other bacteria from the α-proteobacteria phylum such as *Anaplasma* and *Bartonella*. Circulating Ig induced by vaccination impaired blood persistence following challenge.

RESULTS

Long term persistence of *B. melitensis* in the bloodstream.

In the *B. melitensis* intraperitoneal (i.p.) infection model, we have previously observed that bacteria are detectable in the spleen 10 minutes after inoculation (Copin *et al.*, 2012). This result suggests that bacteria must transit freely by the blood after i.p. inoculation to reach the spleen. In order to determine the persistence of bacteria in the blood, we collected samples from wild type C57BL/6 mice infected with different doses ($4x10^4$, 10^6 or $5x10^7$ CFU) of mCherry-expressing *Brucella melitensis* (mCherry-Br) and determined the bacteria titers (Figure 1.A). Bacteria were detectable in the blood as soon as 10 minutes after i.p. inoculation (data not shown). Surprisingly, we observed that bacteria persist at least two weeks in the blood of infected mice (Figure 1.A). When a high dose of bacteria ($5x10^7$ CFU) is inoculated, 100% of mice display a mean of almost 10^5 CFU/ml of blood at 6 days p.i.. Clearance of bacteria appears dose-dependent since at more classical dose ($4x10^4$ CFU) only 50% of mice did not manage to clear the infection from the blood and displayed a mean of 10^2 CFU/ml of blood at 6 days p.i. (Figure 1.A and 1.B).

***Brucella* is found associated with erythrocyte**

While bacteremia is common in human brucellosis (Yagupsky, 1999), to our knowledge, presence of *Brucella* in the blood of infected mice has been rarely reported. In a study of Braude and Coll. in 1951 (Braude, 1951), it was mentioned that approximately 25% of bacteria located outside of blood cells. In order to clarify this question, we centrifuged the blood from mice infected with two different doses ($4x10^4$ or 10^6 CFU) to separate the blood cells from the plasma at different times early after infection (Figure 1.C). At 3h post infection, bacteria were mainly found in the plasma (70 to 75%) and only 20 to 30% of bacteria were found in the pellet. However, 3 days after infection with the higher dose (10^6 CFU), the pellet percentage increased to reach about 80%. In order to confirm these results, the location of mCherry-Br in the blood during the course of infection was examined by fluorescent microscopic analysis (Figure 2). $5x10^7$ CFU of mCherry-Br were i.p. injected in transgenic mice expressing GFP under the control of ubiquitin promoter (Schaefer et al., 2001) and the blood was collected at 3, 24 and 72h post infection. Note that collected blood was immediately diluted in paraformaldehyde for fixation without centrifugation or staining in order to avoid any artifact. Microscopic observations showed that *Brucella* locates mainly

extracellularly at 3h post infection (data not shown). At later time, *Brucella* is mainly found associated with erythrocytes (several examples in Figure 2). Whatever the time tested, we never observed a binding of *Brucella* to nucleated ($DAPI^+$) cells.

In order to rigorously quantify the location of *Brucella* in the blood, we performed an imagestream analysis (Figure 3) on blood samples from wild type infected mice. In a preliminary experiment, we confirmed that imagestream analysis was able, based on morphological and fluorescent parameter, to detect and discriminate between mCherry-Br, blood cells and RAW cells (Figure 3, A, B, C). Uninfected RAW cells and *in vitro* mCherry-Br infected RAW cells were used as internal control to demonstrate that we are able to visualize *Brucella* inside nucleated cells. Kinetic imagestream analysis of infected blood (Figure 3.D) showed that some bacteria were found extracellular (free or adherent to cells) at 3 and 24h post infection but not at 72h post infection. In contrast, the percentage of bacteria found inside cells increased exponentially until 72h post infection (Figure 3.E). Surprisingly, bacteria were mainly detected associated or inside erythrocytes. The percentage of infected erythrocytes is around 1%. Whatever the time tested, we never observed a binding of *Brucella* to nucleated cells or several bacteria inside erythrocytes.

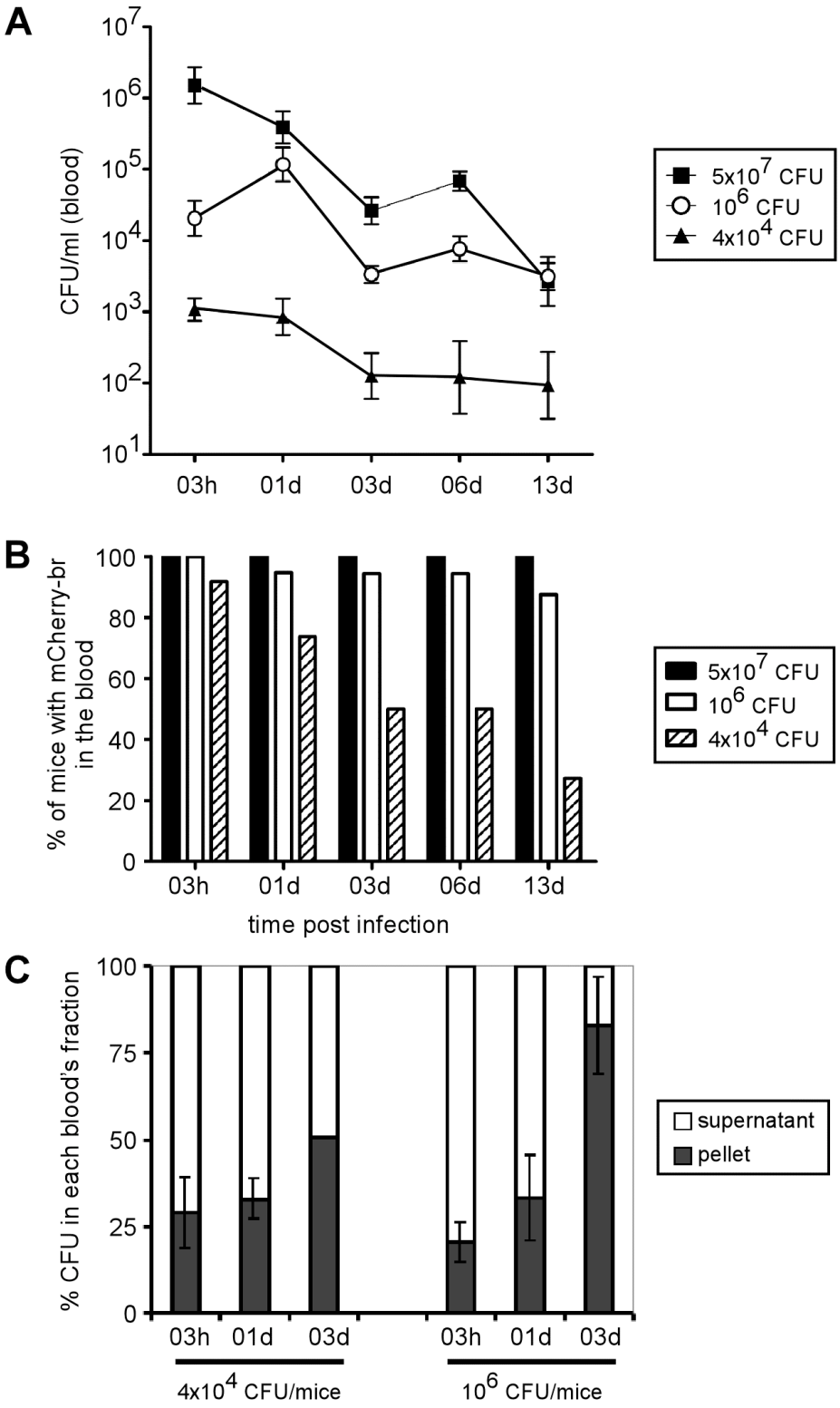

FIGURE 1. Persistence of *B. melitensis* in the blood of mice after i.p. infection. A, B: Mice were injected i.p. with $4x10^4$, 10^6 or $5x10^7$ CFU of mCherry-expressing *B. melitensis* (mCherry-Br) and blood was collected at selected time. Data represent either the mean +/- SEM of mCherry-Br CFU per ml of blood (**A**) or the percentage of mice that have still bacteria in the blood (**B**). Results are representative from two independent experiments. **C**, Mice were injected i.p. with $4x10^4$ or 10^6 CFU of mCherry-Br and blood was collected at indicated time. Samples were diluted and centrifuged to separate blood cells and plasma. Data represent the mean +/- 1 SD of the percentage of CFU in each blood fraction and are representative of two independent experiments.

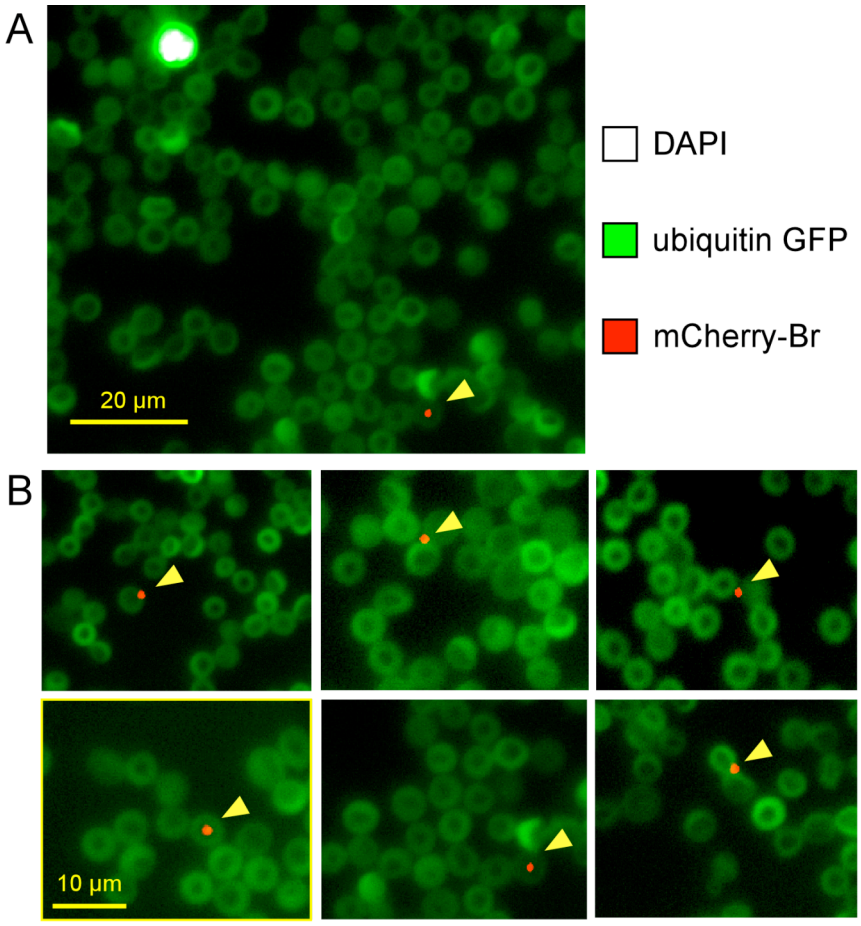

FIGURE 2. Microscopic analysis of blood from infected mice. Ubiquitin-GFP C57BL/6 mice were i.p. infected with $5x10^7$ CFU of mCherry-Br and bleed at 3h and 24h post infection. Blood were fixed and then smeared on slide. After overnight drying, slides were mounted in Fluoro-Gel medium containing DAPI nucleic acid stain (Electron Microscopy Sciences, Hatfield, PA) and examined with a fluorescent microscope. *Brucella* was found associated to erythrocytes mainly at 24h, thus we presented only images from 24h post infection. **A.** White cells ($DAPI^+$) and erythrocytes ($DAPI^-$) with mCherry bacteria. **B.** Some examples of mCherry bacteria associated with erythrocytes ($DAPI^-$). Panels are color-coded according to the antigen examined. Scale bar = 20 or 10 µm, as indicated. Data are representative of at least 3 independent experiments with a total of 86 bacteria observed.

FIGURE 3 (SEE BELOW). Images of *B. melitensis* in the blood of infected mice. Wild type C57BL/6 mice were injected i.p. with $5x10^7$ CFU of mCherry-Br and blood was collected at 3h, 24h and 72h post infection. Blood samples were immediately fixed by 3-fold dilution in paraformaldehyde 4% and then analyzed using multispectral imaging flow cytometry (ImageStream 100). As control, we used fresh culture of wild type *Brucella*, mCherry-Br and a macrophage cell line (RAW cells) *in vitro* infected since 3h with mCherry-Br. **A.** R1 gate is based on area and aspect ratio of events (i.e. the ratio of the length to the breadth of an event). R1 gate is defined in order to exclude aggregate of cells. Numbers indicate the percentage of events among R1 gate. **B.** Comparison of fluorescence intensity among R1 gate in mCherry channel 4 of bacteria culture and blood from control and infected mice. Numbers indicate the percentage of mCherry positive events (gate R2). **C.** Representative image of free bacteria, uninfected and infected cells in channel 2 and 4 among R1 gate. **D, E**. Kinetic analysis of the percentage of extracellular (**D**) and intracellular (**E**) bacteria found among R1 and R2 gate in blood sample from control and infected mice. The location of bacteria is defined using erode mask (see Materials and Methods). These data are representative of three independent experiments.

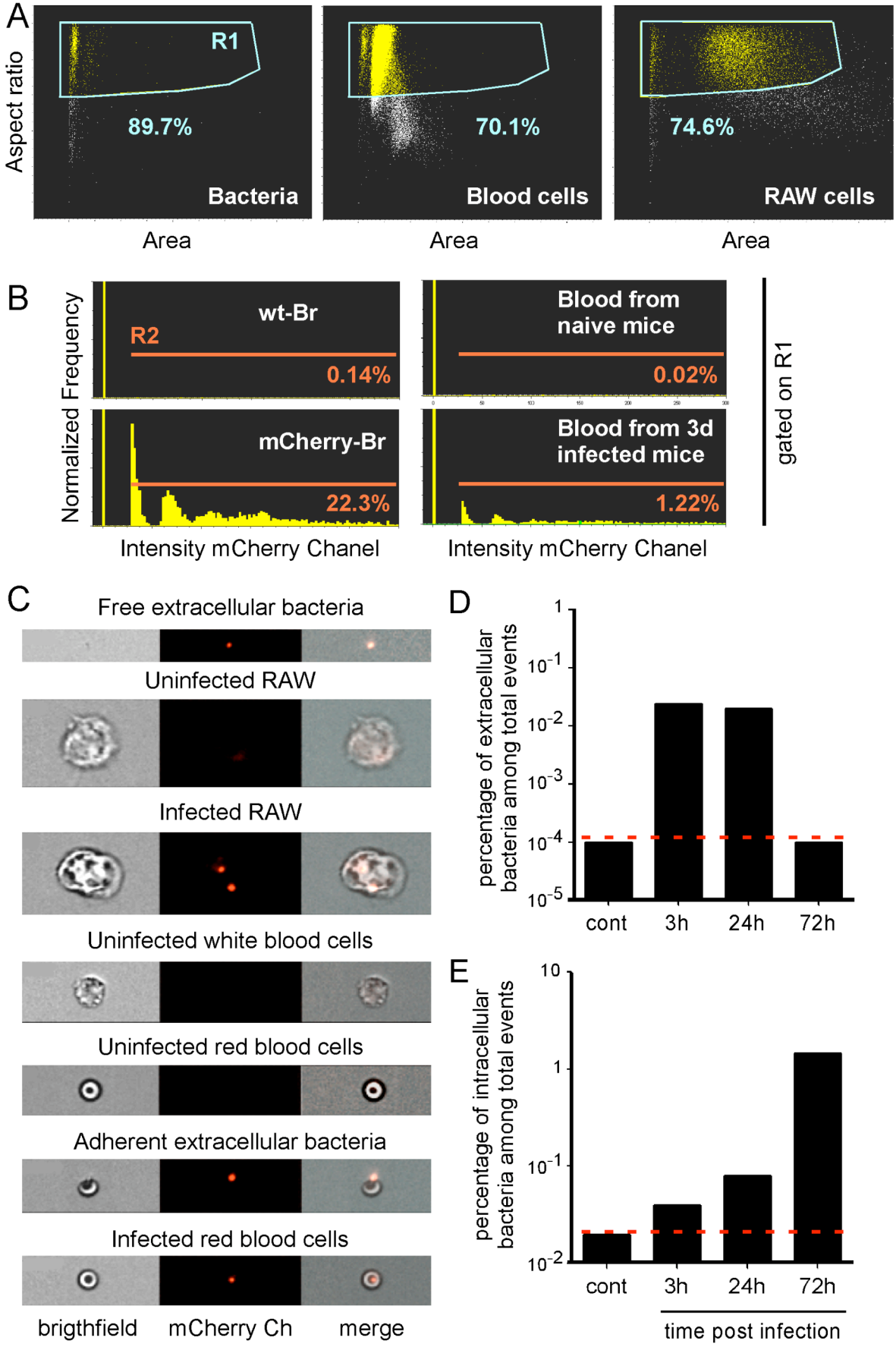
A
Aspect ratio
R1
89.7%
Bacteria
70.1%
Blood cells
74.6%
RAW cells
Area
Area
Area
B
Normalized Frequency
wt-Br
R2
0.14%
mCherry-Br
22.3%
Blood from naive mice
0.02%
Blood from 3d infected mice
1.22%
gated on R1
Intensity mCherry Chanel
Intensity mCherry Chanel
C
Free extracellular bacteria
Uninfected RAW
Infected RAW
Uninfected white blood cells
Uninfected red blood cells
Adherent extracellular bacteria
Infected red blood cells
brigthfield
mCherry Ch
merge
D
percentage of extracellular bacteria among total events
1
10-1
10-2
10-3
10-4
10-5
cont
3h
24h
72h
E
percentage of intracellular bacteria among total events
10
1
10-1
10-2
cont
3h
24h
72h
time post infection

Figure 3

Confocal and electron microscopic analysis confirm the invasion of erythrocyte by *Brucella*

As the image resolution of imagestream analysis is very low, we used electron and confocal microscopic analysis in order to fully confirm the presence of *Brucella* inside erythrocytes. Scanning electron microscopy of blood from infected ubiquitin-GFP (Ubi-GFP) mice showed that *Brucella* is either found free in the blood (Figure 4.A), attached to erythrocyte (Figure 4.B) or inside erythrocyte (Figure 4.C-F) at 3 hours post infection in the blood samples. Using confocal microscopy, we performed a Z-stack analysis of infected erythrocytes. These images were processed for 3D reconstruction by Imaris program. At 24h post infection (Figure 5), we confirmed that a great majority of bacteria were found inside erythrocytes. In order to gain more information on the mechanism of erythrocyte invasion by *Brucella*, we performed transmission electron microscopic analysis on erythrocytes from 24h -infected mice. Surprisingly, we detected intraerythrocytic *Brucella* which were not surrounded by a vacuole membrane, indicating that these bacteria were free in the cytoplasm of erythrocytes (Figure 6). This particular location has been occasionally observed in other intraerythrocytic pathogens like *Bartonella bacilliformis* (Benson *et al.*, 1986) and *Mycoplasma suis* (Groebel *et al.*, 2009).

In order to further characterize the mechanism of erythrocyte invasion by *Brucella*, we tested several *in vitro* protocols to infect erythrocytes from naïve mice. Unfortunately, in conditions where RAW cells were fully infected, we never observed erythrocytes infection (data not shown).

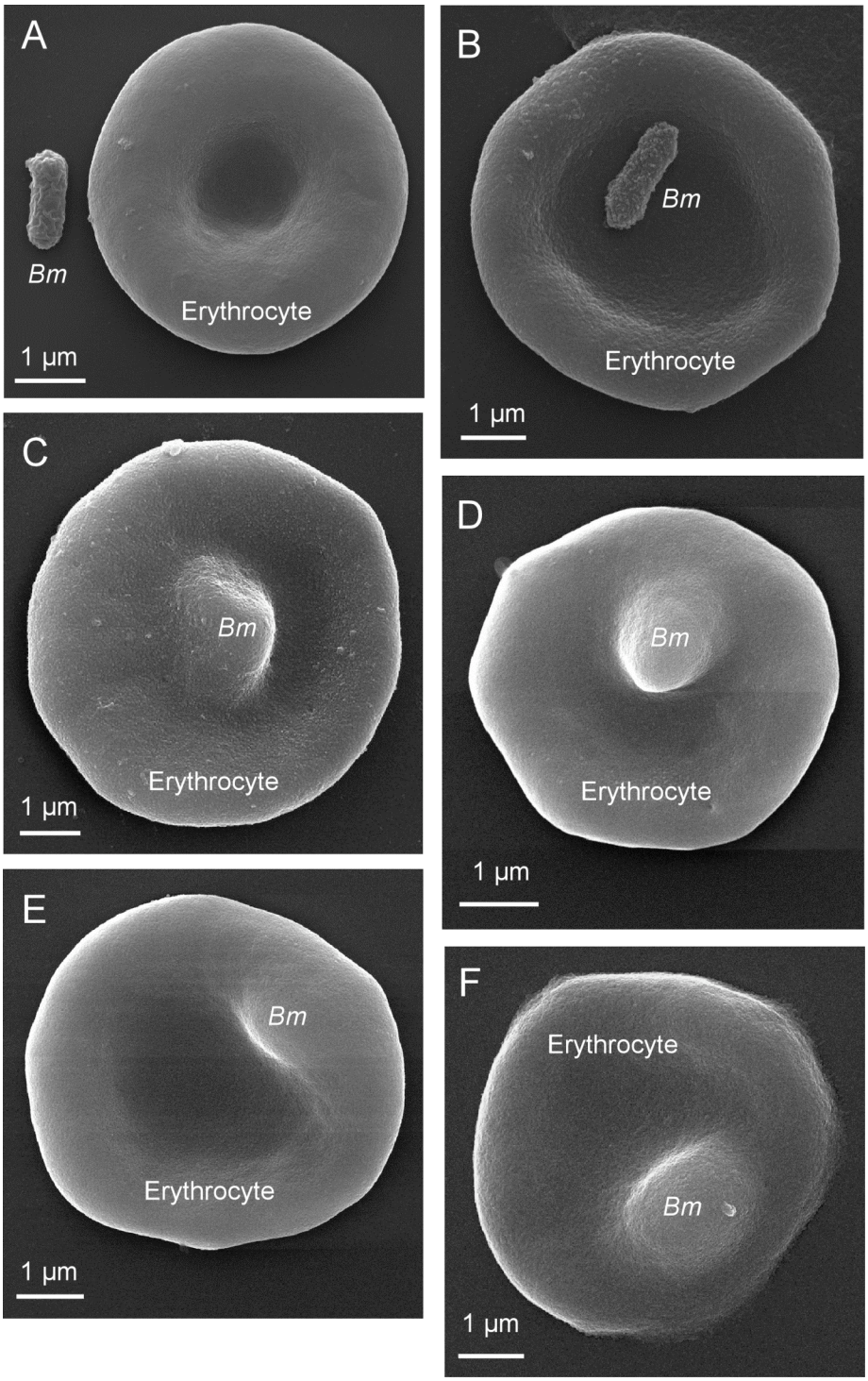

FIGURE 4. *Brucella* is found associated with or inside erythrocytes by scanning electronic microscopy. Scanning electron microscopy images from blood collected in mice infected since 3h by i.p. injection of $5x10^7$ CFU of *Brucella*. Extracellular bacteria (**A**), attachment of *Brucella* to erythrocyte (**B**), *Brucella* inside erythrocyte (**C-F**).

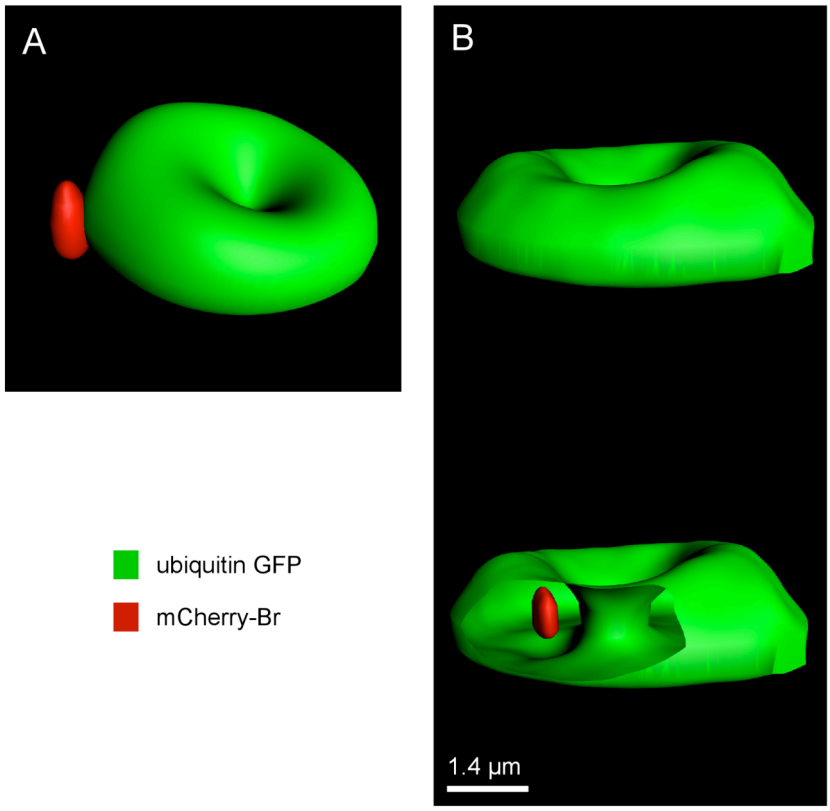

FIGURE 5. Confocal microscopic analysis of *Brucella* infected erythrocytes. Confocal microscopy images from blood collected in mice infected since 24h by i.p. injection of $5x10^7$ CFU of *Brucella*. Attachment of *Brucella* to erythrocyte (**A**), *Brucella* inside erythrocyte (**B**).

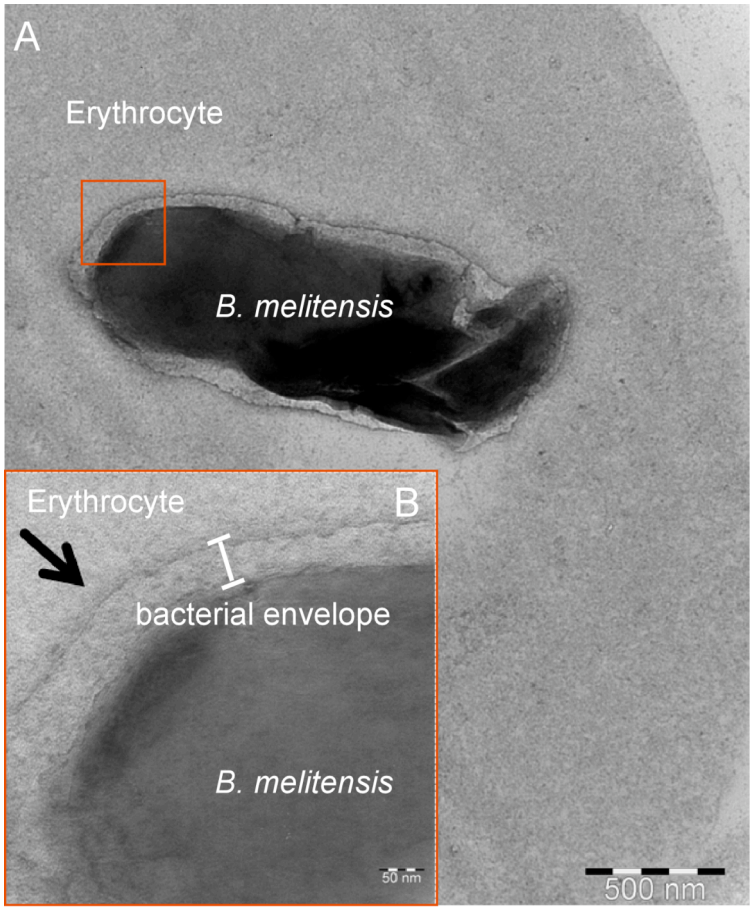

FIGURE 6. *Brucella* is not surrounded by a vacuole membrane in erythrocytes. Transmission electronic microscopic analysis of erythrocytes from mice infected since 24h by i.p. injection of $5x10^7$ CFU of *Brucella* (**A**). Higher magnification of bacterial envelope showing the absence of vacuole surrounding *Brucella* in the erythrocyte (**B**).

Vaccination-induced specific antibodies are able to reduce the persistence of *Brucella* in the bloodstream

Some studies have reported that natural IgM antibodies play a crucial role in early trapping of viral and bacterial particles. This capture reduces pathogen dissemination and infection of organs (Boes, 2000; Ochsenbein et al., 1999). The extracellular location of bacteria during the first 24h of infection suggests that they are potentially exposed to antibodies. In order to determine the impact of circulating antibodies on this persistence, we compared the level of *Brucella* in the blood of wild type and MuMT$^{-/-}$ mice (B-cell deficient) infected with 5x10^7 CFU (Figure 7.A). Results showed that absence of circulating antibodies in MuMT$^{-/-}$ mice did not modify the number of blood bacteria between 1 and 3 days post-infection. In agreement, kinetic analyses of *Brucella*-specific antibodies in serum of infected mice showed specific IgM Abs appearing at 6 days, whereas IgG2 and IgG3 Abs were detectable by 28 days post-infection (Figure 7.B). In striking contrast, when 5x10^7 CFU were injected in mice previously immunized with live virulent bacteria 60 days before and cured of infection with antibiotics, we observed that persistence of *Brucella* in the blood was strongly reduced in wild type mice but not in MuMT$^{-/-}$ mice, demonstrating that specific antibodies are able to impair the access of *Brucella* to erythrocytes.

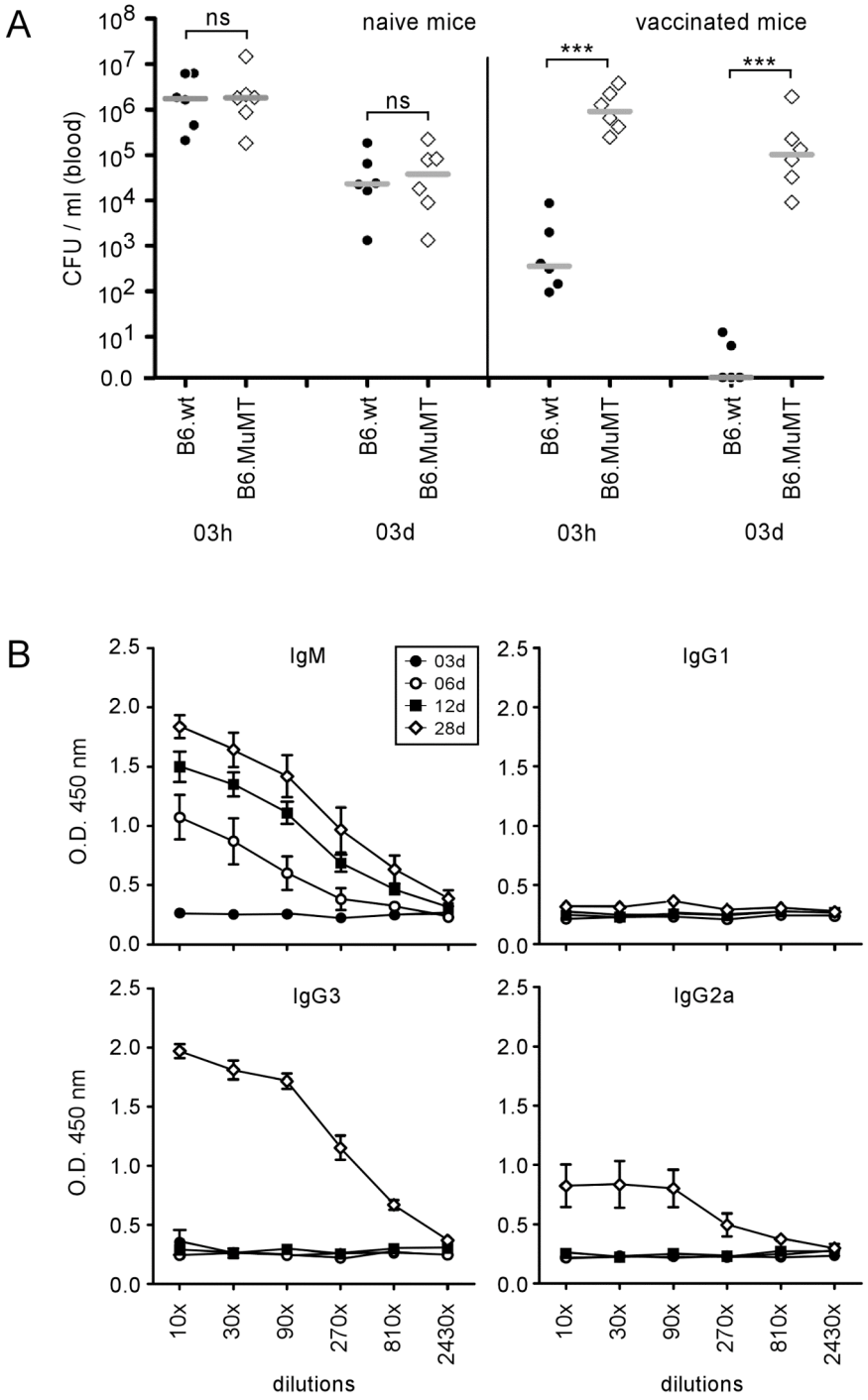

FIGURE 7. Natural antibodies do not prevent *B. melitensis* dissemination in the blood whereas vaccine induced specific antibodies reduce the bacteremia following a re-call infection. A, Wild type and MuMT$^{-/-}$ C57BL/6 mice were immunized i.p. with 4x10^4 CFU of *B. melitensis* (vaccinated group) or injected i.p. with PBS (naive group) and then treated with antibiotics as previously described (in preparation – see Part III of this thesis). After 60 days, all mice were injected i.p. with 5x10^7 CFU of mCherry-Br and blood was collected at indicated time. Data represent the CFU per ml of blood and are representative of three independent experiments. Grey bars represent the median. **B,** Wild-type C57BL/6 mice were infected with 4x10^4 CFU of *B. melitensis*. Sera were collected at indicated times and ELISA was performed to detect isotype distribution of *Brucella*-specific antibodies. Data represent the mean +/- SEM of 6 mice.

DISCUSSION

Using imagestream analysis, confocal laser scanning microscopy, as well as scanning and transmission electron microscopy, we demonstrated for the first time that erythrocyte invasion allows persistence of *B. melitensis* in the bloodstream following intraperitoneal injection. Until now, the virulence of *Brucella in vivo* has been associated with its ability to replicate in phagocytic cells of the innate immune system, such as macrophages and dendritic cells (Archambaud *et al.*, 2010; Billard *et al.*, 2005; Copin *et al.*, 2012). However *in vitro* studies have shown that *Brucella* can invade and replicate in a range of nonphagocytic cells (trophoblasts, epithelial cells, fibroblasts) (Anderson *et al.*, 1986; Detilleux *et al.*, 1990; Holland & Pickett, 1956; Pizarro-Cerda *et al.*, 1998). Persistence of *Brucella* in the blood inside erythrocytes raises many questions.

We have first tried to determine if the control of *B. melitensis* persistence in the blood implicates circulating antibodies and B cells. Some studies have reported an important protective function for natural antibodies in the control of early dissemination of virus (Vesicular Stomatitis Virus, vaccinia virus, Lymphocytic ChorioMeningitis Virus) and facultative intracellular bacteria (*Listeria monocytogenes*) (Boes, 2000; Ochsenbein *et al.*, 1999). Natural antibodies could increase the early trapping of pathogens to secondary lymphoid organs by enhancing phagocytosis. The role of natural antibodies has been previously analyzed in *Brucella* experimental model by several groups with contradictory results. Natural antibodies have been reported to favor the control of attenuated *Brucella* mutant by facilitating its elimination by phagocytosis (Rolan *et al.*, 2009). Many serum transfer experiments have demonstrated a positive role of antibodies in protecting mice against *Brucella* infection (Araya *et al.*, 1989; Montaraz & Winter, 1986; Winter *et al.*, 1989). In this study, we were unable to detect *Brucella* specific antibodies in naïve mice or during the early days following infection. Specific IgM antibodies were first detected at 6 days and IgG Abs at 28 days with a predominance of IgG2a- and IgG3-switched isotype Abs. This observation and the fact that the number of bacteria found in the bloodstream during the course of infection appears similar in wild type and MuMT$^{-/-}$ mice demonstrate that natural antibodies have no or a weak impact on *Brucella* trapping in naïve mice in our experimental conditions. In contrast, we observed that specific circulating antibodies present in wild type vaccinated mice dramatically reduced the count of bacteria persisting in the blood after a

secondary infection. This suggests that blockage of access to erythrocyte reservoir could constitute an important step in protective immune response against *Brucella* infection.

Brucella requires iron to maintain a chronic infection in mice (Paulley *et al.*, 2007) and is able to utilize heme as an iron source *in vitro* and *in vivo* (Ojeda *et al.*, 2012; Paulley *et al.*, 2007). Iron source necessary for virulence of *Brucella* has often been associated with macrophages infection, due to their central role in heme recycling in mammals (Crichton *et al.*, 2002). When the macrophages engulf and degrade senescent erythrocytes (Bratosin *et al.*, 1998), hemoglobin and heme are released into the cell. Moreover macrophages are able to scavenge heme and hemoglobin released from damaged cells to avoid a toxic level of iron in the body (Taketani, 2005). Both the host and the pathogen require iron because this metal is essential in many basic cellular functions and metabolic pathways. As free iron catalyses the formation of free oxygen radicals, it is sequestered within ferritin or as cofactor of heme complexed to hemoglobin within erythrocytes. Iron is thus largely intracellular and any extracellular free iron is rapidly bound by circulating transferrin. This withholding of iron constitutes a first line of defense against bacterial infection and is termed nutritional immunity (Skaar, 2010). Consequently acquisition of sufficient amounts of iron by the pathogen in the iron-restricted environment of the host is a struggle for life, in particular for intracellular bacteria (Schaible & Kaufmann, 2004). The present study indicates that *Brucella* could find the iron required for its virulence by directly infecting erythrocytes, which contain large amounts of heme-bound iron.

Although erythrocytes do not appear to constitute a replication niche for *Brucella* since each cell contained only one single bacterium, they could serve as an important part of the pathogen reservoir. Given the long lifespan of erythrocytes (120 days, (Shemin & Rittenberg, 1946)), *Brucella* could potentially persist hidden in this environment for several months. The undulating fever aspect of human brucellosis and the high risk of relapse also argue for regularly occurring new bacteremia waves. In this context, the failure to replicate within erythrocytes could be advantageous for *Brucella*.

Our data have been obtained in the murine experimental model of infection and their relevance in the natural host remains to be demonstrated. However, bacteremia is common in human brucellosis (Pappas & Papadimitriou, 2007) and an *in vitro* study has reported that *Brucella* is able to bind sialic acid residues on human erythrocytes (del C. Rocha-Gracia *et al.*, 2002), suggesting that erythrocyte invasion may occur in patients. In addition, the unexpected ability of *Brucella* to infect erythrocytes should not be so surprising. Indeed several bacterial pathogens belonging to the α- (*Bartonella spp, Anaplasma marginale*) and γ-

(*Francisella tularensis*) proteobacteria phylum persist inside erythrocytes in the bloodstream during the course of infection and are transmitted to host by ticks or fleas. Moreover, old studies have raised the possibility of *Brucella* transmission by blood-sucking arthropods (ticks and fleas) (Gudoshnik, 1958; Ozsan, 1962; Wellmann, 1951; Wellmann, 1952; Zheludkov & Tsirel'son, 2010).

In conclusion, invasion of erythrocytes could constitute a crucial mean to enhance evasion of host immune defenses and transmission by blood-sucking arthropods. This new trait of *Brucella* pathogenesis opens new areas of research. Since erythrocytes are not capable of endocytosis (Schekman & Singer, 1976), defining the precise mechanism of *Brucella* uptake by the cell and identifying virulence factors necessary for this process should be of great interest. In addition it should be interesting to investigate the homing of infected erythrocytes in tissues and whether they trigger a different immune response than other infected cells. Determining the importance of this mechanism to pathogenesis and transmission of infection thus remains an exciting challenge.

MATERIALS AND METHODS

Ethics Statement

The animal handling and procedures of this study were in accordance with the current European legislation (directive 86/609/EEC) and in agreement with the corresponding Belgian law "*Arrêté royal relatif à la protection des animaux d'expérience du 6 avril 2010 publié le 14 mai 2010*". The complete protocol was reviewed and approved by the Animal Welfare Committee of the Facultés Universitaires Notre-Dame de la Paix (FUNDP, Belgium)(Permit Number: 05-558).

Mice and reagents

MuMT$^{-/-}$ C57BL/6 mice (Kitamura *et al.*, 1991) and C57BL/6 mice transgenic for GFP under the control of ubiquitin C promoter (Ubi-GFP) (Schaefer *et al.*, 2001) were purchased from The Jackson Laboratory (Bar Harbor, ME). Wild type C57BL/6 mice were purchased from Harlan (Bicester, UK) and were used as control. All wild type and deficient mice used in this study were bred in the animal facility of campus Gosselies from the Free University of Brussels (ULB, Belgium).

B. melitensis strain 16M (Biotype1, ATCC 23456) was grown in biosafty level III laboratory facility. Overnight culture grown with shaking at 37°C in 2YT media (Luria-Bertani broth with double quantity of yeast extract) to stationary phase was washed twice in PBS (3500xg, 10 min.) as previously described (Copin *et al.*, 2007). When indicated, we used a strain of *B. melitensis* strain 16M stably expressing the mCherry protein (mCherry-Br), a previously described rapidly maturing variant of the red fluorescent protein DsRed (Shaner *et al.*, 2004), under the control of a strong *Brucella spp.* promoter named PsojA (Kohler *et al.*, 1999). The construction of mCherry-Br strain has been previously described in detail (Copin *et al.*, 2012).

Mouse infection

Mice were injected intra-peritoneally (i.p.) with indicated dose of *B. melitensis* in 500 µl of PBS. Control animals were injected with the same volume of PBS. Infectious doses were validated by plating serial dilutions of inoculums. Mice were bled at selected time intervals and sacrificed at the end of the experiment.

RAW cells infection

RAW cells grown in 6-well tissue culture plates were inoculated with mCherry-Br at a multiplicity of infection (MOI) of 300 in 2 ml of cell culture medium (DMEM 10% FBS). Cells were then centrifuged at 1200 g for 10 minutes at 4°C and placed under 5% CO_2 atmosphere at 37°C. After 1 h, cells were washed twice in PBS and incubated further with cell culture medium supplemented with 50 μg/ml gentamicin until the end of the infection time. After indicated time of incubation, cells were washed three times in PBS, collected and centrifuged at 1200 g for 10 minutes at 4°C. Cells were fixed in 300 μl of PBS/4% PFA for further imagestream analysis.

Bacterial count

For bacterial counts in the blood, 70 μl of blood were collected at the tail with heparinated capillaries at selected time intervals and diluted in PBS/0,1% X-100 triton (Sigma). Serial dilutions in PBS were performed and plated onto 2YT media plates. CFU were counted after 4 days of culture at 37°C.

To separate the blood cells from the plasma, 140 μl of blood were collected and diluted 4 times to allow a better separation. Samples were then centrifugated at 250g for 10 minutes at room temperature (RT) and each blood's fraction was recovered. An additional centrifugation (8000g, 2 minutes, RT) was made for the supernatant of samples from mice infected with a low dose of bacteria ($4x10^4$ CFU). This was necessary to concentrate the bacteria. The supernatant fraction was diluted in PBS when necessary while the pellet fraction was diluted in PBS/0,1% X-100 triton. Each fraction was then plated on 2YT and CFU were counted after 4 days of culture at 37°C.

Immunofluorescence microscopy

Blood from Ubiquitin-GFP C57BL/6 mice infected with $5x10^7$ CFU of mCherry-Br was diluted 1/1 (without preliminary wash or centrifugation) for 15 min in PBS containing 4% paraformaldehyde (pH 7.4) before being smeared on slide. Slide were dried overnight at room temperature and then mounted in Fluoro-Gel medium containing DAPI nucleic acid stain (Electron Microscopy Sciences, Hatfield, PA). Labeled cells were visualized with an Axiovert M200 inverted microscope (Zeiss, Iena, Germany) equipped with high resolution monochrome camera (AxioCam HR, Zeiss). Images, 1384x1036 pixels (0.16μm/pixel), were acquired sequentially for each fluorochrome with LD-Plan-NeoFluar 63x/0.75 N.A. dry

objective and recorded as eight bit grey levels zvi files. Images were exported as TIFF files and figures prepared in Canvas 7 program.

Multispectral Imaging Flow Cytometry

Events were imaged using a ImageStream 100, Amnis Corp., Seattle, WA). At least, 60 000 events were collected from each sample (10^5 for blood sample) and data were analyzed using image analysis IDEAS software (Amnis Corp., Seattle, WA). Cells and bacteria brightfield images were recorded in channel 2 and mCherry bacteria in channel 4. R1 gate is based on area and aspect ratio of events (i.e. the ratio of the length to the breadth of an event). R1 gate includes all cells and bacteria and excludes fragments of cells and cell aggregates. R2 gate is based on mCherry signal and allow the identification of bacteria inside and outside of blood cells. The distinction between intracellular and extracellular bacteria has been performed by a combination of the erode mask applied on the brightfield image and of the intensity mask at 50% in the dedicated mCherry channel and by using the intensity feature of IDEAS software (Amnis Corporation, Seattle, WA). Briefly, bacterium was tagged as intracellular when its fluorescent image overlaps with the brightfield image of blood cell. The intracellular or extracellular location of bacteria was always confirmed by examination of the images.

Confocal microcopy

Confocal analysis were performed with LSM780 confocal system fitted on an Observer Z1 inverted microscope equipped with alpha Plan Apochromat 63x/1.46 N.A. oil immersion objective (Zeiss, Iena, Germany). The 488 nm excitation wavelength of the Argon/2 laser, a main dichroic HFT 488 and a band-pass emission filter (BP500-530 nm) were used for selective detection of the eGFP fluorochrome. The 543nm excitation wavelength of the HeNe1 laser, a main dichroic HFT 488/543/633 and a long-pass emission filter (BP580-650 nm) were used for selective detection of the mCherry fluorochrome. Stacks of one micron thick (i.e. 1 Airy Unit for the red fluorophore) optical sections (volume sampled: 27 x 27 x 5.2 microns, 512 by 512 pixels, scaling: X & Y: 0.053 micron, Z: 0.370 micron) were collected sequentially for each fluorochrome throughout the thickness of the object and stored as 16-bits *.lsm files. Files Stacks were processed for deconvolution using Huyghens ® software (Scientific Volume Imaging BV, Hilversum, The Netherlands) and 3D rendering was performed using Imaris ® software (Bitplane AG, Zurich, Switzerland). Images were exported as 8 bits uncompressed *.TIF image files.

Scanning electron microcopy

Samples were fixed over night at 4°C in PFA 4%, 0.1M cacodylate buffer (pH 7.2), and post-fixed in OsO4 (2%) in the same buffer. After serial dehydration, samples were dried at critical point and coated with platinum by standard procedures. Observations were made in a Tecnai FEG ESEM QUANTA 200 (FEI) and images processed by SIS iTEM (Olympus) software.

Transmission electron microcopy

Infected red blood cells were fixed overnight in 2,5% glutaraldehyde in cacodylate buffer, postfixed in 2% osmium tetroxide, serially dehydrated in ethanol and embedded in epoxy resin (Agar 100 resin, Agar scientific, UK) Ultrathin sections of 70 nm were obtained, mounted on copper-formvar-carbon grids (EMS, UK) and stained with uranyl acetate and lead citrate by standard procedures. Observations were made on a Tecnai 10 electron microscope (FEI, Eindhoven) and images were captured with a Veleta CCD camera and processed with AnalySIS and Adobe Photoshop softwares.

ELISA

Specific murine IgM, IgG1, IgG2a and IgG3 isotypes were determined by enzyme – linked immunosorbent assay (ELISA). Polystyrene plates (Nunc 269620) were coated with heat-killed *B. melitensis* (10^7 CFU/ml). After an overnight incubation at 4°C, plates were blocked for 2 hours at room temperature (RT) with 200 µl of PBS-3,65% casein. Then plates were incubated for 1 h at RT with 50 µl of serial dilutions of the serum in PBS-3,5% casein. The sera from naive mice were used as negative control. After 4 washes with PBS, isotype-specific goat anti-mouse horseradish peroxidase conjugates were added (50µl/well) at appropriate dilutions (αIgM from Sigma; LO-MG1-13 HRPO, LO-MG2a-9 HRPO, LO-MG3-13 HRPO from LO-IMEX). Note that LO-MG2a-9, recognizing mouse gamma 2a heavy chain, binds to all allotypes, including the IgHla allotype expressed in BALB/c mice and the IgHlb allotype expressed in C57BL mice. After 1 h of incubation at room temperature, plates were washed 4 times in PBS, and 100 µl/well of substrate solution (BD OptEiA) was added to each well. After 10 minutes of incubation at room temperature in the dark, the enzyme reaction was stopped by addition of 25 µl/well of 2N H_2SO_4, and the absorbance was measured at 450 nm.

Statistical analysis

We have used a (Wilcoxon-) Mann-Whitney test provided by GraphPad Prism program to statistically analyze our results. Each group of deficient mice was compared to wild type mice. Values of $p < 0.05$ were considered to represent a significant difference. *, **, *** denote $p<0.05$, $p<0.01$, $p<0.001$, respectively.

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3 Part III : Humoral immunity and CD4$^+$ Th1 cells are both necessary for a sterilizing immune response in the spleen upon secondary infection with *Brucella melitensis*

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In preparation

Immunological memory is a central feature of the adaptive immune system and relies on the maintenance of antigen-experienced B and T cells. Memory cells mediate better protection of the host upon re-infection because of their increased frequency, preferential localization in peripheral tissues and improved effector functions such as antibody production and cytokines secretion. It is usually assumed that activated effectors during recall infections are the same key players than the ones expressed during a primary immune response and that they provide a qualitatively and quantitatively stronger response. In this context, selection of new vaccine candidates against brucellosis is frequently based on their ability to induce effectors that have been described during the course of primary chronic response. However some works provide evidence that, upon re-infection, different classes of effectors, not observed during primary infection, can mediate protection of the host (Khader *et al.*, 2007; Lin *et al.*, 2009; Narni-Mancinelli *et al.*, 2011; Schito *et al.*, 1998; Seo *et al.*, 2010).

Brucellosis is a zoonotic disease that leads to chronic infection of the host, causing serious clinical manifestations if left untreated (Colmenero *et al.*, 1996; Godfroid *et al.*, 2005; Young, 1995). Currently, live attenuated strains of *Brucella* are used to immunize cattle, goats and sheep (Schurig *et al.*, 2002). These vaccines do not cover humans and animals other than these domestic ruminants, including wildlife, and display some drawbacks in terms of safety and diagnostic. There is so an increasing interest in developing new vaccines. To this end, there is an urgent need to improve our understanding of the development of a memory response in brucellosis. Indeed, the nature of the memory response elicited by the

virulent strain of *Brucella* has rarely been investigated in brucellosis and little is known about the activation of immune protective effectors upon re-infection by *Brucella*.

Therefore the objectives of the third part of this work were to evaluate the respective contributions of humoral response, T cell subsets and signaling pathways in the development of a protective immune response upon re-infection by *B. melitensis*. For this purpose, we compared the protection status and the secondary immune response induced by live virulent *B. melitensis* or by heat-killed bacteria. These parameters were characterized in several compartments (blood, peritoneal cavity and spleen) and at different times after re-call infection with virulent *B. melitensis*. In order to identify the lymphocyte populations and signaling pathways associated with protection, we used a large panel of mice rendered genetically deficient for key elements of the immune response[1].

Our results demonstrate that both HK and live vaccines induce drastic early control of bacteria dissemination in the blood but that only live vaccines are associated with late complete elimination of bacteria in the spleen. In contrast with the detrimental role of B cells described during a primary response (Goenka *et al.*, 2011; Goenka *et al.*, 2012; Vitry *et al.*, 2012), specific antibodies appear critical for both protection levels upon re-infection. Humoral immunity able to restrict bacteria dissemination in the bloodstream does not require MyD88/IL-12 signaling pathways, $CD4^+$ T cells or even the activation-induced deaminase (AID)-mediated class switching. However MyD88/IL-12 signaling pathways and IFN-γ-producing $CD4^+$ T cells are essential for elimination of bacteria in the spleen. These results also highlight the novel and recently described (Alugupalli *et al.*, 2004; Racine & Winslow, 2009; Racine *et al.*, 2011) implication of IgM in intracellular bacterial infection since *B. melitensis* infection elicits a protective, long-term IgM response.

[1] Additional results on genetically deficient mice tested during this thesis can be found in the Appendix section (chapter 2). Because they have not been always replicated or display variability, these results are only shown for guidance and will not been discussed here.

Manuscript

The following manuscript is still in preparation and is not submitted to a journal nor published. Therefore this is not a final version.

Humoral immunity and $CD4^+$ Th1 cells are both necessary for a sterilizing immune response in the spleen upon secondary infection with *Brucella melitensis*

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Running title: Protective immune response against secondary infection by *Brucella*

ABSTRACT

Brucella spp. are intracellular bacteria that cause brucellosis, one of the most common zoonoses in the world. Given the serious medical consequences of this disease, a safe and effective human vaccine is urgently needed. Efforts to develop this vaccine have been hampered by our lack of understanding of what constitutes a protective memory response against *Brucella.* Here, we characterize the cells and signaling pathways implicated in the generation of a protective immune memory response following the injection of heat-killed (HK) or live *Brucella melitensis* 16M. Using a panel of genetically-deficient mice, we demonstrated that the *Brucella*-specific humoral response and $CD4^+$ Th1 cells act together to induce a sterilizing immune response in the spleen after a secondary *B. melitensis* infection. Humoral immunity able to restrict bacteria dissemination in the bloodstream is induced by the inoculation of both HK and live bacteria and its development does not require T cells, MyD88/IL-12p35 signaling pathways or an activation-induced deaminase-mediated isotype switch. In striking contrast, the presence of memory IFN-γ-producing $CD4^+$ Th1 cells requires the administration of live bacteria and functional MyD88/IL-12p35 pathways. In summary, our work identifies several immune markers closely associated with protective immune memory and could help to define a rational strategy to obtain an effective human vaccine against brucellosis.

INTRODUCTION

Brucella (α-proteobacteria) are small, non-motile, non-spore-forming, facultative intracellular Gram-negative coccobacilli that infect humans as well as domestic (cattle, sheep, swine, camels, etc.) and wild-type (deer, bison, etc.) mammals. Animal infection leads to abortion in pregnant females and orchitis and epididymitis in males, resulting in infertility (Anderson *et al.*, 1986; Enright, 1990). Human brucellosis is a zoonotic infection transmitted through ingestion, inhalation, or contact with conjunctiva or skin lesions (Godfroid *et al.*, 2005). Although it is rarely fatal, it is a severe and debilitating chronic disease without prolonged antibiotic treatment (Colmenero *et al.*, 1996; Young, 1995). Despite significant progress, the incidence of human brucellosis remains very high in endemic areas, with more than 500,000 new human cases reported annually (Pappas *et al.*, 2006b), and this number is considered to be largely underestimated (Seleem *et al.*, 2010). In addition, *Brucella* species are considered as potential biological warfare agents and have been "weaponized" by several governments (Pappas *et al.*, 2006a). Since *Brucella* are classed as category B threat agents (Pappas *et al.*, 2006a), their use in bioterrorist attacks must be taken seriously and response plans should be designed.

As the complete eradication of *Brucella* would be unpractical due to its presence in a large range of wild mammals (Gregoire *et al.*, 2012; Zheludkov & Tsirel'son, 2010) and because antibiotic treatment is costly and patients frequently suffer from resurgence of the bacteria (Solera *et al.*, 1998), vaccination remains the only rational strategy to confer protection to populations living in endemic countries. Unfortunately, there is currently no available vaccine against human brucellosis as a great majority of commercially available animal vaccines are based on live attenuated strains of *Brucella* (*B. melitensis* Rev.1, *B. abortus* S19, *B. abortus* RB51) (Ficht *et al.*, 2009; Oliveira *et al.*, 2011) that cause disease in humans. Little real progress in the field of *Brucella* vaccination has been reported in recent decades. One clear cause seems be the empirical nature of research on the *Brucella* vaccine. Indeed, the vast majority of publications report only the protective ability of candidate vaccines, limiting their analysis to present CFU counts in the spleen after challenge (Arenas-Gamboa *et al.*, 2011; Kahl-McDonagh *et al.*, 2007). Evaluations of the ability of vaccines to induce IFN-γ producing cells, detected *in vitro* after re-stimulation, and/or a humoral response are also often reported (Gonzalez *et al.*, 2008; Kahl-McDonagh & Ficht, 2006; Pasquevich *et al.*, 2009). Rare studies (Araya *et al.*, 1989; Elzer *et al.*, 1994; Winter *et al.*, 1989; Zhan *et al.*,

1995) have tried to characterize the nature of the protective immune response induced by vaccination and thus identify potential protective immune markers for the development of a rational strategy to select candidate vaccines. These markers cannot be deduced from studies of the primary immune response against *Brucella*, because, as shown in other infectious models (Khader *et al.*, 2007; Lin *et al.*, 2009; Nakayama *et al.*, 2010; Narni-Mancinelli *et al.*, 2011; Schito *et al.*, 1998; Seo *et al.*, 2010; Soudja *et al.*, 2012), primary and secondary immune responses frequently implicate different classes of effectors.

Live vaccines are widely accepted to be superior to inactivated vaccines for protection against brucellosis (Montaraz & Winter, 1986; Zhan *et al.*, 1993; Zhan *et al.*, 1995), suggesting that the localization and persistence of *Brucella* antigens are key factors in the development of protective immunity. However, there is no consensual explanation for this fundamental difference. The use of heat-killed (HK) preparations of *Brucella* as adjuvants to induce a Th1 response has been described by some authors (Huang *et al.*, 1999; Huang *et al.*, 2001; Huang *et al.*, 2003; Huang *et al.*, 2005) while others have demonstrated that HK *Brucella* failed to induce the desirable Th1 protective response (Zhan *et al.*, 1993; Zhan *et al.*, 1995). Transfer experiments suggest that antibodies, $CD4^+$ and $CD8^+$ T cells could be protective (Araya *et al.*, 1989; Araya & Winter, 1990; Elzer *et al.*, 1994; Montaraz *et al.*, 1986; Winter *et al.*, 1989), but these results are subject to multiple interpretations in the context of a chronic infection due to the half-life of the transferred antibodies and cells. The fact that both cell-mediated immunity and antibodies have been reported to independently protect mice against brucellosis may explain why a broad collection of immunogens have been described to elicit a protective response, with sometimes substantial variability in the protocol used (Grillo *et al.*, 2012).

To increase our understanding of the nature of protective mechanisms induced by live vaccines, we developed an original model to compare and analyze in detail the level of protection in the blood and spleen induced by the intraperitoneal (i.p.) injection of heat-killed (HK) and live virulent strains of *B. melitensis* 16M. The protection levels and elicited immune responses were characterized in several compartments (blood, peritoneal cavity and spleen) and at different times after the i.p. challenge with live *B. melitensis*. In this model, we observed that both HK and live vaccines induce drastic early control of bacteria dissemination in the blood, but that only live vaccines mediate late complete elimination of bacteria in the spleen. Using mice rendered genetically deficient for key elements of the immune response, we tried to identify the lymphocyte populations and signaling pathways associated with these early and late protections. Our results demonstrate that specific antibodies are critical for both

protection levels. Humoral immunity able to restrict bacteria dissemination in the bloodstream does not require MyD88/IL-12 signaling pathways, $CD4^+$ T cells or even an activation-induced deaminase (AID)-mediated class switch. However, MyD88/IL-12 signaling pathways and IFN-γ-producing $CD4^+$ T cells are needed to eradicate the bacteria from the spleen. On the whole, these results identify potential preliminary markers of protective immune response against *B. melitensis* and could thus help to develop a rational strategy to identify protective live vaccines against human brucellosis.

RESULTS

Administration of both killed and live *Brucella melitensis* induces a protective memory state that limits bacteria dissemination in the blood, but only live-infected mice acquire a sterilizing immune response in the spleen

It is well documented that live vaccines induce better protection against *Brucella* infection compared to killed vaccines (Montaraz & Winter, 1986; Zhan *et al.*, 1993; Zhan *et al.*, 1995). However, the efficacy of these protocols is rarely compared in the same study and there is no precise or consensual explanation for this fundamental difference. To increase our understanding of this phenomenon, we performed here a detailed analysis of the protective state, including the humoral and cellular immune responses induced by the injection of heat-killed (HK) or live fully virulent *B. melitensis* 16M in mice. In addition, as C57BL/6 and BALB/c mice have been reported to display different levels of resistance to *Brucella* infection (Baldwin & Parent, 2002; Copin *et al.*, 2007; Fernandes *et al.*, 1996) and are frequently used in *Brucella* vaccination studies (Grillo *et al.*, 2012), we performed our comparison in both strains of mice.

Mice were injected i.p. with PBS (the control, naive mice group), $4x10^4$ CFU of live *B. melitensis* (termed here "live-immunized group") or $5x10^7$ CFU of HK *B. melitensis* (termed here "HK-immunized group"). In order to avoid the impact of persistent chronic infection in mice injected with live bacteria, all groups were treated 21 days post-injection with antibiotics (rifampicin and streptomycin) for 3 weeks and then left resting for at least 3 weeks before challenge with a high ($5x10^7$ CFU) or moderate (10^5 CFU) dose of live bacteria. See Figure S1.A for a detailed schematic representation of this protocol. As expected, antibiotic treatment completely eliminated *Brucella* in the spleens of wild-type mice after generally 8 days (Figure S1.B), but 3 weeks of treatment was necessary to eliminate *Brucella* from the spleens of several deficient mice displaying high susceptibility to infection, such as $MyD88^{-/-}$ and $Il\text{-}12p35^{-/-}$ mice (data not shown).

All naive control mice injected with a high ($5x10^7$ CFU) dose of *B. melitensis* (termed here the “primo infected group”) displayed clearly detectable counts of bacteria in the blood after 3 hours (Figure 1.A). We used this blood persistence to quantify the ability of the immunized group to control dissemination of the bacteria. In striking contrast to naive mice, both the HK and live-immunized groups, when challenged with the same dose, displayed a drastic and highly similar reduction of CFU counts in the blood, with elimination of approximately 99.99% of bacteria from the blood. This demonstrated that both groups possess

effector mechanisms able to rapidly limit the blood dissemination of *Brucella*. In agreement, these two groups also presented a significant reduction of CFU counts in the spleen at 1 day post-challenge compared to naive control mice (Figure 1.B). Histological analysis of spleen sections from infected mice challenged with a mCherry-expressing strain of *B. melitensis* showed that the bacteria are located in the same zone and cells in both the primo-infected and live-immunized groups of mice (Figure S2). As described in detail by our group in a previous study (Copin *et al.*, 2012), these cells are mainly red pulp macrophages ($F4/80^+$, Figure S2) and marginal zone macrophages ($MOMA\text{-}1^+$, not shown). At 6 days post-challenge, the live-immunized group displayed highly-significant better control of the bacteria count in the spleen compared with the HK-immunized group. Similar results were obtained in BALB/c and C57BL/6 mice (Figures 1.A and 1.B).

In order to investigate in greater detail the ability of the live and HK-immunized groups to develop sterilizing immunity in the spleen over the long term, we also challenged these mice with a lower and more physiological dose (10^5 CFU) of *B. melitensis* (Figures 2.A and 2.B). Kinetic analysis of the bacterial load in the spleen showed that mice handle the infection differently according to the immunization protocol used (Figure 2.A). In approximately 80% of primo-infected C57BL/6 mice, bacteria escape the immune response and persist in the spleen until 50 days post-infection (Figure 2.B). In contrast, only 30% of C57BL/6 mice from the live-immunized group conserved detectable CFU counts in the spleen at 50 days post-infection. Surprisingly, this reduction was not observed in the live-immunized group of BALB/c mice or in the HK-immunized groups of both strains of mice (Figure 2.B).

On the whole, these results demonstrate that, though injections of killed or live bacteria greatly reduce the bacteria count disseminated by blood circulation after a challenge, only live bacteria induce a sterilizing immune response in peripheral organs such as the spleen. We also observed that the strain of mice used to investigate this phenomenon is critical as C57BL/6 mice display sterilizing immunity in the spleen, unlike BALB/c mice. In order to identify immune parameters associated with resistance to infection, we compared the development of humoral and cellular immune responses in both the HK- and live-immunized groups.

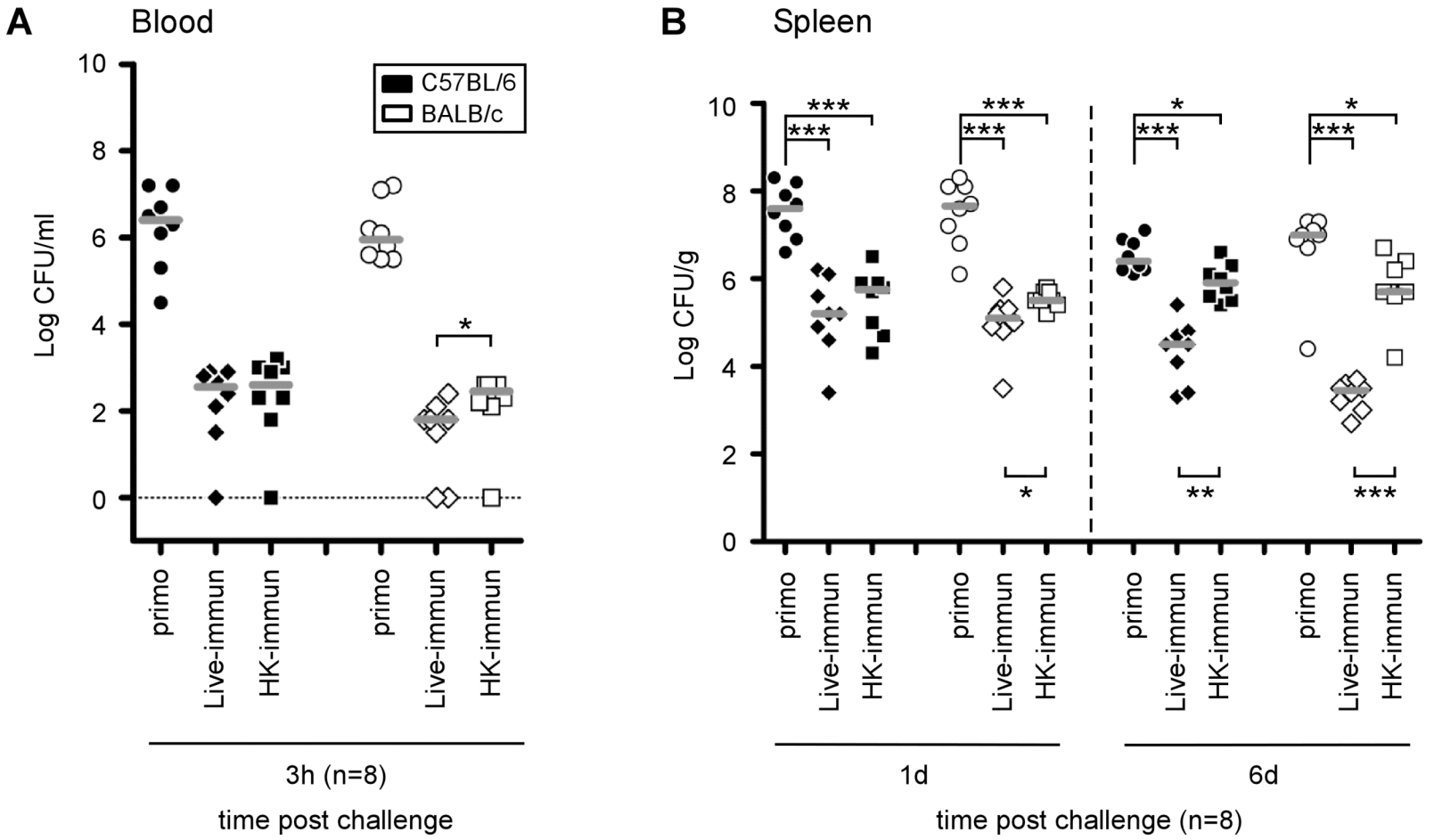


FIGURE 1. Comparison of protective immunity induced by a high-dose challenge in C57BL/6 or BALB/c mice immunized previously with live or heat-killed (HK) *B. melitensis*. C57BL/6 and BALB/c WT mice were immunized i.p. either with live ($4x10^4$ CFU, Live-immunized group) or HK bacteria (10^8 CFU, HK-immunized group), as indicated. All mice were treated with antibiotics as described in the Materials and Methods and then challenged with a high dose of live bacteria ($5x10^7$ CFU) and bled or sacrificed for spleen harvesting at the selected time. The data represent the CFU per ml of blood (**A**) or the CFU per gram of spleen (**B**). Grey bars represent the median. These results are representative of two independent experiments. Significant differences are denoted by an asterisk (*). *, **, *** denote $p<0.05$, $p<0.01$, $p<0.001$, respectively.

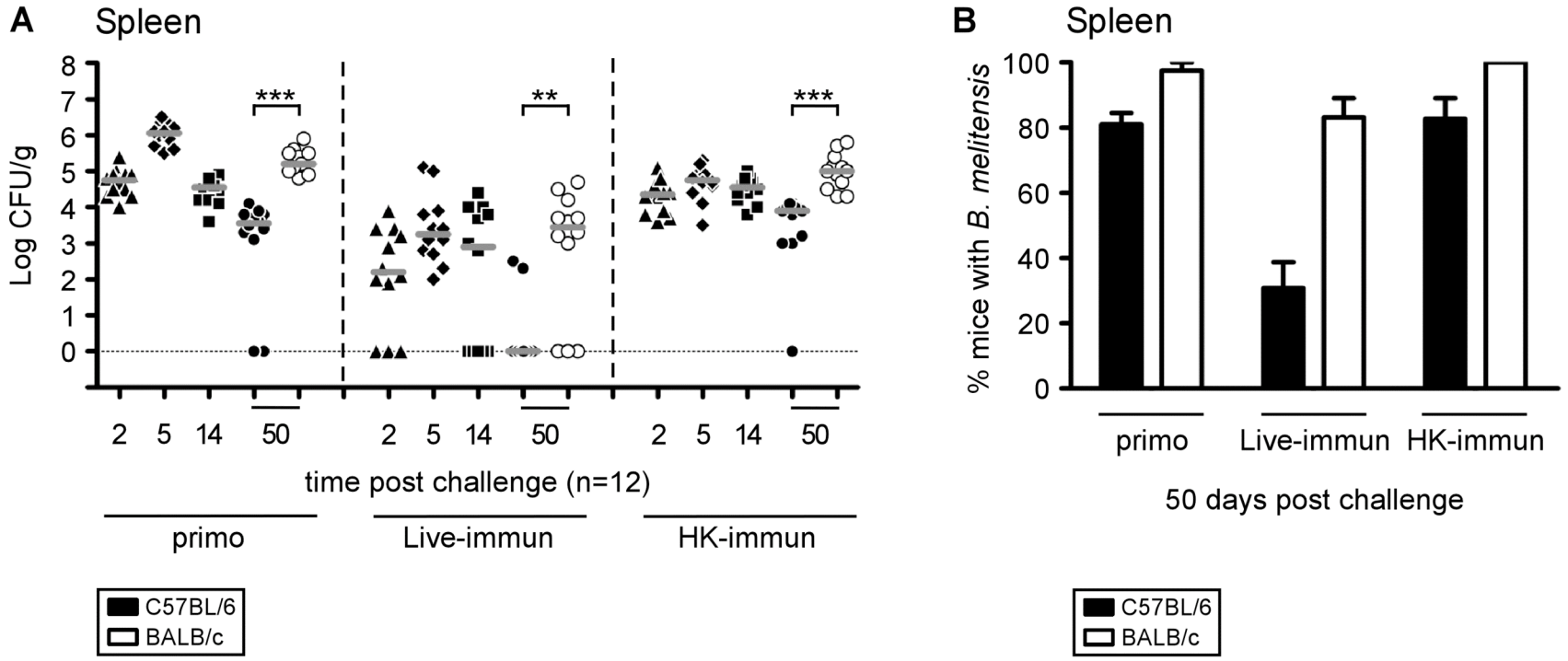


FIGURE 2. Comparison of protective immunity induced by a low-dose challenge in C57BL/6 or BALB/c mice immunized previously with live or heat-killed (HK) *B. melitensis*. C57BL/6 and BALB/c WT mice were immunized i.p. either with live ($4x10^4$ CFU, Live-immunized group) or HK bacteria (10^8 CFU, HK-immunized group). All mice were treated with antibiotics as described in the Materials and Methods and then were challenged with a low dose of bacteria (10^5 CFU) and sacrificed at the selected time. (**A**) The data represent the CFU per gram of spleen from one representative experiment**.** Grey bars represent the median The mean +/- SEM of the percentage of mice that are still positive for *Brucella* in the spleen 50 days post challenge is represented in (**B**). These data are pooled from at least two independent experiments, each including a minimum of 15 mice. Significant differences are denoted by an asterisk (*). **, *** denote $p<0.01$, $p<0.001$, respectively.

Administration of killed or live *Brucella melitensis* induces specific circulating antibodies able to reduce the blood dissemination of *Brucella* infection

The presence of specific immunoglobulins (Ig) against *Brucella* antigens in the serum of the HK- and live-immunized groups of C57BL/6 mice was investigated by ELISA two days before challenge (Figure S3). The results showed that both groups displayed high levels of specific IgM, IgG1 and IgG3 antibodies (Abs) against *Brucella* antigens. It is interesting to note that *Brucella*-specific IgG2a were detected only in the live-immunized group.

In order to determine the importance of these circulating Ig during a challenge with live *Brucella*, we compared the ability of live-immunized groups of wild-type, $RAG1^{-/-}$, $MuMT^{-/-}$ (B cell-deficient) and $AID^{-/-}$ (deficient in isotype-switched Abs, B cells produce only IgM) C57BL/6 mice to control *Brucella*. When challenged with a high dose ($5x10^7$ CFU) of *B. melitensis*, $RAG1^{-/-}$ and $MuMT^{-/-}$ mice appeared unable to control *Brucella* dissemination as demonstrated by much higher blood CFU counts detected at 3 hours and 3 days compared to wild-type and $AID^{-/-}$ live-immunized groups of mice (Figure 3.A). Following a low-dose challenge (10^5 CFU), we also observed that $MuMT^{-/-}$ mice displayed higher CFU counts in the spleen at 50 days (Figure 3.B) and a reduced frequency of these mice displayed a sterilizing response (Figure 3.C) compared to wild-type mice. These results demonstrate that circulating antibodies are the main effectors limiting early dissemination of *Brucella* in the blood of live-immunized groups and suggest that this early control is a critical step allowing a sterilizing response in the spleen. In addition, the ability of $AID^{-/-}$ mice to control blood dissemination strongly suggests that IgM alone are sufficient to perform this task.

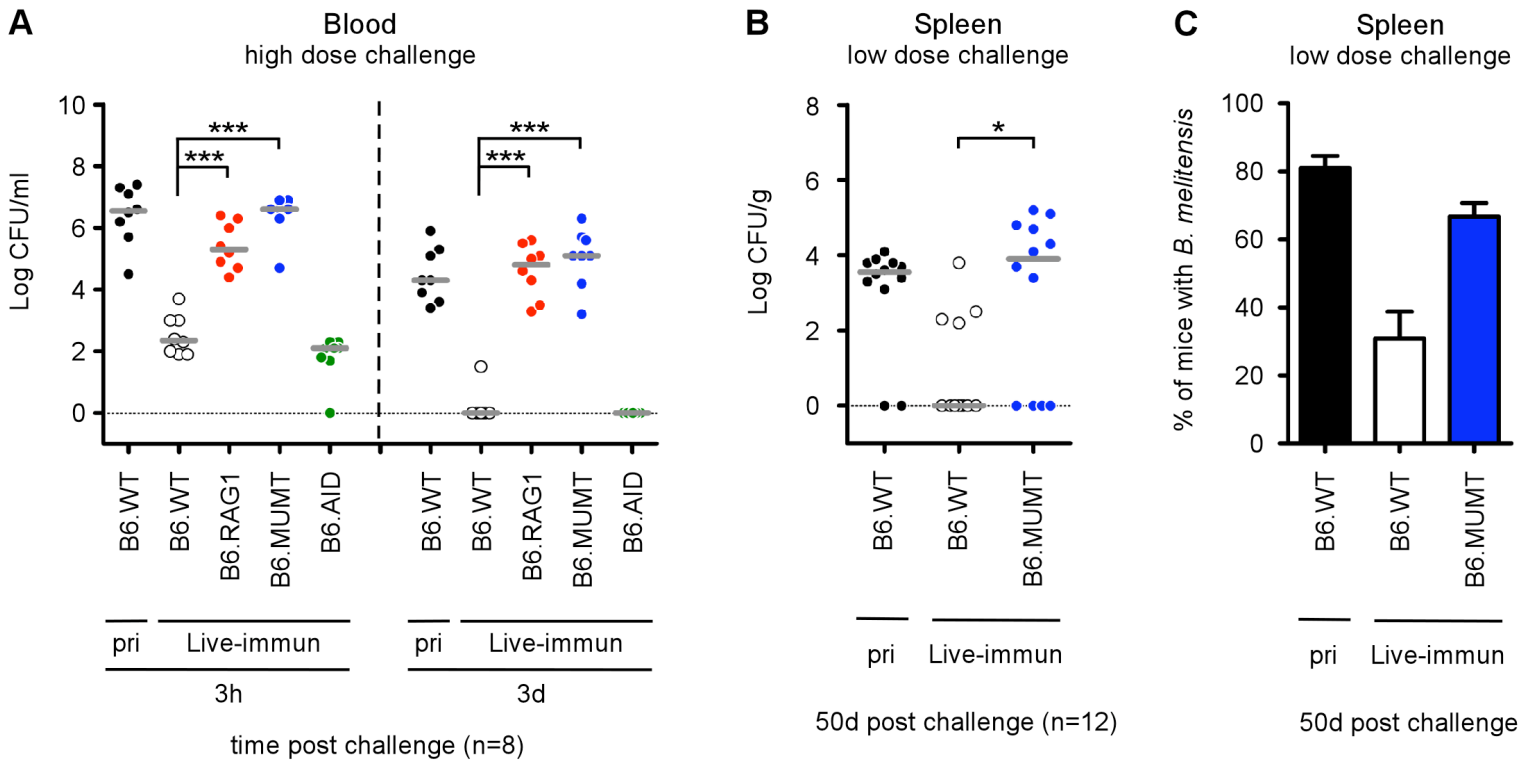


FIGURE 3. Comparison of protection between wild-type, RAG1$^{-/-}$, MuMT$^{-/-}$ and AID$^{-/-}$ C57BL/6 mice immunized previously with live *B. melitensis*. Wild-type (WT), RAG1$^{-/-}$, MuMT$^{-/-}$ and AID$^{-/-}$ C57BL/6 mice were immunized i.p. with live *B. melitensis* ($4x10^4$ CFU) and were treated with antibiotics as described in the Materials and Methods. **A,** Mice were challenged with a high dose of *B. melitensis* ($5x10^7$ CFU) and bled at the selected time. The data represent the CFU per ml of blood. **B, C**, Mice were challenged with a low dose of *B. melitensis* (10^5 CFU) and sacrificed 50 days post-challenge. (**B)** represents the CFU per gram of spleen. These data are representative of three independent experiments. Grey bars represent the median. (**C)** displays the mean +/- SEM of the percentage of mice that are still positive for *B. melitensis* in the spleen. These data are pooled from two independent experiments, each including a minimum of 10 mice. Significant differences are denoted by an asterisk (*). *, **, *** denote $p<0.05$, $p<0.01$, $p<0.001$, respectively. "Pri" means primo group.

Injection of live but not killed *Brucella melitensis* induces the development of a $CD4^+$ T cell memory population able to rapidly produce IFN-γ in response to *Brucella* infection

We and others (Baldwin & Parent, 2002; Copin *et al.*, 2007; Ko *et al.*, 2002; Murphy *et al.*, 2001; Stevens *et al.*, 1992; Vitry *et al.*, 2012; Zhan & Cheers, 1993) have shown that IFN-γ is a key cytokine-regulating protective cellular immune response against primary *Brucella* infection. IFN-γ is produced by Natural Killer (NK) cells, $CD4^+$ T and $CD8^+$ cells (Copin *et al.*, 2007; Vitry *et al.*, 2012) and is crucial for the development of inducible Nitric Oxide Synthase (iNOS) positive granulomas that limit *B. melitensis* infection in the spleen and the liver (Copin *et al.*, 2012). Here, we analyzed by flow cytometry the phenotype of IFN-γ producing cells in the peritoneal cavity and in the spleen of HK- and live-immunized groups of C57BL/6 mice challenged with low (10^5 CFU) and high ($5x10^7$ CFU) doses of live *B. melitensis*. IFN-γ-producing cells were analyzed at 12, 24 and 48 hours post-challenge.

With the low-dose challenge (10^5 CFU), in the absence of *in vitro* stimulation, an elevated frequency of IFN-γ positive cells was detected at 12 hour in the peritoneal cell population (Figure 4.A-B) and at 24 and 48 hours (data not shown) in the spleen cells from the live-immunized group. In contrast, in the primo-infected or HK-immunized groups, only a weak IFN-γ signal was detected in both the peritoneal cavity (Figure 4.A-B) and spleen (data not shown) during the first 48 hours. The specificity of the IFN-γ signal was confirmed using IFN-$\gamma^{-/-}$ C57BL/6 mice (data not shown).

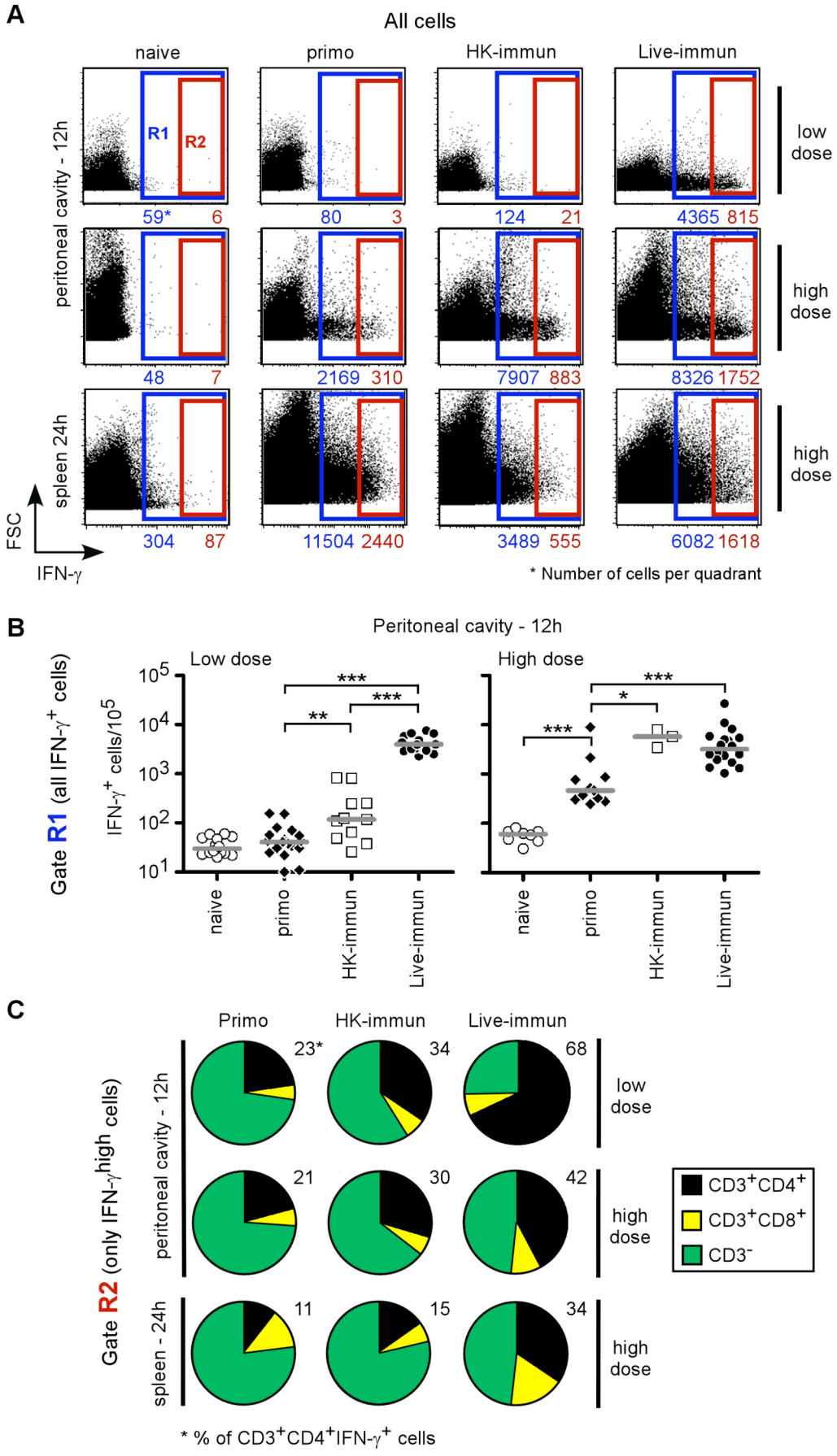
The majority of high IFN-γ producers in the peritoneal cavity in the live-immunized group were $CD4^+$ T cells since a mean of 68% of highly IFN-γ-positive cells were found to co-express CD3ε and CD4 markers (Figure 4.C). These cells also expressed higher levels of CD25, CD69 (activation marker) and Ly-6C (memory T cells marker) (Figure S4). As expected, the live-immunized group of BALB/c mice displayed a ten-fold reduction of the frequency of IFN-γ positive peritoneal cells compared to C57BL/6 mice (Figure 5A-B). When stimulated overnight *in vitro* with HK *B. melitensis*, only peritoneal cells from the live-immunized group displayed IFN-γ producing $CD4^+$ T cells. This result demonstrates that this group contained *Brucella*-specific memory $CD4^+$ T cells in the peritoneal cavity before challenge (Figure S5).

When infected with a high-dose ($5x10^7$ CFU) challenge, high counts of IFN-γ signal were observed in the peritoneal cavity (Figure 4.A-B) and in the spleen (Figure 4.A) of the primo-infected, HK- and live-immunized groups. In both the HK- and live-immunized groups, a large proportion of highly-positive IFN-γ-producing cells (+/-75% and +/-50%,

respectively) from the peritoneal cavity and the spleen were non-T cells ($CD3\varepsilon^-$ cells) (Figure 4.C), suggesting that this challenge dose induces mainly non-specific production of IFN-γ by innate immune cells. In addition, the highest frequency of IFN-γ positive cells was observed in the spleen of the primo-infected groups of both strains of mice (Figure 5.C-D). Thus, we conclude that the high-dose challenge model is not well adapted to compare the ability of HK- and live-immunized groups to produce IFN-γ in response to a *B. melitensis* challenge.

All together, the data from the low-dose challenge model suggest that only injection of live *B. melitensis* induces high-affinity IFN-γ-producing $CD4^+$ memory T cells able to rapidly react *in vivo* to a second *Brucella* infection.

FIGURE 4. (SEE BELOW) Comparison of IFN-γ^+ cell frequency after challenge in C57BL/6 mice immunized previously with live or HK *B. melitensis*. C57BL/6 mice were immunized i.p. either with live ($4x10^4$ CFU, Live-immunized group) or HK bacteria (10^8 CFU, HK-immunized group). All mice were treated with antibiotics as described in the Materials and Methods and then were challenged with either a low dose (10^5 CFU) or a high dose ($5x10^7$ CFU) of live bacteria, as indicated, and sacrificed at the selected time. Peritoneal and spleen cells were collected and analyzed by flow cytometry. **A,** Cells were analyzed for Forward Size Scatter (FSC) versus IFN-γ production. The figure shows representative dot plots from individual peritoneal cavities in each group. Numbers under the line of plots indicate the number of cells in gate R1 (blue) or gate R2 (red) out of 10^5 (peritoneal cavity) or $1.5x10^6$ (spleen) cells acquired. **B,** The graphs represent the number of IFN-γ positive cells per 10^5 peritoneal cells acquired in gate R1. Each data point represents the value obtained from an individual peritoneal cavity and the data are representative of two independent experiments. Grey bars represent the median. Significant differences are denoted by an asterisk (*). *, **, *** denote $p<0.05$, $p<0.01$, $p<0.001$, respectively. **C,** Highly IFN-γ-positive cells (gate R2) in each group were analyzed for CD3, CD4 and CD8α expression. Numbers next to the circles indicate the percentage of $CD3^+CD4^+IFN\text{-}\gamma^+$ T cells in gate R2.


A
All cells
naive
primo
HK-immun
Live-immun
R1
R2
peritoneal cavity - 12h
spleen 24h
low dose
high dose
high dose
59* 6
80 3
124 21
4365 815
48 7
2169 310
7907 883
8326 1752
304 87
11504 2440
3489 555
6082 1618
FSC
IFN-γ
* Number of cells per quadrant
B
Peritoneal cavity - 12h
Low dose
High dose
Gate R1 (all IFN-γ+ cells)
IFN-γ+ cells/10^5
naive
primo
HK-immun
Live-immun
C
Primo
HK-immun
Live-immun
Gate R2 (only IFN-γhigh cells)
peritoneal cavity - 12h
spleen - 24h
23*
34
68
21
30
42
11
15
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low dose
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high dose
CD3+CD4+
CD3+CD8+
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* % of CD3+CD4+IFN-γ+ cells

Figure 4

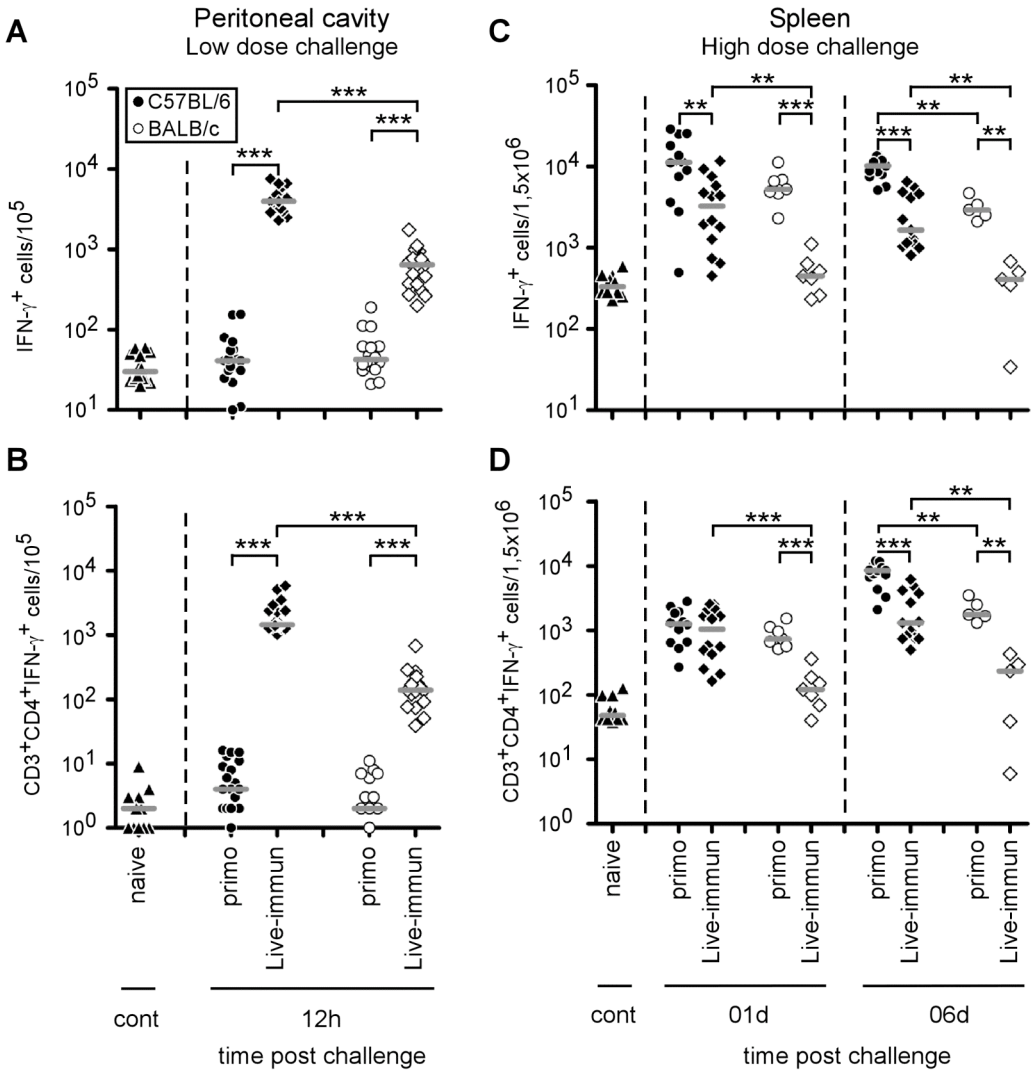

FIGURE 5. IFN-γ$^+$ peritoneal or spleen cell frequency after challenge in C57BL/6 or BALB/c mice immunized previously with live *B. melitensis*. C57BL/6 and BALB/c mice were immunized i.p. with live *B. melitensis* (4x10^4 CFU, Live-immunized) and were treated with antibiotics as described in the Materials and Methods. **A, B,** Mice were then challenged with a low dose of *B. melitensis* (10^5 CFU) and sacrificed at 12 hours post challenge. Peritoneal cells were collected and analyzed by flow cytometry. Cells were first analyzed for Forward Size Scatter (FSC) versus IFN-γ production and then for cell surface markers. The data represent the number of IFN-γ positive cells (**A**) or CD3$^+$CD4$^+$IFN-γ$^+$ T cells (**B**) per 10^5 peritoneal cells acquired. **C, D,** Mice were challenged with a high dose of *B. melitensis* (5x10^7 CFU) and sacrificed at the selected time. Spleen cells were collected and analyzed by flow cytometry as described above. The data represent the number of IFN-γ positive cells (**C**) or CD3$^+$CD4$^+$IFN-γ$^+$ T cells (**D**) per 1.5x10^6 spleen cells acquired. Each data point represents the value obtained from an individual spleen and the data are representative of two independent experiments. Grey bars represent the median. Significant differences are denoted by an asterisk (*). **, *** denote $p<0.01$, $p<0.001$, respectively.

MHC-II and MyD88/IL-12 pathways are crucial for the development of sterilizing immunity in the spleen of mice immunized with live *Brucella melitensis*

In order to confirm the link between sterilizing immunity in the spleen and the presence of IFN-γ-producing $CD4^+$ memory T cells observed in the live-immunized group, we analyzed the impact of the absence of $CD4^+$ T cells or of IFN-γ-inducing pathways using several genetically-deficient mouse strains. Live-immunized groups of wild-type, $MyD88^{-/-}$, $IL\text{-}12p35^{-/-}$ and $MHC\text{-}II^{-/-}$ C57BL/6 mice were challenged with high ($5x10^7$ CFU) or low (10^5 CFU) doses of live *B. melitensis* and their ability to control *Brucella* dissemination in the blood and confer sterilizing immunity in the spleen was assessed (Figure 6).

MyD88, IL-12p35 and MHC-II deficiencies did not impair the ability of live-immunized groups to display lower *Brucella* CFU counts in the blood following a high-dose challenge compared to naive infected mice (Figure 6.A). A comparative analysis of the humoral immune response in these deficient mice was performed two days before challenge and showed that all groups displayed normal levels of *Brucella*-specific IgM but extremely variable levels of different *Brucella*-specific IgG isotypes (Figure S6). In particular, $MHC\text{-}II^{-/-}$ mice presented very low levels of *Brucella*-specific IgG1, IgG2a and IgG3. These results demonstrate that MyD88/IL-12p35 signaling pathways are not implicated in the early control of *Brucella* dissemination. They also suggest that, as previously observed with $AID^{-/-}$ mice (figure 3.A), specific IgM alone could suffice to perform this task.

In striking contrast, we observed that MyD88, IL-12p35 and MHC-II deficiencies strongly impacted the ability of live-immunized groups to eliminate *Brucella* from the spleen after a low-dose challenge (Figure 6.B-C). Impaired sterilizing immunity in the spleen of various deficient mouse strains was found to be associated with a drastic reduction in IFN-γ-producing cells at 12 hours post-challenge in the peritoneal cavity (Figure 7).

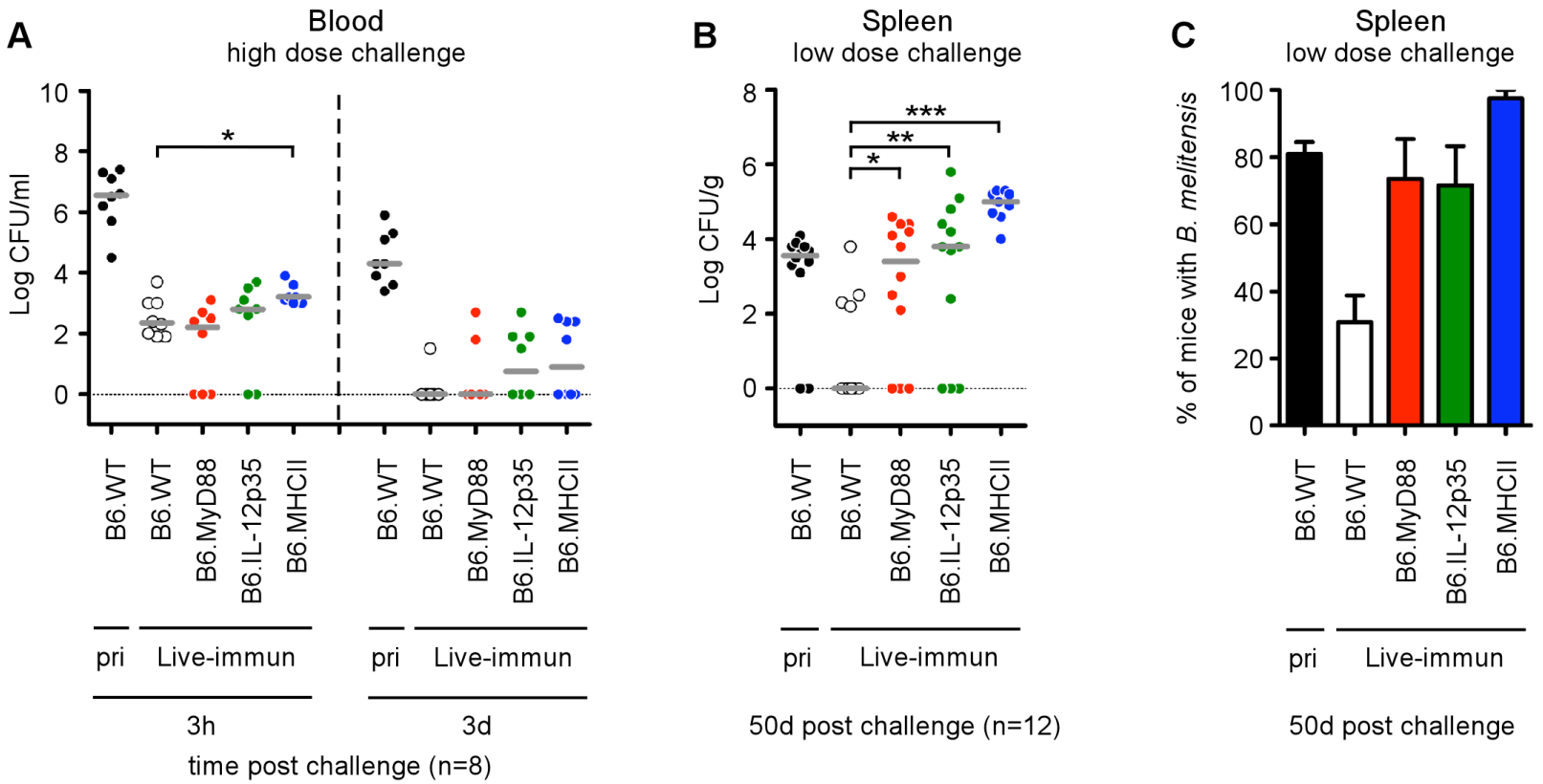

FIGURE 6. Comparison of protection in wild-type and deficient C57BL/6 mice immunized previously with live *B. melitensis*. Wild-type (WT), MyD88$^{-/-}$, IL-12p35$^{-/-}$ and MHCII$^{-/-}$ C57BL/6 mice were immunized i.p. with live *B. melitensis* ($4x10^4$ CFU) and were treated with antibiotics as described in the Materials and Methods. **A**, Mice were challenged with a high dose of *B. melitensis* ($5x10^7$ CFU) and bled at the selected time. The data represent the CFU per ml of blood. **B, C**, Mice were challenged with a low dose of *B. melitensis* (10^5 CFU) and sacrificed at 50 days post challenge. **B**, The data represent the CFU per gram of spleen and are representative of two independent experiments. Grey bars represent the median. The percentage of mice that are still positive for *Brucella* in the spleen at 50 days post challenge is represented in (**C**). These results are pooled from at least two independent experiments, each including a minimum of 10 mice. Significant differences are denoted by an asterisk (*). *, **, *** denote $p<0.05$, $p<0.01$, $p<0.001$, respectively. "Pri" means primo group.

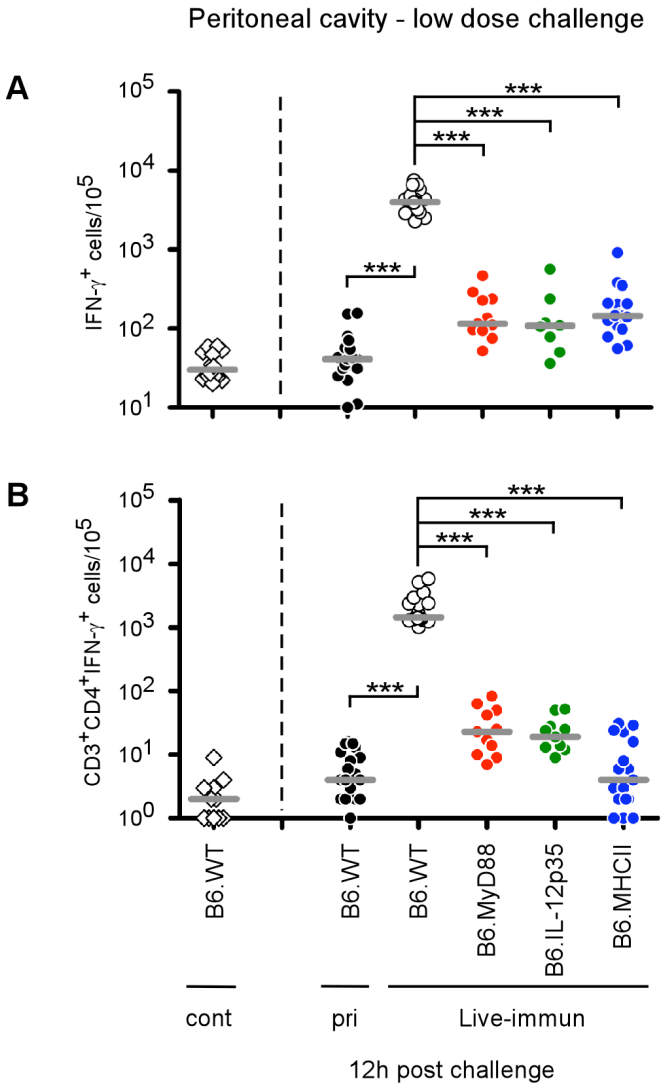

FIGURE 7. Comparison of IFN-γ$^+$ cell frequency after challenge in wild-type and deficient C57BL/6 mice immunized previously with live *B. melitensis*. WT, MyD88$^{-/-}$, IL-12p35$^{-/-}$ and MHCII$^{-/-}$ C57BL/6 mice were immunized i.p. with live *B. melitensis* (4x10^4 CFU) and were treated with antibiotics as described in the Materials and Methods. Mice were then challenged with a low dose of *B. melitensis* (10^5 CFU) and sacrificed at the selected time. Peritoneal cells were collected and analyzed by flow cytometry. Cells were first analyzed for Forward Size Scatter (FSC) versus IFN-γ production and then for cell surface markers. The data represent (**A**) the number of IFN-γ positive cells and (**B**) the number of CD3$^+$CD4$^+$IFN-γ$^+$ T cells per 10^5 peritoneal cells acquired. Each data point represents the value obtained from an individual spleen and the data are representative of two independent experiments. Grey bars represent the median. Significant differences are denoted by an asterisk (*). *** denote p<0.001. "Pri" means primo group.

Finally, as BALB/c mice displayed reduced IFN-γ production in both the peritoneal cavity and the spleen (Figure 5) and impaired sterilizing immunity in the spleen (Figure 2.A-B), we analyzed the impact of IL-4/IL-13 receptor signaling pathways neutralisation in the live-immunized group of BALB/c mice. Despite similar frequencies of IFN-γ producing cells in the peritoneal cavity at the time point tested (Figure 8.A-B), the live-immunized group of STAT-$6^{-/-}$ mice displayed lower CFU counts (Figure 8.C) and significantly better elimination of *B. melitensis* in the spleen (Figure 8.D) compared to wild-type mice. This suggests that sterilizing immunity in the spleen is negatively affected by IL-4/IL-13 signaling in BALB/c mice.

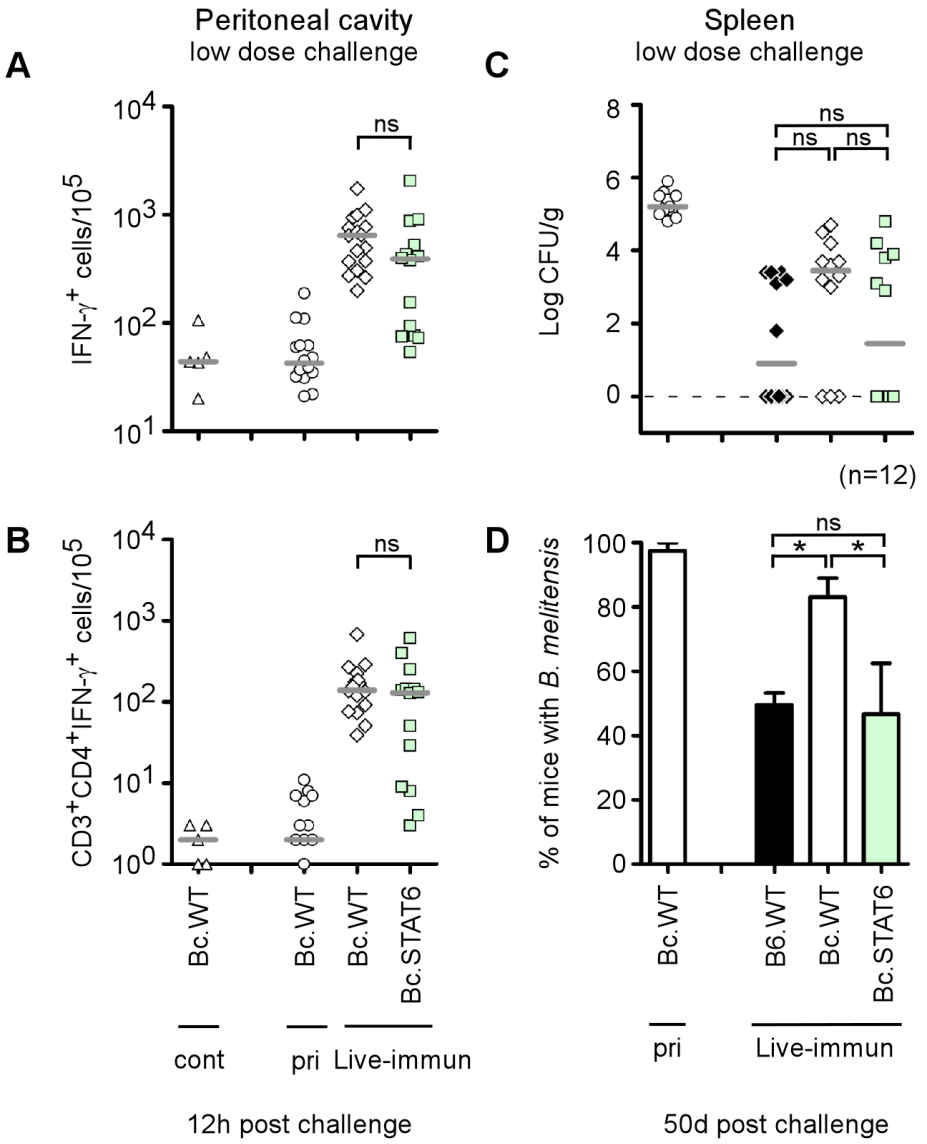

FIGURE 8. Comparison of protection and IFN-γ$^+$ cell frequency after challenge in wild-type or STAT6$^{-/-}$ BALB/c mice immunized previously with live *B. melitensis*. Wild-type (WT) and STAT6$^{-/-}$ BALB/c mice were immunized i.p. with live *B. melitensis* ($4x10^4$ CFU) and were treated with antibiotics as described in the Materials and Methods. Immunized C57BL/6 WT mice were used as the control. Mice were then challenged with a low dose of *B. melitensis* (10^5 CFU) and sacrificed at the selected time. **A, B,** To characterize the elicited immune response, mice were sacrificed at 12 hours post-challenge and peritoneal cells were collected and analyzed by flow cytometry. Cells were first analyzed for Forward Size Scatter (FSC) versus IFN-γ production and then for cell surface markers. The data represent (**A**) the number of IFN-γ positive cells and (**B**) the number of CD3$^+$CD4$^+$IFN-γ$^+$ cells per 10^5 peritoneal cells acquired. **C, D,** To estimate the elicited protection, mice were sacrificed at 50 days post-challenge and the spleens were harvested. **C**, The data represent the CFU per gram of spleen and are representative of two independent experiments. Grey bars represent the median. The mean +/- SEM of the percentage of mice that are still positive for *B. melitensis* in the spleen is represented in (**D**). These results are pooled from two independent experiments, each including a minimum of 12 mice. Significant differences are denoted by an asterisk (*). * denote $p<0.05$. "Pri" means primo group.

DISCUSSION

Brucellae seem perfectly well adapted to their mammalian hosts. They furtively infect mammals, causing only minor inflammation, modify the vesicular environment of phagocytic cells to safely grow intracellularly and disseminate in all tissues (Martirosyan *et al.*, 2011). However, though *Brucella* infection remains largely silent, brucellosis induces potentially serious complications over the long term (Colmenero *et al.*, 1996; Cutler *et al.*, 2005; Godfroid *et al.*, 2005). As antibiotic-treated patients frequently display bacteria resurgence (Ariza *et al.*, 1995; Solera *et al.*, 1998), the development of a safe protective vaccine remains the only realistic strategy to protect exposed populations. Empirical research has failed to develop a safe protective vaccine for humans (Oliveira *et al.*, 2011; Perkins *et al.*, 2010) and, despite a plethora of publications on the murine model of brucellosis, our understanding of the secondary immune response against *Brucella* is currently very poor. Immune markers used to determine the efficacy of vaccination are commonly based on the primary immune response against *Brucella*. However, it has been often observed in other infectious models (Khader *et al.*, 2007; Lin *et al.*, 2009; Nakayama *et al.*, 2010; Narni-Mancinelli *et al.*, 2011; Schito *et al.*, 1998; Seo *et al.*, 2010; Soudja *et al.*, 2012) that the primary and secondary responses use distinct classes of effector mechanisms. In a recent study (Vitry *et al.*, 2012) using a large panel of genetically-deficient mice, we attempted to clearly identify the effector cells and signaling pathways implicated in the primary immune response against *B. melitensis* infection. We showed that IFN-γ-producing $CD4^+$ Th1 cells play a crucial role in the control of bacteria, but that a deficiency in $CD8^+$ T cell, B cell, Th2 and Th17 responses does not qualitatively affect the course of the infection. We also demonstrated that Th1 induction requires functional TLR9/MyD88/IL-12p35 signaling pathways (Copin *et al.*, 2007; Copin *et al.*, 2012; Vitry *et al.*, 2012). In the present study, we have developed an original model to characterize the effector mechanisms involved in the control of a secondary infection by *B. melitensis*. Mice were injected with HK or live virulent *Brucella melitensis* 16M and were treated with antibiotics after 21 days. After a resting phase, the mice were challenged with live bacteria. Protection was analyzed at two distinct levels. Early protection was measured by the ability of the immune response to reduce bacterial load in the bloodstream. The late sterilizing protection was scored by the proportion of animals that were not able to completely eradicate bacteria from their spleen at 50 days post-challenge. The spleen was chosen as the control because *Brucella* has been shown to persist for long periods of time (up to 100 days)

in this organ (Durward *et al.*, 2012). In our model, the absence of bacteria in the spleen has been always correlated with complete elimination of bacteria in the liver (data not shown). However, a reservoir in other tissues cannot be formally excluded.

Control of intracellular pathogens such as bacteria and protozoa usually requires $CD4^+$ T cell-, IFN-γ- and/or tumor necrosis factor (TNF)-dependent activation of macrophages. This leads to an upregulation of antimicrobial effector mechanisms, including the acidification of phagolysosomes and the expression of inducible nitric oxide synthase (iNOS, NOS2 (Chakravortty & Hensel, 2003)). Although antibodies are frequently regarded as irrelevant for the control of intracellular bacteria and protozoa, more recent studies demonstrate that they may contribute both to development of the disease as well as to its control (Casadevall & Pirofski, 2006). Antibody-mediated aggravation of infections with intracellular pathogens might be due to Fc-receptor-mediated facilitation of entry of the pathogen into the host cell or to macrophage deactivation conveyed by inhibitory Fc receptors (Buxbaum & Scott, 2005; Kima *et al.*, 2000; Padigel & Farrell, 2005). Conversely, antibody-dependent control of intracellular microbes may result from antibody binding to the pathogen during intermittent extracellular phases, leading to opsonization and classical complement activation (Bitsaktsis *et al.*, 2007).

In our model, we observed that humoral immunity is necessary for full protection upon secondary infection (see Table 1). Circulating specific antibodies are crucial to control the early dissemination of *Brucella* by the blood stream following challenge by intraperitoneal injection. They are also critical for the development of sterilizing immunity in the spleen at 50 days post-challenge. Thus, though B cells appear to be dispensable (Vitry *et al.*, 2012) or even detrimental (Goenka *et al.*, 2011; Goenka *et al.*, 2012) during primary infection, they play an important positive role in the control of secondary infection. Surprisingly, our results demonstrate that $CD4^+$ T cells, MyD88/IL-12p35 signaling pathways and even the AID-mediated class switch are dispensable to obtain protective circulating antibodies able to restrict *Brucella* dissemination in the blood. No other isotype seems to play a crucial role in this step as deficiency in $CD4^+$ T cells, MyD88 and IL-12p35 affects various IgG isotypes, but does not reduce the early control of infection. Interestingly, *Brucella*-specific IgM are maintained in the absence of chronic infection, as antibiotic-treated mice remained protected for 3 months against a challenge infection (data not shown). IgM-mediated immunity is usually considered to be short-lived and only effective during the early stages of infection. Our findings indicate that IgM may be of greater utility during chronic bacterial infections than previously thought. Other researchers have also provided evidence for long-term IgM

responses, although such reports are relatively rare (Racine & Winslow, 2009). Similar results have been reported in experimental models of infection by intracellular bacteria such as *Borrelia hermsii* (Alugupalli *et al.*, 2004) and *Ehrlichia muris* (Racine *et al.*, 2011). As T cell-independent activation of B cells is generally dependent on pattern recognition receptors (PRRs) (Defrance *et al.*, 2011), we can hypothesize that *Brucella* pathogen-associated molecular patterns (PAMPs) are implicated in the activation of *Brucella*-specific B cells and that PRRs recognizing these PAMPs may act by a MyD88-independent signaling pathway. Our observations that long-lived protective IgM responses can be generated *in vivo* by *Brucella* infection suggest that it may be feasible to target IgM production as part of vaccination strategies.

Early protective immunity mediated by *Brucella*-specific circulating antibodies developed following inoculation of both HK and live bacteria. In striking contrast, development of late sterilizing immunity in the spleen required previous injection of live bacteria. This late protection level is closely correlated with the presence of both circulating *Brucella*-specific antibodies and peritoneal Th1 $CD4^+$ T cells able to quickly produce high IFN-γ counts after *Brucella* challenge (see Table 1). The absence of antibodies ($MuMT^{-/-}$ mice) or CD4+ T cells ($MHC\text{-}II^{-/-}$ mice) leads to persistence of the bacteria in the spleen, demonstrating that both effector mechanisms must act together to eradicate *Brucella* from peripheral tissues. HK *Brucella* administration fails to induce peritoneal $CD4^+$ T cells able to produce high IFN-γ counts after *Brucella* challenge, suggesting that the dynamics of intracellular infection are critical to induce this effector mechanism. As expected, analysis of genetically-deficient mice showed that the development of IFN-γ producing $CD4^+$ T cells is strictly dependent on MyD88/IL-12p35 signaling pathways. Failure of HK *Brucella* immunization to induce these cells could explain the absence of IgG2a in the serum of the HK-immunized group, as the development of this isotype is well known to be dependent on IFN-γ (Finkelman *et al.*, 1988).

Interestingly, IFN-γ-producing $CD4^+$ T cells are not replaced by IFN-γ-producing $CD8^+$ T cells in the absence of MHC-II-dependent antigen-presenting pathways, as was previously observed during the *Brucella* primary response (Vitry *et al.*, 2012). This demonstrates that primary *Brucella* infection induces low-quality responding $CD8^+$ T cells unable to participate in the secondary immune response. Several recent reports suggest that the failure of the immune system to maintain a $CD8^+$ T cell response during chronic brucellosis results from bacterial evasion dependent on the virulence factor, TcpB (Durward

et al., 2012), and show *in vitro* that *Brucella* induces intracellular retention of the MHC-I down-modulating cytotoxic $CD8^+$ T cell response (Barrionuevo *et al.*, 2012).

Several reports in the *Mycobacterium tuberculosis* infectious model suggest that the ability of memory Th1 $CD4^+$ T cells to fight intracellular bacteria could be dissociated from IFN-γ production (Cowley & Elkins, 2003; Gallegos *et al.*, 2011; Goldsack & Kirman, 2007; Mittrucker *et al.*, 2007). As IFN-$\gamma^{-/-}$ mice succumb rapidly to *Brucella* infection (Brandao *et al.*, 2012; Murphy *et al.*, 2001), we have not been able to test this hypothesis in our *Brucella* model. However, we have shown previously that IFN-γ-producing $CD8^+$ T cells fail to protect mice during primary *Brucella* infection (Vitry *et al.*, 2012), suggesting that IFN-γ production is not the only property of $CD4^+$ T cells implicated in the control of *Brucella*. The nature of any such additional factors in our model has not yet been determined. Recent studies (Narni-Mancinelli *et al.*, 2007; Narni-Mancinelli *et al.*, 2011) on the *L. monocytogenes* infectious model suggest that the ability of T cells to regulate the local recruitment of innate effector cells can be crucial to the protective secondary response. Comparison of chemokine production by $CD4^+$ and $CD8^+$ T cells during brucellosis could provide interesting new areas of investigation.

In this study, we were unable to confer sterilizing protection in the spleen of naive mice by the transfer of serum or peritoneal cells from the live-immunized group (data not shown). We hypothesize that it may have been due to failed homing of the transferred $CD4^+$ T cells or to the absence of other unidentified synergic cell populations.

C57BL/6 and BALB/c mice are equally used in vaccination studies. However, the efficacy of vaccines is rarely compared with both mice strains in the same study. Our results demonstrate that, following HK or live immunization, C57BL/6 and BALB/c mice display a similar efficacy to control early dissemination of *Brucella* after challenge but differ significantly in their ability to develop a sterilizing immune response in the spleen. Unlike in C57BL/6 mice, the injection of live bacteria in BALB/c mice does not improve their capacity to eradicate bacteria from the spleen. This phenomenon could be correlated with the reduced frequency of IFN-γ-producing cells in the live-immunized group of BALB/C mice compared to C57BL/6 mice after challenge in the peritoneal cavity. These results demonstrate that the mice strain chosen to test the efficacy of a vaccination protocol is crucial.

It has been hypothesized that IL-4 production in BALB/c mice reduces IFN-γ production and adversely affects the protective immune response to *Brucella* (Fernandes *et al.*, 1996). We have demonstrated previously that IL-4 receptor deficiency (Vitry *et al.*, 2012)

or neutralization of IL-4/IL-13 receptor signaling pathways with STAT-6 deficiency (data not shown) does not improve the ability of C57BL/6 and BALB/c mice to control primary *Brucella* infection. In this study, we observed that STAT-6 deficiency in the live-immunized group of BALB/c mice significantly increases the rate of *Brucella* elimination after secondary infection. Indeed, STAT-$6^{-/-}$ BALB/c mice display a level of control similar to wild-type C57BL/6 mice. This surprising result suggests that sterilizing immunity in the spleen is affected by IL-4/IL-13 in BALB/c mice. As IFN-γ-producing cell frequency in the peritoneal cavity after challenge of STAT-$6^{-/-}$ BALB/c does not seem to be higher, we hypothesize that IL-4 and/or IL-13 could act on other unidentified crucial effector mechanisms. This interesting phenomenon requires further study.

Though previous studies on *Brucella* vaccination have reported the importance of the induction of specific antibodies (Araya *et al.*, 1989; Araya & Winter, 1990; Montaraz *et al.*, 1986) and $CD4^+$ T cells (Araya *et al.*, 1989; Araya & Winter, 1990; Zhan *et al.*, 1995) in protection, our study is the first, to our knowledge, to (i) formally demonstrate using genetically-deficient mice the complementary role played by both humoral immunity and Th1 $CD4^+$ T cells in the clearance of *Brucella* during secondary infection and (ii) identify the signaling pathways implicated in the development of these effector mechanisms. These results could improve our ability to develop protective vaccines or therapeutic treatments against brucellosis. Our observations suggest that the development of protective vaccines requires the selection of a vaccination protocol favoring humoral immunity, antigen presentation to $CD4^+$ T cells and IL-12 production. As functional Th1 $CD4^+$ T cells only developed following the administration of live bacteria in our model, live vaccines seem to remain the easiest and most potent tools for the production of candidate protective vaccines. However, live-attenuated strains retain generally unacceptable levels of virulence for human vaccination. Gamma-irradiated *Brucella* do not divide but conserve metabolic activity and protect mice against virulent bacterial challenge without signs of residual virulence (Magnani *et al.*, 2009). Thus, inactivated, yet metabolically active, microbes could represent a promising strategy for safe vaccination against human brucellosis.

Table 1. Impact of vaccination protocol and various immune deficiencies on the ability of immune system from C57BL/6 mice to control *B. melitensis* challenge.

C57BL/6 mice	**WT primo**	**WT HK-immun**	**WT Live-immun**	**MuMT$^{-/-}$ Live-immun**	**MyD88$^{-/-}$ Live-immun**	**IL-12p35$^{-/-}$ Live-immun**	**MHCII$^{-/-}$ Live-immun**
Circulating IgM	-	+++	+++	-	++	++++	++
Circulating IgG	-	++	+++	-	++	+++	-
Control of bacteria dissemination in the blood	-	+++	+++	-	+++	+++	++
IFN-γ^{+} cells (peritoneal cavity) *	-	+	+++	+++	+	+	+
IFN-γ^{+}CD4^{+} T cells (peritoneal cavity) *	-	+	+++	+++	+	+	-
% of mice displaying a sterilizing immunity in the spleen**	**19**	**17**	**69**	**33**	**26**	**28**	**2**

* Data considered for IFN-γ production concern the analysis of peritoneal cells 12h after a low dose challenge (10^5 CFU of *Brucella*), without restimulation. IFN-γ production of MuMT$^{-/-}$ mice are in data not shown.
**Numbers indicate the mean of the percentage of mice that are still positive for *Brucella* in the spleen 50 days post challenge. Mean was calculated with data from at least two independent experiments, each including a minimum of 10 mice.

SUPPLEMENTAL MATERIAL

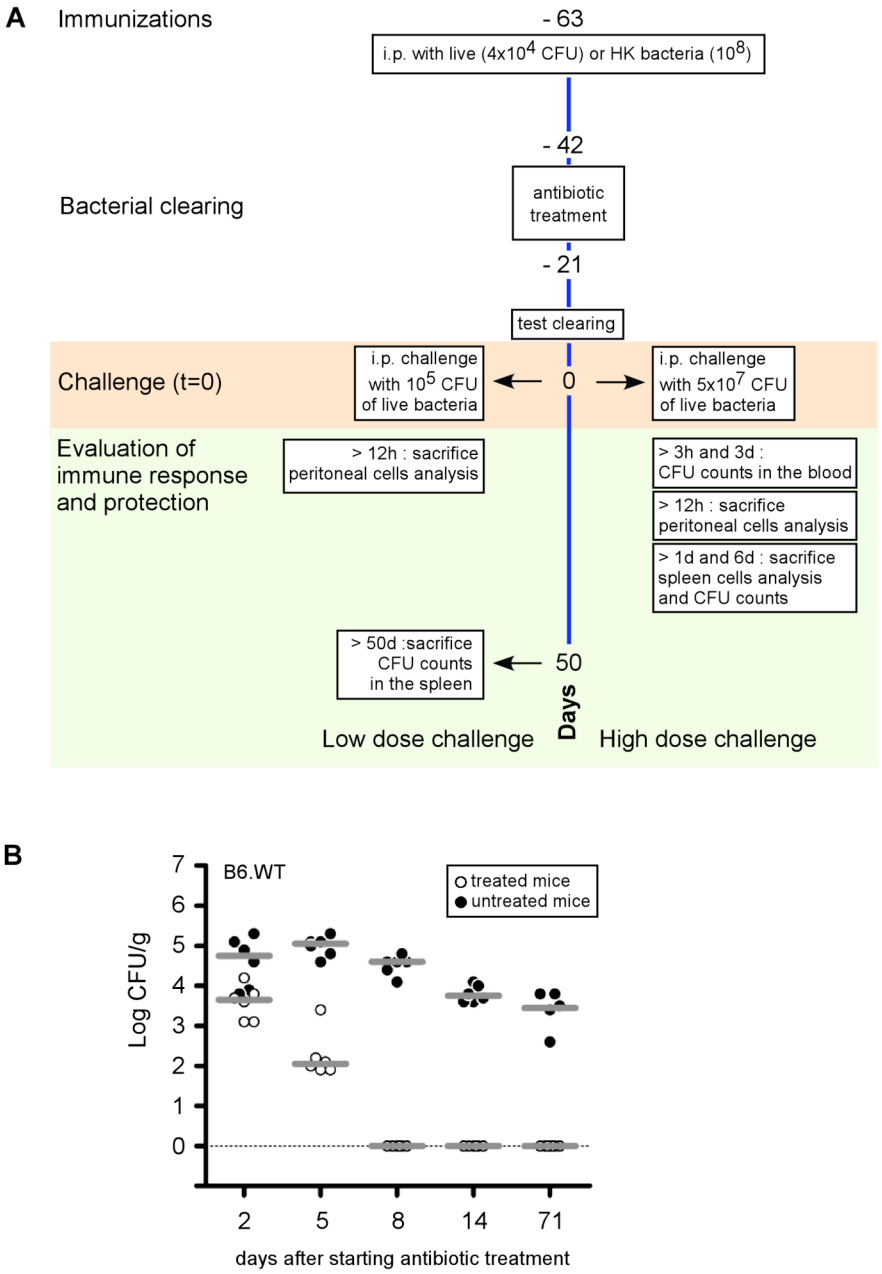

FIGURE S1. Experimental design and antibiotic treatment efficacy. A, Mice were immunized i.p. on day -63 with live ($4x10^4$ CFU) or heat-killed (HK) (10^8 CFU) *B. melitensis*. Three weeks after immunization, antibiotic treatment was administered daily for 3 weeks, starting on day -42. To ensure that the antibiotic treatment was effective, some mice were sacrificed 2 weeks after the end of the treatment and the splenic CFU were evaluated. Following the end of the treatment, after resting for 3 weeks, the mice were challenged on day 0 with either a low dose (10^5 CFU) or a high dose of *B. melitensis* ($5x10^7$ CFU). After a low-dose challenge, the immune response developed in the peritoneal cavity was analyzed at 12 hours post-challenge by flow cytometry. To evaluate the elicited protection, mice were sacrificed 50 days later and the splenic CFU were evaluated. Following a high-dose challenge, the immune response was characterized in the peritoneal cavity at 12 hours and in the spleen at 1 and 6 days post-challenge. The early dissemination of *Brucella* was evaluated by CFU counts in the blood at 3 hours and 3 days post-challenge, and the protection by CFU counts in the spleen at 1 and 6 days. **B,** The kinetics of antibiotic treatment efficacy was evaluated by following the elimination of *B. melitensis* in the spleen of C57BL/6 mice that received the treatment, started at 6 days post-infection. The data represent the CFU per gram of spleen. Grey bars represent the median. These results are representative of two independent experiments.

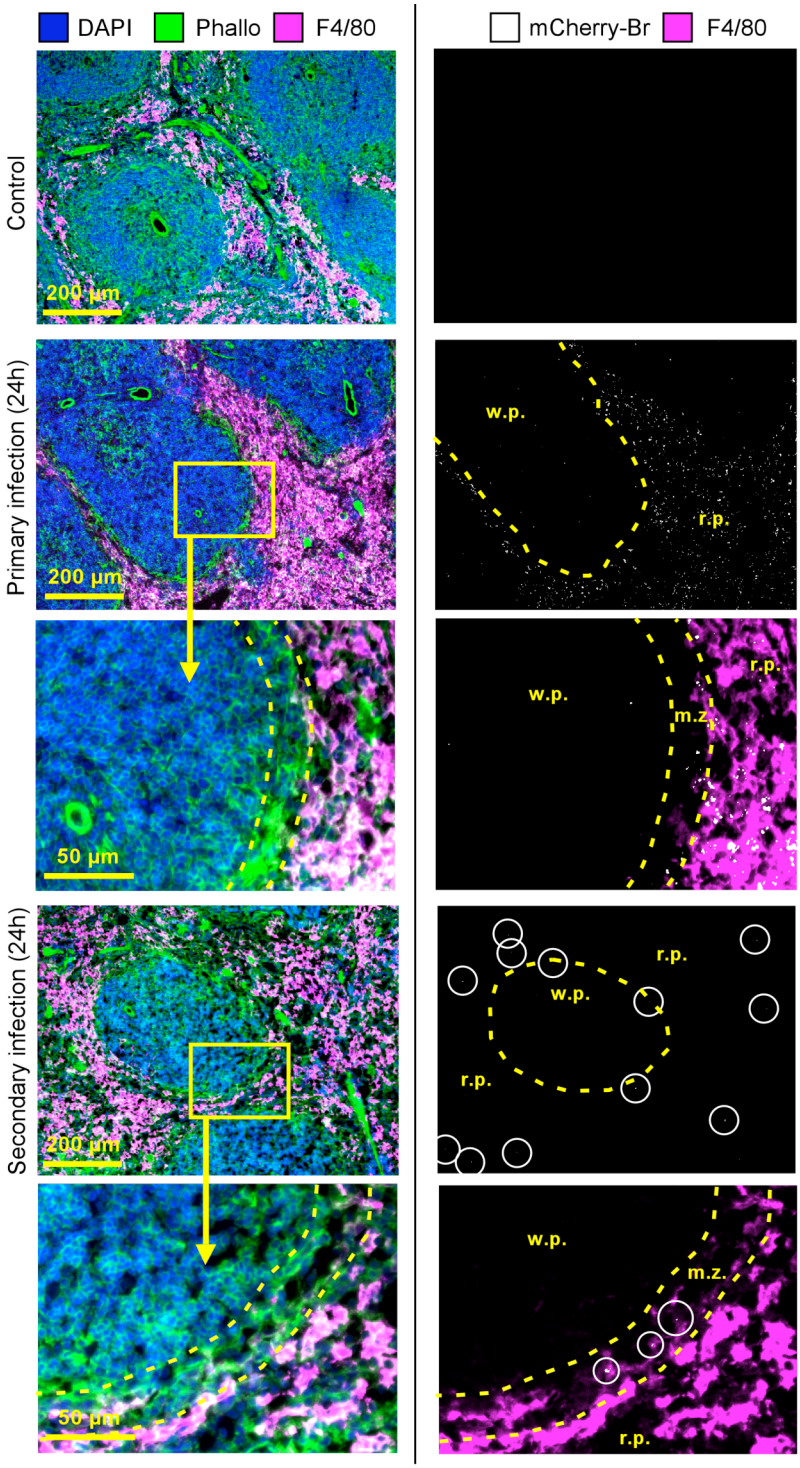

FIGURE S2. Phenotype of infected spleen cells following secondary infection. Wild-type C57BL/6 mice were immunized i.p. with live *B. melitensis* ($4x10^4$ CFU). Mice were treated with antibiotics as described in the Materials and Methods and were then challenged with a high dose of bacteria ($5x10^7$ CFU) and sacrificed at the selected time. Naive mice were infected with the same dose to compare the cell phenotypes between secondary and primary infection. The spleens were harvested and fixed. Frozen sections were examined by immunohistofluorescence for bacteria (mCherry signal) and F4/80-expressing cells at 24 hours post-infection. The insert in the middle panels is shown in the bottom panel at high magnification. The panels are color-coded by antigen as indicated. Scale bar = 200 or 50 µm. r.p.: red pulp; w.p.: white pulp; m.z.: marginal zone. The data are representative of 2 independent experiments.

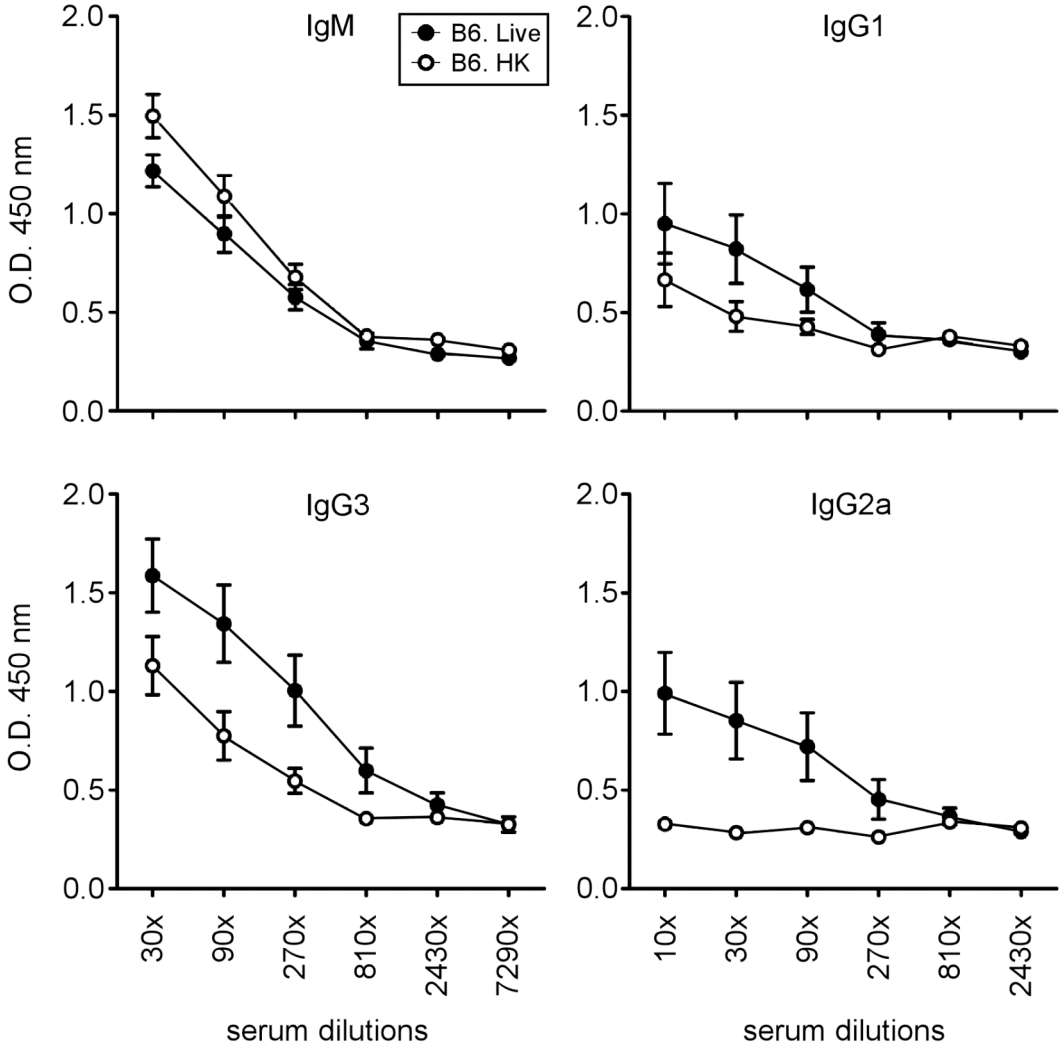

FIGURE S3. Analysis of *Brucella*-specific antibodies in the serum of C57BL/6 mice immunized previously with live or heat-killed *B. melitensis*. Mice were immunized i.p. with live ($4x10^4$ CFU) or heat-killed (HK) (10^8 CFU) *B. melitensis* and were treated with antibiotics as described in the Materials and Methods. Sera were collected 2 days before challenge and ELISA was performed to detect the isotype distribution of *Brucella*-specific antibodies. The data represent the mean +/- SEM of 8 mice.

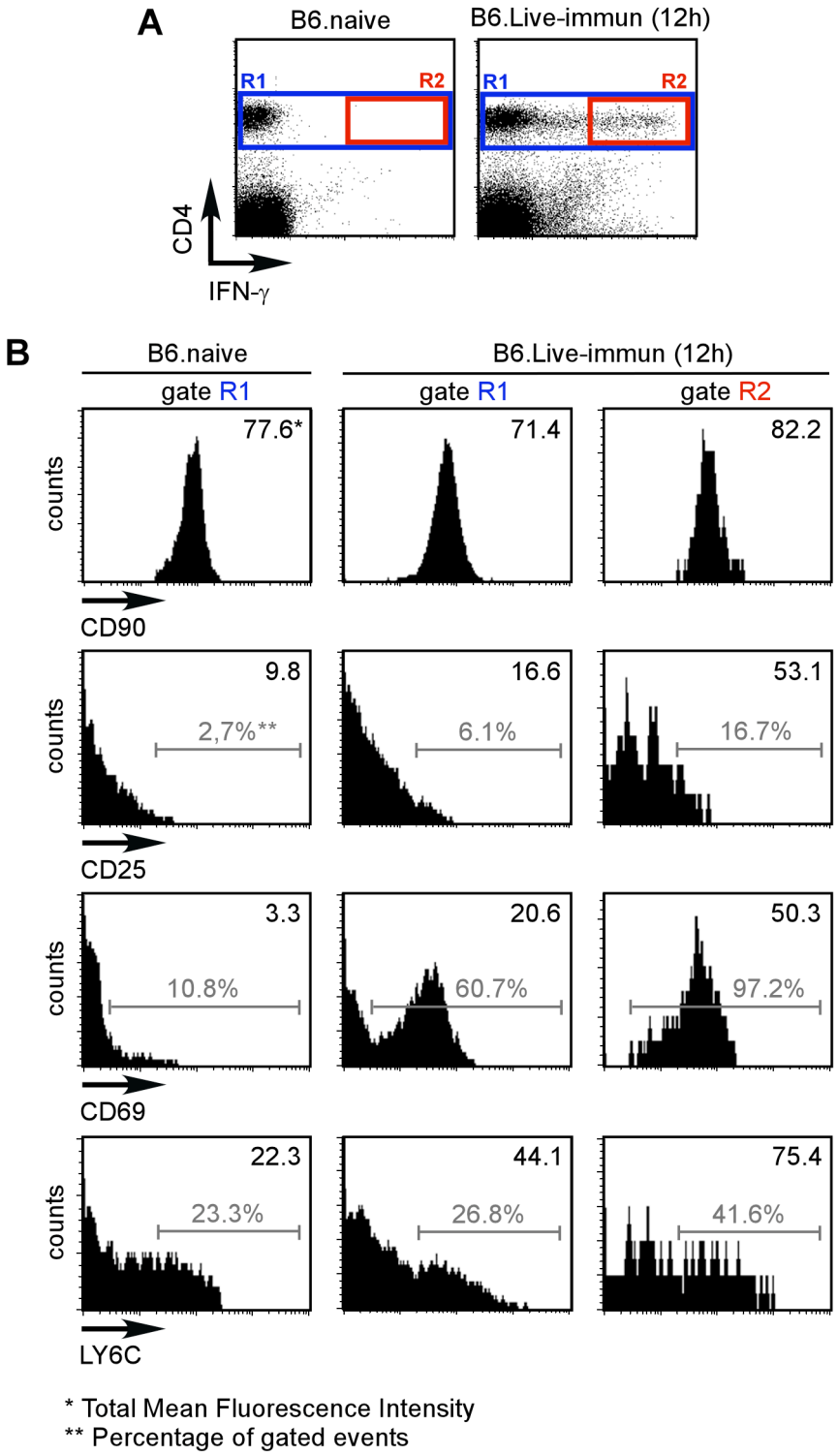

FIGURE S4. Phenotype of CD4$^+$IFN-γ$^+$ peritoneal T cells. C57BL/6 mice were immunized i.p. with live *B. melitensis* (4x10^4 CFU) and were treated with antibiotics as described in the Materials and Methods. Mice were then challenged with a low dose of *B. melitensis* (10^5 CFU) and sacrificed at 12 hours post-challenge. Peritoneal cells were collected and analyzed by flow cytometry. CD4$^+$ (gate R1) or CD4$^+$IFN-γ$^+$ peritoneal T cells (gate R2) were selected (**A**) and analyzed for the expression of a panel of activation markers: CD90, CD25, CD69 and LY6C (**B**). The data are represented for each group by the total Mean Fluorescence Intensity (mfi). The percentage of positive gated events is also shown for each marker.

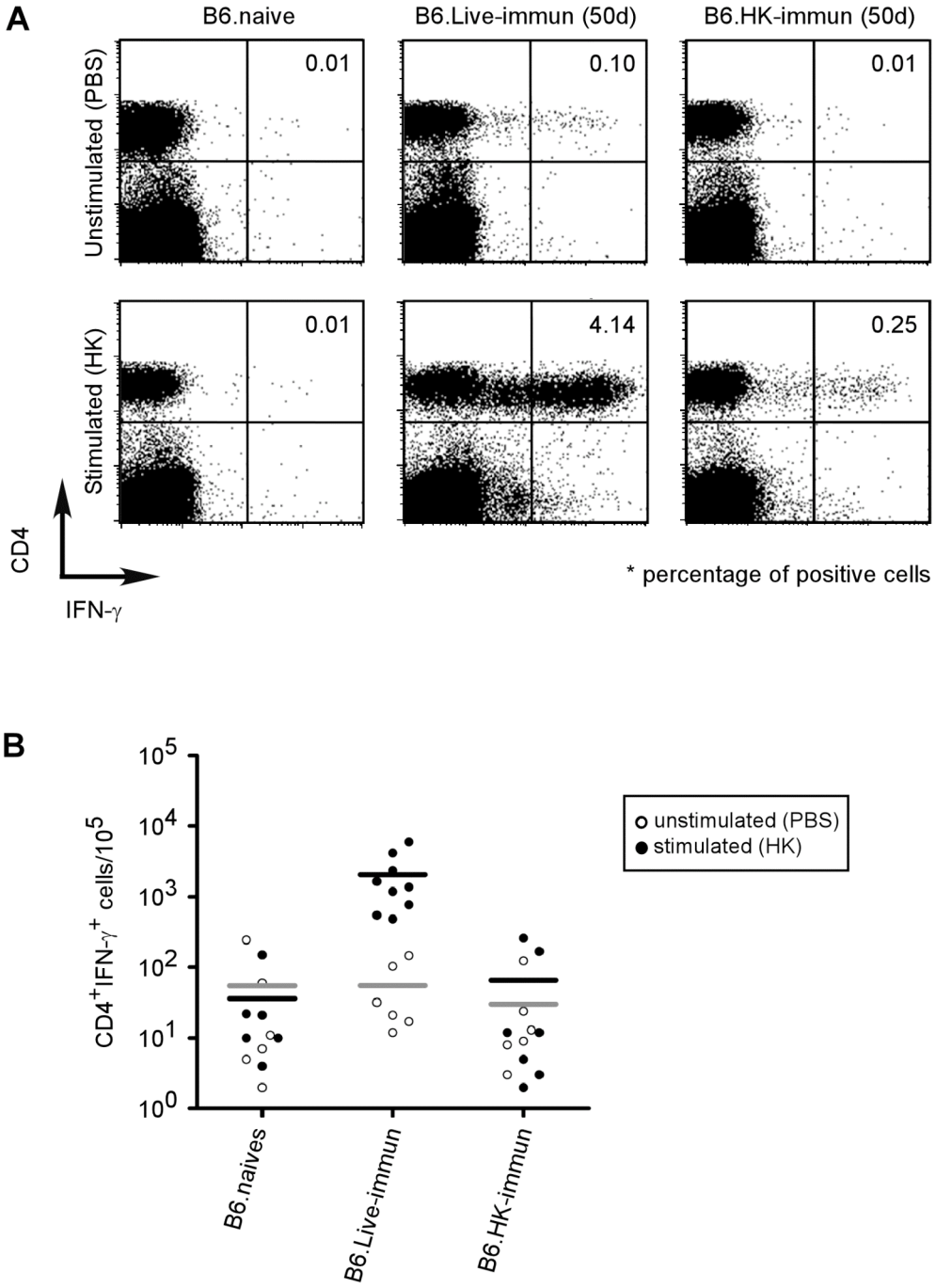

FIGURE S5. ***In vitro*** **stimulation of peritoneal cells from wild-type C57BL/6 mice immunized previously with live or HK** ***B. melitensis*****.** Mice were immunized i.p. with live ($4x10^4$ CFU) or heat-killed (HK) (10^8 CFU) *B. melitensis* and were treated with antibiotics as described in the Materials and Methods. Instead of an *in vivo* challenge, mice were sacrificed 60 days after immunization and peritoneal cells were recovered for *in vitro* stimulation with PBS (unstimulated) or HK *B. melitensis* (10^7 CFU) for 20 hours. Cells were then analyzed by flow cytometry. **A**, Cells were analyzed for CD4 expression versus IFN-γ production. The figure shows representative dot plots from a pool of two peritoneal cavities in each group. Numbers indicate the percentage of IFN-γ producing $CD4^+$ T cells. **B**, The graph represents the number of $CD4^+IFN\text{-}\gamma^+$ cells per 10^5 peritoneal cells acquired. Each data point represents the value obtained from an individual peritoneal cavity and the data are representative of two independent experiments. Grey bars represent the median of unstimulated groups whereas black bars represent the median of stimulated groups.

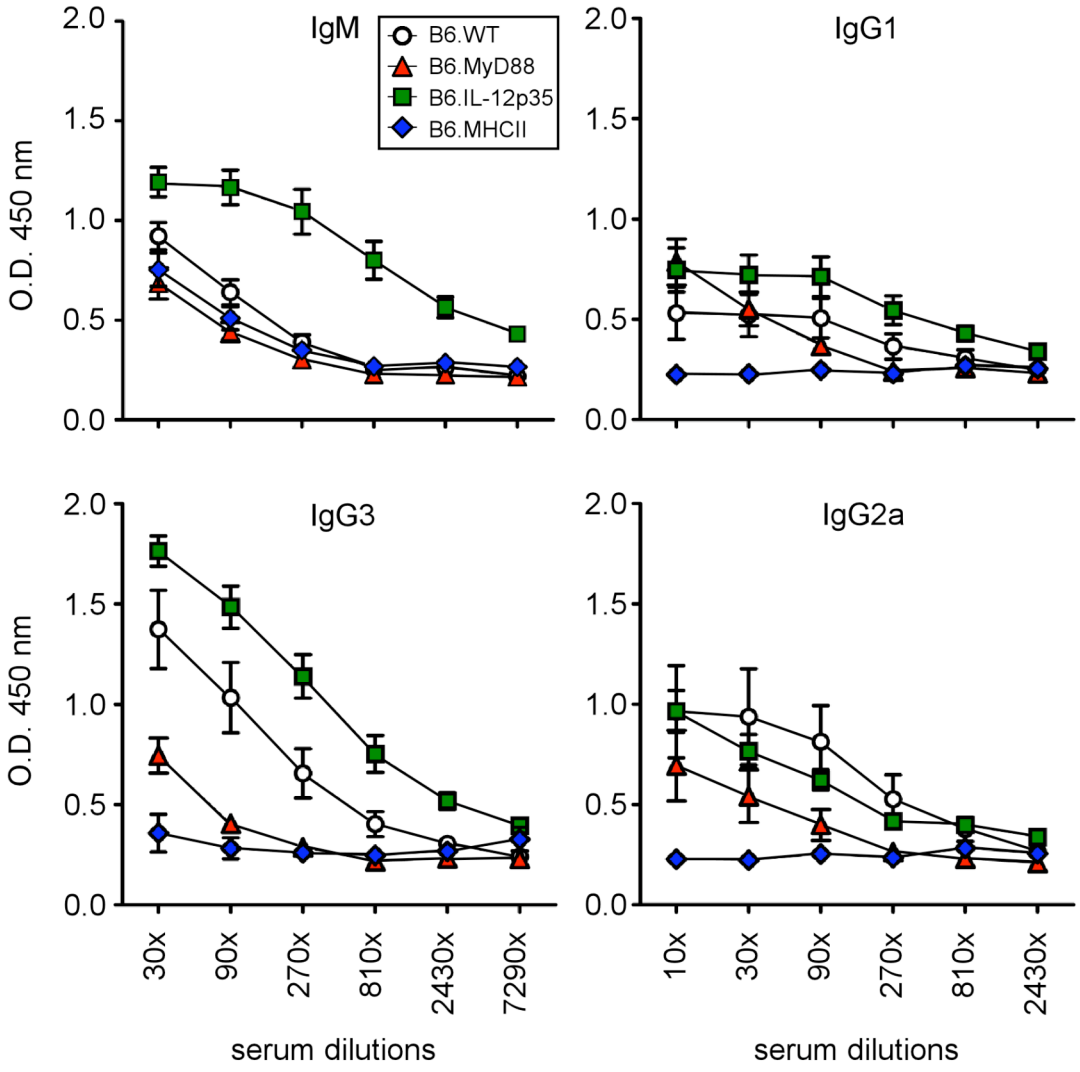

FIGURE S6. Analysis of *Brucella*-specific antibodies in the serum of wild-type, MyD88$^{-/-}$, IL-12p35$^{-/-}$ and MHCII$^{-/-}$ C57BL/6 mice immunized previously with live *B. melitensis*. Wild-type and deficient mice were immunized i.p. with live *B. melitensis* (4x10^4 CFU) and were treated with antibiotics as described in the Materials and Methods. Sera were collected 2 days before challenge and ELISA was performed to detect the isotype distribution of *Brucella*-specific antibodies. The data represent the mean +/- SEM of 8 mice.

ACKNOWLEDGEMENTS

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MATERIALS AND METHODS

Ethics Statement

The animal handling and procedures of this study 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 complete protocol was reviewed and approved by the Animal Welfare Committee of the Facultés Universitaires Notre-Dame de la Paix (FUNDP, Belgium) (Permit Number: 05-558).

Mice and reagents

MyD88$^{-/-}$ C57BL/6 mice (Kawai *et al.*, 1999) were obtained from Dr. S. Akira (Osaka University, Japan). IL-12p35$^{-/-}$ C57BL/6 mice (Mattner *et al.*, 1996) from Dr. B. Ryffel (University of Orleans, France). AID$^{-/-}$ C57BL/6 mice (Muramatsu *et al.*, 2000) from Dr. H Jacobs (The Netherlands Cancer Institute, The Netherlands). MHCII$^{-/-}$ C57BL/6 mice (Cosgrove *et al.*, 1991) from Jörg Reimann (University of Ulm, Ulm, Germany). RAG1$^{-/-}$ C57BL/6 mice (Mombaerts *et al.*, 1992) from Dr. S. Goriely (Université Libre de Bruxelles, Belgium). STAT6$^{-/-}$ BALB/c mice (Stamm *et al.*, 1998), MuMT$^{-/-}$ C57BL/6 mice (Kitamura *et al.*, 1991) were purchased from The Jackson Laboratory (Bar Harbor, ME). Wild-type C57BL/6 and BALB/c mice were purchased from Harlan (Bicester, UK) and were used as controls. All wild-type and deficient mice used in this study were bred in the animal facility of the Gosselies campus of the Université Libre de Bruxelles (ULB, Belgium).

B. melitensis strain 16M (Biotype1, ATCC 23456) was initially isolated from an infected goat and grown in biosafety level III laboratory facilities. Overnight cultures grown with shaking at 37°C in 2YT media (Luria-Bertani broth with double quantity of yeast extract) to stationary phase were washed twice in PBS (3500xg, 10 min.) before use for mice inoculation as previously described (Copin *et al.*, 2007). When indicated, we used a strain of *B. melitensis* 16M stably expressing the mCherry protein (mCherry-Br), a previously-described rapidly maturing variant of the red fluorescent protein DsRed (Shaner *et al.*, 2004), under the control of the strong *Brucella spp.* Promoter, PsojA (Kohler *et al.*, 1999). Construction of the mCherry-Br strain has been described previously in detail (Copin *et al.*, 2012).

To prepare heat-killed *B. melitensis*, bacteria from an overnight liquid culture in 2YT media were washed twice in PBS (3500xg, 10 min.) before heating at 80°C for 1 hour. To confirm the killing, an aliquot was plated onto 2YT medium.

Mice immunization and challenge

Mice were injected intra-peritoneally (i.p.) with $4x10^4$ CFU of live or 10^8 CFU of heat-killed (HK) *B. melitensis* in 500 µl of PBS. Control animals were injected with the same volume of PBS. Infectious doses were validated by plating serial dilutions of inoculums. 3 weeks after immunization, mice were given antibiotics for 3 weeks to clear the infection. After resting for an additional 3 weeks, they were challenged i.p. with either a low dose (10^5 CFU) or a high dose of *B. melitensis* ($5x10^7$ CFU). At the selected time after challenge, mice were bled or sacrificed by cervical dislocation. Immediately after sacrifice, peritoneal or spleen cells were collected for bacterial count, flow cytometry and microscopic analyses.

Antibiotic treatment

Mice were injected i.p. with $4x10^4$ CFU of live or 10^8 CFU of HK *B. melitensis* in 500 µl of PBS. Control animals were injected with the same volume of PBS. After resting for 3 weeks, antibiotic treatment was administered to both immunized and control mice for 3 weeks. The oral treatment was a combination of rifampicin (12 mg/kg) and streptomycin (450 mg/kg) (adapted from (Sathiyaseelan *et al.*, 2006)) prepared fresh daily and given in the drinking water. An additional i.p. treatment was given and consisted of 5 injections of streptomycin (300 mg/kg/day) throughout the 3 weeks of oral treatment (Lang *et al.*, 1993). The mice were not in distress. To ensure that the antibiotic treatment was effective, some mice from each group were sacrificed 1 week prior to the challenge and the CFU counts were evaluated in the spleen.

Bacterial count

Spleens were recovered in PBS/0.1% X-100 triton (Sigma). We performed successive serial dilutions in PBS to get the most accurate bacterial count and plated them onto 2YT medium. The CFU were counted after 4 days of culture at 37°C. For bacterial counts in the blood, 70 µl of blood was collected from the tail with heparinated capillaries at selected time points and diluted in PBS/0.1% X-100 triton (Sigma). Serial dilutions in PBS were performed and plated onto 2YT medium. The CFU were counted after 4 days of culture at 37°C.

Cytofluorometric analysis

As previously described (Copin *et al.*, 2007), spleens were harvested, cut in very small pieces and incubated with a cocktail of DNAse I fraction IX (Sigma-Aldrich Chimie SARL, Lyon, France) (100 µg/ml) and 1.6 mg/ml of collagenase (400 Mandl U/ml) at 37°C for 30 min. After washing, spleen cells were filtered and first incubated in saturating doses of purified 2.4G2 (anti-mouse Fc receptor, ATCC) in 200 µl PBS 0.2% BSA 0.02% NaN3 (FACS buffer) for 20 minutes on ice to prevent antibody binding to Fc receptor. 3-5x10^6 cells were stained on ice with various fluorescent mAb combinations in FACS buffer and further collected on a FACScalibur cytofluorometer (Becton Dickinson, BD). We purchased the following mAbs from BD Biosciences: Fluorescein (FITC)-coupled 145-2C11 (anti-CD3ε), Phycoerythrin (PE)-coupled RM4-5 (anti-CD4), Phycoerythrin (PE)-coupled 53-6.7 (anti-CD8α), Fluorescein (FITC)-coupled 53-2.1 (anti-CD90), Fluorescein (FITC)-coupled 7D4 (anti-CD25), Fluorescein (FITC)-coupled H1.2F3 (anti-CD69), Biotin-coupled AL-21 (anti-LY6C) and Fluorescein (FITC)-coupled avidin. The cells were analyzed on a FACScalibur cytofluorometer. Cells were gated according to size and scatter to eliminate dead cells and debris from the analysis.

Intracellular cytokine staining

For the intracellular staining, after DNase-collagenase treatment, spleen cells were incubated for 4 h in RPMI 1640 (Gibco Laboratories) 10% FCS with 1 µl/ml Golgi Stop (BD Pharmingen) at 37°C, 5% CO2. The cells were washed with FACS buffer and stained for cell surface markers before fixation in PBS/1% PFA for 15-20 min on ice. These cells were then permeabilized for 30 min using a saponin-based buffer (10X Perm/Wash, BD Pharmingen in FACS buffer) and stained with allophycocyanin-coupled XMG1.2 (anti-IFN-g; BD Biosciences). After final fixation in PBS/1% PFA, cells were analyzed on a FACScalibur cytofluorometer. No signal was detectable with control isotypes.

Immunofluorescence microscopy

Spleens were fixed for 6 hour at 4°C in 2% paraformaldehyde (pH 7.4), washed in PBS, incubated overnight at 4°C in a 20% PBS-sucrose solution under shaking, and washed again in PBS. Tissues were embedded in the Tissue-Tek OCT compound (Sakura), frozen in liquid nitrogen, and cryostat sections (5µm) were prepared. Tissues sections were rehydrated in PBS, then incubated successively in a PBS solution containing 1% blocking reagent

(Boeringer) (PBS-BR 1%) and in PBS-BR 1% containing any of the following mAbs or reagents: DAPI nucleic acid stain, Alexa Fluor 350 or 488 phalloidin (Molecular Probes), Alexa Fluor 647-coupled BM8 (anti-F4/80, Abcam). Slides were mounted in Fluoro-Gel medium (Electron Microscopy Sciences, Hatfield, PA). Labeled tissue sections were visualized with an Axiovert M200 inverted microscope (Zeiss, Iena, Germany) equipped with a high resolution monochrome camera (AxioCam HR, Zeiss). Images (1384x1036 pixels, 0.16μm/pixel) were acquired sequentially for each fluorochrome with A-Plan 10x/0.25 N.A. and LD-Plan-NeoFluar 63x/0.75 N.A. dry objectives and recorded as eight-bit grey-level *.zvi files. At least 3 slides per organ were analyzed from 3 different animals and the results are representative of 2 independent experiments.

In vitro stimulation of peritoneal cells

Mice were injected i.p. with $4x10^4$ CFU of live or 10^8 CFU of heat-killed *B. melitensis* in 500 μl of PBS and treated with antibiotics as described above. Control animals were injected with the same volume of PBS. Peritoneal cells from naive or immunized mice were harvested 60 days later by washing the peritoneal cavity with 10 ml of cold RPMI 1640. Cells were centrifuged and then cultured in RPMI 1640 supplemented with 10% FCS, 1% L-Glutamine, 1% Non Essential Amino Acids, 1% Pyruvate Sodium and 0.1% gentamycin, in 6-well plates with 10^7 cells/well in a volume of 2 ml. For stimulation, a concentration of $2x10^7$ bacteria/ml of HK *B. melitensis* was used. Cells were then incubated for 7 hour at 37°C, 5% CO2. After adding 1 μl/ml Golgi Stop (BD Pharmingen), the incubation was continued for an additional 13 hour at 37°C, 5% CO2. Cells were then washed and stained as described above.

ELISA

Specific murine IgM, IgG1, IgG2a and IgG3 isotypes were determined by enzyme-linked immunosorbent assay (ELISA). Polystyrene plates (Nunc 269620) were coated with heat-killed *B. melitensis* (10^7 CFU/ml). After incubation overnight at 4°C, plates were blocked for 2 hours at room temperature (RT) with 200 μl of PBS-3.65% casein. Then plates were incubated for 1 hour at room temperature with 50 μl of serial dilutions of the serum in PBS-3.5% casein. The sera from unimmunized mice were used as the negative control. After 4 washes with PBS, isotype-specific goat anti-mouse horseradish peroxidase conjugates were added (50 μl/well) at appropriate dilutions (αIgM from Sigma; LO-MG1-13 HRPO, LO-

MG2a-9 HRPO, LO-MG3-13 HRPO from LO-IMEX). Note that LO-MG2a-9, recognizing mouse gamma 2a heavy chain, binds to all allotypes, including the IgHla allotype expressed in BALB/c mice and the IgHlb allotype expressed in C57BL mice. After 1 hour of incubation at room temperature, plates were washed 4 times in PBS, and 100 µl of substrate solution (BD OptEiA) was added to each well. After 10 minutes of incubation at room temperature in the dark, the enzyme reaction was stopped by adding 25 µl/well of 2N H_2SO_4, and absorbance was measured at 450 nm.

Statistical analysis

We used a (Wilcoxon-) Mann-Whitney test provided by 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 $p<0.05$, $p<0.01$, $p<0.001$, respectively.

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Discussion & conclusions

1 Context

Brucellosis is a world widespread zoonotic disease caused by *Brucella* species. The human disease represents an important cause of morbidity worldwide whereas animal brucellosis is associated with serious economical losses caused mainly by abortion and infertility in ruminants (Corbel, 1997; Cutler *et al.*, 2005; Godfroid *et al.*, 2005). Currently, there is no available vaccine against human brucellosis and a great majority of commercially available animal vaccines are based on live, attenuated strains of *Brucella* (Ficht *et al.*, 2009; Schurig *et al.*, 2002) and display some drawbacks in terms of safety and diagnostic (Ashford *et al.*, 2004; Oliveira *et al.*, 2011b).

Throughout the last years scientists have made efforts to develop improved vaccines against brucellosis. But there is presently little knowledge on the immunological mechanisms that could mediate the efficacy of these candidate vaccines. Evaluation of these candidates is usually based on the knowledge of the primary immune response, using as reference the IFN-γ-mediated Th1 immune response. However there are still controversial results on the relative roles played by B cells, $CD4^+$ and $CD8^+$ T cells, probably originated from the use of variable strains of mice or species of *Brucella* (reviewed in (Baldwin & Goenka, 2006)). Moreover several studies have reported that various innate or adaptive immune deficiencies do not seem to alter drastically the outcome of *Brucella* infection in mice (**Appendix - 1**), suggesting that complex immune regulatory and compensatory events may take place during murine brucellosis (Goenka *et al.*, 2011; Martirosyan *et al.*, 2011). Together these data demonstrate the urgent need to clarify the contribution of each lymphocyte subset in the control of *Brucella* infection.

In other infectious models, several studies have demonstrated that primary and secondary responses can use distinct classes of effector mechanisms (Khader *et al.*, 2007; Lin *et al.*, 2009; Nakayama *et al.*, 2010; Narni-Mancinelli *et al.*, 2011; Schito *et al.*, 1998; Seo *et al.*, 2010; Soudja *et al.*, 2012). In the *Brucella* model of infection rare studies have tried to characterize the nature of protective immune response induced by immunization with a

virulent strain of *Brucella* (Araya *et al.*, 1989; Elzer *et al.*, 1994; Winter *et al.*, 1989; Zhan *et al.*, 1995). It is thus crucial to identify the immune markers closely associated to protective immune memory in order to provide a rational strategy for the evaluation of candidate vaccines.

Using a large panel of mice rendered genetically deficient for key elements of immune response, the main objectives of this thesis were (i) to evaluate the contributions of humoral response and T cell subsets during the primary and secondary immune response against *B. melitensis*, and (ii) identify the signaling pathways that are critical to elicit protective immune memory responses.

2 Primary immune response against *B. melitensis*

2.1 Lymphocyte subsets controlling brucellosis

In our first preliminary study (Vitry *et al.*, 2012), we clearly confirmed the key function played by IFN-γ-producing $CD4^+$ Th1 cells in the control of *B. melitensis* primary infection. Our study also revealed that Th2, Th17, IFN-γ-producing $CD8^+$ T cells or B cell-mediated humoral immunity only play a modest role in the clearance of bacteria. However some results raise novel questions that are discussed here.

2.1.1 IFN-γ production must be associated with activated $CD4^+$ T cells rather than $CD8^+$ T cells to be protective

Our study has revealed that in MHC-$II^{-/-}$ mice (that are deficient for $CD4^+$ T cells), IFN-γ production is taken over by $CD8^+$ T cells. When we compare MHC-$II^{-/-}$ mice and TAP-$1^{-/-}$ mice (that are deficient for $CD8^+$ T cells), we found that both infected strains display similar frequencies of IFN-γ producing $CD3^+$ T cells during the plateau phase of infection. However only MHC-$II^{-/-}$ mice failed to control *Brucella* infection during the chronic steady phase of infection, displaying a 10-fold increased bacterial load at 28 days p.i., suggesting that $CD4^+$ T cells cannot be successfully replaced by $CD8^+$ T cells.

Interestingly NK cells are also able to transiently compensate the lack of IFN-γ producing T cells in RAG-$1^{-/-}$ mice by producing a considerable amount of IFN-γ during the first week of infection. However RAG-$1^{-/-}$ mice are unable to control *Brucella* infection at any time but are nevertheless more resistant than IFN-$\gamma^{-/-}$ mice ((Copin *et al.*, 2012), see **Appendix-3**). This later data suggests that, despite the absence of a clearly established function for NK cells in murine brucellosis (Fernandes *et al.*, 1995), these cells could provide a temporary barrier limiting *Brucella* infection in immunodeficient hosts. Similar data have been observed in the *M. tuberculosis* model of infection and have revealed a critical role for NK cells in the T-independent IFN-γ response to the infection (Feng *et al.*, 2006).

Together, these results suggest that resistance to *Brucella* is not only restricted to IFN-γ production but also requires functional $CD4^+$ T cells. The observation that IFN-γ production

must be associated with activated $CD4^+$ T cells to be protective could be explained by the fact that $CD4^+$ T cells (i) provide other additional protective cofactors or (ii) are located in a more suitable place than $CD8^+$ T cells for the IFN-γ-dependent elimination of *Brucella*.

- *(i) $CD4^+$ T cells could provide other additional protective cofactors*

CD40L-CD40 ligation

Several studies have demonstrated that, independently of IFN-γ production, $CD4^+$ T cells *per se* exert contributing mechanisms in the activation of bactericidal effector functions of macrophages. Among them, activation of macrophages through direct contact via the interaction between CD40 on macrophages and CD40L on activated lymphocytes (Shu *et al.*, 1995) leads to NO and IL-12 production (Grewal *et al.*, 1997; Tian *et al.*, 1995). However in our work we have shown that CD40 deficiency does not affect IFN-γ production by $CD4^+$ T cells (Vitry *et al.*, 2012) or control of *Brucella* infection (**Appendix - 2**), suggesting that this way of macrophages activation is not selected during *Brucella* infection.

TNF-α secretion

In addition to CD40-dependent mechanisms of macrophages activation, $CD4^+$ T cells produce TNF-α, lymphotoxin-α and lymphotoxin-β, including membrane-associated forms of these mediators (Ware *et al.*, 1992). TNF-α may be a promising candidate because mice deficient for TNF receptor ($TNFR^{-/-}$) are more susceptible to *Brucella* infection than WT mice and they display a severe deficiency in IL-12 and NO production (Zhan & Cheers, 1998). This is in agreement with the fatal susceptibility observed in IRF-$1^{-/-}$ mice (Ko *et al.*, 2002a; Ko *et al.*, 2002b), which are defective in IFN-γ and TNF-α production (Senaldi *et al.*, 1999). In other words IRF-$1^{-/-}$ mice lack both IFN-γ-dependent as well as independent mechanisms to activate the phagocytic functions of macrophages. In agreement IRF-$1^{-/-}$ mice display more severe symptoms than SCID (Morfitt *et al.*, 1991), nude (Cheville *et al.*, 1995) or IFN-$\gamma^{-/-}$ mice (Murphy *et al.*, 2001b) and die only 2 weeks after *B. abortus* S2308 infection ($5x10^5$ CFU) (Ko *et al.*, 2002a).

Other mediators of inflammation

In *Listeria monocytogenes* model of infection, clearance of bacteria is mediated primarily by activated macrophages and later by T cells. Cytolysis, IFN-γ and TNF-α secretion were previously considered as the major effector mechanisms of memory $CD8^+$ T cells required for immunological protection *in vivo*. However Narni-Mancinelli *et al* (Narni-Mancinelli *et al.*, 2007) have found that none of these effectors was sufficient to protect against secondary infection. They demonstrated that CCL3 derived from re-activated memory $CD8^+$ T cells is required for efficient killing of WT bacteria by innate inflammatory mononuclear phagocytic cells. CCL3 (or macrophage inflammatory protein-1α, MIP-1α) is an important mediator of inflammation in vivo (Cook *et al.*, 1995). Cook *et al.* (Cook *et al.*, 1999) had demonstrated that this chemokine was produced by $CD8^+$ T cells and that this production was required for clearance of *L. monocytogenes* but the biological function of CCL3 was unknown until the studies of Narni-Mancinelli (Narni-Mancinelli *et al.*, 2007). Such scenario could occur in the *Brucella* model of infection. It should be interesting to compare gene expression in $CD4^+$ and $CD8^+$ T cells during the infection in order to identify the differential expression profile of cytokines and chemokines of each T cell population. For example, the Affymetrix GeneChip system has already been effective in identifying Th1- or Th2-expressed transcripts, for both human and mouse Th1 and Th2 cells (Chtanova *et al.*, 2001; Chtanova *et al.*, 2004), or more recently the transcriptomic signature of infected cells in the context of *Leishmania* infection (Rabhi *et al.*, 2012).

- *(ii) $CD4^+$ T cells are located in a more suitable place than $CD8^+$ T cells for the IFN-γ dependent elimination of Brucella*

By using immuno-fluorescence microscopy techniques, our team ((Copin *et al.*, 2012), see **Appendix-3**) has recently shown that *Brucella*-infected cells in the spleen were found clustered in granulomas at 5 days p.i.. Those granulomas were constituted of mature monocytes $CD11b^+F4/80^+Ly\text{-}6G^-MHC\text{-}II^+$ cells surrounded by $CD90.2^+$ cells (T cells) and $Ly\text{-}6G^+$ cells (granulocytes). Some of the $CD11b^+F4/80^+Ly\text{-}6G^-MHC\text{-}II^+$ infected cells clustered in granulomas were also $CD11c^+$ $iNOS^+$, suggesting that these cells were inflammatory DCs. In a previous report from our lab (Copin *et al.*, 2007), these inflammatory DCs were identified as the main iNOS-producing cells during *Brucella* infection. In addition NO production was shown to be strictly dependent on IFN-γ. As proposed in other infectious model (Kang *et al.*,

2008), IFN-γ appears to be essential for the building of fully functional granulomas. Moreover a causal link between the presence of fully functional granulomas and *Brucella* growth control has been suggested ((Copin *et al.*, 2012), see **Appendix-3**), rising granulomas as the "spearhead" of the immune response against *Brucella*.

We could hypothesize that $CD90^+$ T cells found in granulomas could be preferentially $CD4^+$ T cells rather than $CD8^+$ T cells. This particular localization of $CD4^+$ T cells could explain why IFN-γ production has to be associated with $CD4^+$ T cells: the proximity of IFN-γ producing cells with monocytes in granulomas allows the maturation of these cells in iNOS-producing DCs and the subsequent killing of bacteria inside this complex structure. It should be interesting to extend histological analysis to the localization of IFN-γ-producing T cells in C57BL/6 WT, TAP-$1^{-/-}$ and MHC-$II^{-/-}$ mice.

2.1.2 $CD8^+$ T cells are not able to sustain IFN-γ production during the plateau phase of infection

During the acute phase of *Brucella* infection, IFN-γ is synthesized by NK cells, $CD4^+$ T and $CD8^+$ T cells (Copin *et al.*, 2007; Vitry *et al.*, 2012). Later (12 days and beyond), $CD4^+$ T cells become the major producing cells and deficiency in MHC-II antigen presentation leads to enhanced susceptibility to the infection (Vitry *et al.*, 2012). However in MHC-$II^{-/-}$ mice, $CD8^+$ T cells remain unexpectedly able to produce IFN-γ, even if this production is not associated with protection, as mentioned above. These observations raise the following question: why do $CD8^+$ T cells cease to produce IFN-γ in WT mice two weeks after *Brucella* infection ?

- *(i) Antigen presentation by infected cells*

The study of Copin *et al* ((Copin *et al.*, 2012), see **Appendix-3**) has revealed that the phenotype of infected cells present a drastic evolution between 1d and 12d post infection in the spleen. Early after i.p. inoculation of 10^8 CFU, first infected cells are mainly (80%) macrophages ($F4/80^+CD11b^-$) located in the red pulp of the spleen. The other (20%) infected cells are marginal zone (m.z.) metallophilic macrophages (MOMA-1^+). Between 3 and 6h after inoculation, the frequency of m.z. macrophages decrease strongly and $CD11b^+CD11c^+$ dendritic cells progressively became infected (30% of total infected cells). At 5d, $CD11b^+$Ly-$6G^-$ monocytes constituted 30-40% of the infected cells

and were mainly found clustered in granulomas. Moreover, while granulomas are mainly detected in the red pulp at 5d, later on (12d) they are mostly located in the white pulp and specifically in T cell zones (Copin *et al.*, 2012). Therefore we could hypothesize that the phenotype's evolution of infected cells also impacts on the antigen presentation to T cells. The first infected cells could present antigens in both MHC-I and MHC-II, inducing IFN-γ production by $CD8^+$ and $CD4^+$ T cells, whereas later on infected cells could preferentially present in MHC-II, restricting IFN-γ production to $CD4^+$ T cells.

- *(ii) Immune modulation by Brucella*

Recently, Durward *et al* have observed a deficit in responding $CD8^+$ T cells during chronic brucellosis (Durward *et al.*, 2012). The authors suggest that the pathogenic protein TcpB of *B. melitensis* could actively suppress the expression of IFN-γ by $CD8^+$ T cells at certain times over the course of infection. TcpB contains a membrane translocation domain that permits its movement from the *Brucella* containing vesicle to the cytosol of infected cells (Radhakrishnan & Splitter, 2010). The authors hypothesize that by blocking specific intracellular pathways, TcpB would affect the immunological synapse between the infected cell and the $CD8^+$ T cell and protect cells expressing *Brucella* peptides in the context of MHC-I from specific $CD8^+$ T cell killing (Fooksman *et al.*, 2009). In agreement it has recently been shown that *B. abortus* induces intracellular retention of the MHC-I molecules in human macrophages down-modulating cytotoxic $CD8^+$ T cell response (Barrionuevo *et al.*, 2012). Durward *et al* also suggest that the membrane translocation domain of TcpB would allow its trafficking to responding $CD8^+$ T cells where it could disrupt the differentiation pathways leading to the generation of the memory phenotype. These actions could regulate the fate of the responding $CD8^+$ T cells, therefore explaining the resulting decline in functionality for these cells (Durward *et al.*, 2012). The study of Durward described mainly the chronic phase of infection (i.e. from 60 to 270 days) but we could hypothesize that such events can occur earlier. Nevertheless this hypothesis implicates that the immune modulation exerted by TcpB is abrogated in MHC-$II^{-/-}$ mice, allowing IFN-γ production by $CD8^+$ T cells.

2.1.3 On the different ability of IL-12p35- and IL-12p40-deficient C57BL/6 mice to control Brucella infection

We have observed that C57BL/6 mice lacking the IL-12p40 subunit are more susceptible to *Brucella* infection than mice lacking the IL-12p35 subunit. This suggests that other p40-dependent molecules act as mediators of protection in brucellosis. The differential ability of mice lacking either the p35 or the p40 subunit to control infection has been reported in infectious diseases caused by various pathogens such as *Mycobacterium tuberculosis* (Cooper *et al.*, 2002), *Toxoplasma gondii* (Lieberman *et al.*, 2004), *Salmonella enterica* (Schulz *et al.*, 2008) and *Cryptococcus neoformans* (Decken *et al.*, 1998).

As the p40 subunit is shared by IL-23 (composed of p19 and p40 subunits), most studies in the field have characterized the involvment of this cytokine in the control of the infection and have demonstrated that IL-23 provides a limited mechanism of resistance in the absence of IL-12p70 (composed of p35 and p40 subunits).

In the *Mycobacterium tuberculosis* (Mtb) model of infection, authors have observed that when IL-12p70 is lacking (as is the case in IL-12p35$^{-/-}$ mice), then IL-23 is required for the generation of IFN-γ-producing CD4^{+} T cells and control of bacterial growth (Khader *et al.*, 2005). Mice lacking IL-23p19 control bacterial growth with comparable kinetics as WT mice but when both IL-12p35 and IL-23p19 are lacking, mice are as susceptible as IL-12p40$^{-/-}$ mice. However, the IL-23-dependent compensatory response developed in IL-12p35$^{-/-}$ mice is not as potent as the IL-12p70-induced response since these mice are unable to control experimental infection. These results indicate that while IFN-γ-producing CD4^{+} Th1 cells are required for control of bacterial growth, other T cell subsets are induced by Mtb infection including Th17 cells. Although both Th17 and Th1 cells are induced following primary infection with Mtb, the protective response is significantly altered in the absence of Th1 cells but not in the absence of Th17 cells (Khader *et al.*, 2005).

In contrast with our study, Mtb infection induced antigen-specific IFN-γ-producing cells in IL-12p35$^{-/-}$ mice, although less potent than the response seen in WT mice, but much more vigorous than that seen in the IL-12p40$^{-/-}$ mice (Cooper et al., 2002). Noteworthy in our model a slight difference in the frequency of TCRβ^{+}CD4^{+}IFN-γ^{+} was detected at 12 days post infection, with IL-12p35$^{-/-}$ mice displaying a higher number of positive cells than IL-12p40$^{-/-}$ mice (figure 5.B, Part I).

Induction of Th17 response during primary immune response is not devoid of interest. Khader *et al*. demonstrated that in vaccinated animals the absence of memory Th17 cells results in loss of both the accelerated memory Th1 response and protection. Authors proposed that vaccination induces IL-17-producing $CD4^+$ T cells that populate the lung and, after challenge, trigger the production of chemokines that recruit $CD4^+$ T cells producing IFN-γ, which ultimately restrict bacterial growth (Khader *et al.*, 2007; Khader & Cooper, 2008).

Therefore it could be interesting to investigate more deeply the implication of the Th17 response during the course of a primary and secondary infection by *Brucella*. Indeed we have detected a small and short upregulation of IL-17A mRNA expression at 5 days post-infection, suggesting that IL-17-producing cells are induced by primary *Brucella* infection.

2.2 *B. melitensis* persists in the blood of infected mice and infects erythrocytes

The second part of our work has focused on mechanisms of *Brucella* dissemination throughout the blood following intraperitoneal infection of C57BL/6 mice (Results section, Part II). Our study revealed that *Brucella* persists several weeks in the blood of the murine host and exhibits a brief extracellular phase in this fluid, likely thanks to its ability to resist complement and cationic peptides (Eisenschenk *et al.*, 1999; Freer *et al.*, 1996; Martinez de Tejada *et al.*, 1995). By using MuMT$^{-/-}$ mice, we showed that absence of adaptive humoral immunity does not modify the number of blood bacteria between 1 and 3 days post-infection, demonstrating that natural antibodies are not involved in the early control of *Brucella* dissemination, in contrast with what has been proposed in other models of infection (Ochsenbein *et al.*, 1999). However circulating antibodies induced by vaccination impair blood persistence following challenge. Unexpectedly, we found that 1 day after infection, bacteria were mainly detected inside erythrocytes. This surprising location is discussed here.

2.2.1 Intraerythrocytic infection is not so scarce

Several bacterial pathogens belonging to the α- (*Bartonella spp, Anaplasma marginale*) and γ- (*Francisella tularensis*) proteobacteria phylum cause bacteremia and are able to establish intraerythrocytic infections. Moreover they are transmitted by ticks or fleas, raising the idea that *Brucella* persistence in the blood could constitute a means of transmission by bloodsucking insects. Similarities among them are even troubling. Some of their characteristics are presented below.

Bartonella spp

Bartonella spp. are facultative intracellular bacteria that exploit hemotropism and arthropod-mediated transmission as common parasitic strategies. The hallmark of *Bartonella* infection is the long-lasting intraerythrocytic and asymptomatic bacteremia (Schulein *et al.*, 2001). Several species of the genus *Bartonella* are human pathogens and pet cats or wild-living animals constitute a large reservoir for this zoonotic disease, with infection rates ranging between 15 to 30% of cats and higher than 50% for wild-living mammals.
Following transmission by an arthropod vector, *Bartonella* colonizes the primary niche, which probably involves entry into migratory cells (whose identity is still controversial) and

transport to the vascular endothelium, where the bacteria persist intracellularly. From the primary niche, the bacteria are seeded into the bloodstream (3-4 days after inoculation, (Schulein *et al.*, 2001)), where they invade erythrocytes and re-infect the primary niche. After limited replication inside the red blood cells, they persist in the intraerythrocytic niche competent for transmission by a bloodsucking arthropod. Depending on the species of *Bartonella*, the proportion of infected erythrocytes ranges from well below 1% to around 60% (reviewed in (Harms & Dehio, 2012)). By passive immune evasion and active immuno-modulation, *Bartonella* promotes a moderated inflammatory response profile and a reduced host immune response. Colonization of erythrocytes is made in 3 steps: adhesion, that is a critical determinant for host specificity (Vayssier-Taussat *et al.*, 2010), deformation characterized by indentations and invaginations at the site where *Bartonella* attached and finally invasion that has been described as a "forced endocytosis" allowing the bacteria to locate in a membrane-bound compartment (Benson *et al.*, 1986). Interestingly a recent study (Deng *et al.*, 2012) investigating the role of the spleen in *Bartonella spp.* infection reveals that this organ does not constitute a sanctuary for chronic infection but rather help to reduce bacterial load by retaining and filtering infected erythrocytes. Bacteremia is 10-fold higher in splenectomized mice than in normal mice and lasted 2 weeks longer (Deng *et al.*, 2012).

Anaplasma marginale

Anaplasma marginale is a tick-borne rickettsial pathogen of cattle, causing a hemolytic disease (Kocan *et al.*, 2003). In cattle, *A. marginale* is an obligate intraerythrocytic bacterium that multiplies only within membrane-bound inclusions in the cytoplasm, and 70% or more of the erythrocytes may become infected during acute infection. However in ticks this pathogen is able to develop in several tissues (gut cells, salivary gland cells, etc.) and undergoes a complex development cycle coordinated with the tick feeding cycle. Infected erythrocytes are subsequently phagocytized by bovine reticuloendothelial cells, resulting in the development of mild to severe anemia, sometime leading to death. Cattle that survive acute infection develop persistent infections characterized by cyclic low-level rickettsemia due to the emergence of antigenic variants (French *et al.*, 1999).

Francisella tularensis

Francisella tularensis causes a variety of naturally occuring human infections that can be acquired by inhalation, arthropod bites, oropharyngeal exposure, or contact (Sjostedt, 2007). Infection can result in the severe disease tularemia whose symptoms vary depending on how the bacteria enter the body (pneumonia, skin ulcer, etc.). *F. tularensis* replicates in phagocytic cells of the innate immune system, such as macrophages (Meibom & Charbit, 2010) but is also able to invade a range of nonphagocytic host cells (alveolar epithelial cells, kidney epithelial cells, hepatocytes, fibroblasts) (Hall *et al.*, 2008). Shortly after entry into the macrophage, *F. tularensis* evades the normal killing mechanisms, escaping from the phagosome into the cytoplasm, where it replicates freely (Sjostedt, 2006). *F. tularensis* also display a significant extracellular phase in the blood of infected mice and this distribution is observed irrespective of size of inoculum, route of inoculation, time after inoculation or virulence of the infecting strain. (Forestal *et al.*, 2007). The recent study of Horzempa *et al.* (Horzempa *et al.*, 2011) has revealed that *F. tularensis* is able to invade erythrocytes *in vivo* during infection of mice. Bacterium is located in the cytosol of the erythrocyte but does not replicate, suggesting that *F. tularensis* may be dormant in this environment. However intraerythrocytic bacteria maintain virulence since infected erythrocytes induce rapid morbidity when transferred to naive mice intravenously. Authors speculate that *F. tularensis* virulence could be modulated by the high hemoglobin content (and so the high iron concentration) in erythrocytes because mice transferred with infected erythrocytes died

earlier than mice challenged with free bacteria. They also propose that complement and heat-stabile components of serum facilitate the initial bacteria-host cell interaction but that entry inside erythrocyte should implicate other mechanisms (Horzempa *et al.*, 2011).

2.2.2 The mystery of invasion process

Erythrocytes are not capable of endocytosis (Schekman & Singer, 1976), so defining the precise mechanism of *Brucella* uptake by the cell remains a challenge.

An interesting hypothesis is that *Brucella* could infect erythrocyte progenitors and then enter the bloodstream coincident with their maturation. This has been proposed in *Bartonella* studies because this bacterium can invade *in vitro* hematopoietic progenitor cells (Mandle *et al.*, 2005). However others have demonstrated *in vivo* that *Bartonella* was able to invade mature erythrocyte (Schulein *et al.*, 2001), indicating that the active part of cell entry is performed by the bacterium.

To gain further insight on this question, we have tried to infect *in vitro* erythrocytes from naive mice but, in conditions where RAW cells were fully infected, we never observed erythrocytes infection (data not shown). Therefore we cannot assert that *Brucella* invades mature erythrocytes *in vivo*. Another explanation for the failure of this experiment could be that, as *Bartonella*, a preceding period of residence in a primary niche is necessary to make the bacteria and/or the host competent for colonization of erythrocytes. However we observed *Brucella* in the blood only 10 minutes after i.p. inoculation (data not shown), whereas the bacteremic phase of *Bartonella* synchronously began at 4-5 days post infection (Schulein *et al.*, 2001). This strongly suggests that *Brucella* is competent for erythrocyte infection as soon as it reaches the bloodstream.

The mechanism of erythrocyte invasion by bacteria has not been resolved so far, even in the other infectious models presented above. In the *Bartonella* model of infection, several studies have focused on the molecular mechanisms underlying this phenomenon, bringing food for thought. Authors described erythrocyte invasion by *Bartonella* as a 3-steps process, including adhesion, deformation and invasion (figure 18) (reviewed in (Harms & Dehio, 2012)). These steps are described here and compared with the *Brucella* infectious model.

Adhesion to erythrocytes : *Bartonella* interacts with the α and β subunits of spectrin, band 3 protein, glycophorin A, and monomeric as well as dimeric glycophorin B from erythrocytes (Buckles & McGinnis Hill, 2000). Adhesion of *Bartonella* to erythrocytes is also mediated by the type IV secretion system (T4SS) Trw. Interestingly, a mutant devoid of a functional Trw system was found to appear in the bloodstream but was cleared shortly after infection. The Trw T4SS is upregulated during the primary niche infection, allowing *Bartonella* to rapidly infect erythrocytes and spend the shortest possible time in the hostile environment that is blood (Dehio, 2004; Vayssier-Taussat *et al.*, 2010). In the case of the *Brucella* model of infection, one *in vitro* study has reported that *Brucella* is able to bind sialic acid residues on human and animal erythrocytes (del C. Rocha-Gracia *et al.*, 2002). Authors showed that adhesion is mediated by a protein of 29 kDa, referred to as SP29 that corresponds to a *Brucella melitensis* periplasmic protein highly homologous to a ribose-binding protein present in *Yersinia pestis* (Parkhill et al., 2001) (i.e. another gram-negative bacteria belonging to the γ-proteobacteria phylum and infecting erythrocytes (Feodorova & Devdariani, 2002)).

Intracellular survival and virulence of *Brucella* requires the virB T4SS that is involved in controlling the maturation of the *Brucella* containing vacuole (BCV) into a replication-permissive organelle (endoplasmic reticulum-associated vacuole) in nucleated cells (Celli, 2006; O'Callaghan *et al.*, 1999). Therefore it should be of great interest to evaluate the blood persistence of the VirB mutant of *Brucella* to test whether this T4SS is involved in erythrocyte adhesion and invasion.

Deformation of erythrocytes : During *Bartonella* infection, erythrocytes develop progressing indentations and invaginations at the sites where *Bartonella* attached (figure 18) (Benson *et al.*, 1986). This process probably provides entry sites for the bacterium and involves the deformin, a secreted bacterial factor whose molecular identity, mode of action and secretion mechanisms remain elusive (Harms & Dehio, 2012; Xu *et al.*, 1995). *Brucella*-infected erythrocytes images obtained in this work showed that intracellular bacterium seem “molded” by the erythrocyte membrane at 3h post infection. Apart from this, at the time analysed, we did not observe deformations of the erythrocyte surface that would provide entry sites for *Brucella*. As molded bacteria are easier to detect during microscopic analysis, we cannot assert that all infected erythrocytes display such deformations.

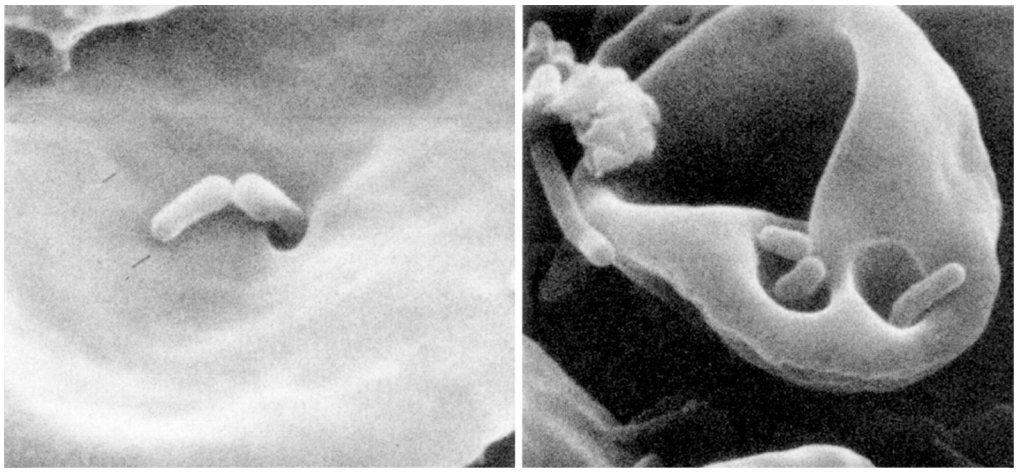

Figure 18. Entry of *Bartonella* into human erythrocytes (from (Benson *et al.*, 1986)**).** The scanning electron micrographs show *B. bacilliformis* first inducing indentations in the erythrocyte surface (left) and then invading at the resulting pits (right).

Invasion of erythrocytes : As mature erythrocytes are nonendocytic, *Bartonella* invasion has been described as "forced endocytosis" (Benson *et al.*, 1986). Pathogenic parasites, including *Plasmodium* (Deguercy *et al.*, 1990; Fiori *et al.*, 1997; Hanspal *et al.*, 2002) use a common strategy that consists in disconnecting the spectrin-actin cytoskeletal network from the membrane in order to locally weaken surface integrity. Such processes have also been proposed for *Bartonella* (Harms & Dehio, 2012). Moreover some species of *Bartonella* that does not harbor the Trw T4SS are flagellated and are thus motile. Flagella are well established as bacterial virulence factors and have been described to play an important role in the invasion of erythrocytes (Scherer *et al.*, 1993). Interestingly Benson *et al*. observed that bacteria were pushing into surface indentations with a "boring or twisting motion", behaving like a drill (Benson *et al.*, 1986). Consistently antiflagellin antibodies decreased erythrocyte attachment but much more invasion of *Bartonella* (Scherer *et al.*, 1993).

Under certain growth conditions, *Brucella melitensis* expresses flagellar genes and produces a polar sheathed flagellum (Ferooz & Letesson, 2010; Fretin *et al.*, 2005), although no motility has been reported. The function of this putative flagellar apparatus is still unknown but mutants of flagellar components have been reported to be attenuated in mice during the chronic steady phase of infection by several studies (Fretin *et al.*, 2005; Lestrate *et al.*, 2003; Petersen *et al.*, 2011), suggesting that flagellar genes are required for *Brucella*

persistence within its host. Interestingly, infection of BALB/c mice with a flagellar mutant strain results in an attenuated phenotype in which the bacteria fail to fully disseminate and instead localize at the site of infection (Petersen *et al.*, 2011). Therefore it should be interesting to investigate the persistence in the blood of such mutants. The flagellum of *Brucella* could serve for erythrocyte invasion and consecutively dissemination in the host.

3 Secondary immune response against *B. melitensis*

3.1 On differences between primary and secondary immune response

The third part of this work aimed to characterize the molecular and cellular mechanisms implicated in a secondary immune response against *B. melitensis*. Interestingly this study revealed that effector mechanisms selected for a secondary immune response were different from those activated during a primary immune response. We discuss here the differential but complementary implication of B and T cells.

3.1.1 B cells are dispensable for controlling primary B. melitensis infection but are essential in a secondary infection

We demonstrated that, during a secondary infection, circulating antibodies play an essential role in the control of *B. melitensis* early dissemination by blood stream and that absence of B cells affects the sterilizing immunity later in the spleen. This is in agreement with other studies showing that serum from infected donors can confer variable levels of protective immunity by passive transfer experiments (Araya *et al.*, 1989; Araya & Winter, 1990; Elzer *et al.*, 1994; Montaraz & Winter, 1986; Montaraz *et al.*, 1986; Winter *et al.*, 1989). However we demonstrate here that humoral response alone is not sufficient to provide a long-term sterilizing protection in the spleen. IFN-γ producing $CD4^+$ T cells are also required.

Different B cell subtypes serve distinct functions

As demonstrated by us (Vitry *et al.*, 2012) and studies from Goenka et al (Goenka *et al.*, 2011; Goenka *et al.*, 2012), primary B cell response to *Brucella* seems dispensable or even detrimental to the host and, in agreement, B cell functions seem to be subverted by *Brucella*. Indeed, the latter study (Goenka *et al.*, 2012) has demonstrated that *Brucella* utilizes the $CD19^+$ B cell niche for survival and imparts a regulatory character to these cells (i.e. production of TGFβ-1). This process may facilitate chronic bacterial infection by dampening host immune response that mediates clearance of infection. But, interestingly,

our study revealed that B cells are able to mount a memory response that is required for the control of a second *Brucella* infection, suggesting that different B cell subtypes serve distinct functions during *Brucella* infection.

Which Ig isotype can be considered as a immunological marker of protection for murine brucellosis ?

In the context of a secondary infection, we demonstrated with MuMT$^{-/-}$ mice (lacking antibodies) that antibodies are necessary for the early control of *Brucella* dissemination in the blood and late sterilizing immunity in the spleen. Strikingly, early control of *Brucella* by antibodies does not specially rely on IgG; IgM are sufficient to assume this task. Indeed our results demonstrate that CD4^{+} T cells, MyD88/IL-12p35 signaling pathways and even AID-mediated class switch are dispensable to obtain protective circulating antibodies able to restrict bacteria dissemination in the blood. However we cannot exclude a role for IgG in the later control of infection since we do not have the protection status in the spleen of AID$^{-/-}$ mice 50 days post-challenge. Complete lack of protective immunity in the spleen is correlated to the absence of IgG in MHC-II$^{-/-}$ mice. This confirms that CD4^{+} T cells are implicated in the generation of specific IgG during *Brucella* infection. Nevertheless, implication of CD4^{+} T cells does not appear to be limited to B cell help since MuMT$^{-/-}$ mice (that lack antibodies but not IFN-γ producing CD4^{+} T cells) display a better sterilizing immunity in the spleen than MHC-II$^{-/-}$ mice (that lack IgG and IFN-γ producing CD4^{+} T cells). We are thus tempted to postulate that humoral immunity and CD4^{+} T cells can develop independently and that humoral immunity rely on IgM. Both effector functions are necessary for a sterilizing immunity in the spleen : on the one hand antibodies-mediated protection may consist in early trapping of bacteria in order to limit their dissemination (eliminating approximately 99,99% of bacteria from the blood), and on the other hand IFN-γ producing CD4^{+} T cells may mediate intracellular killing of the remaining *Brucella* (the precise mechanisms remain to be determined).

Accordingly, preliminary results recently obtained in our lab indicate that AID$^{-/-}$ mice immunized by intranasal route with *B. melitensis* develop a sterilizing immunity (similar to wild-type mice) in the spleen, the liver and the lungs following an intranasal challenge. This suggests that high affinity IgG generated in germinal centers are not necessary to provide a protective secondary immune response against *Brucella* and that IgM are sufficient for the

protection. An experiment intended to confirm the ability of $AID^{-/-}$ mice to develop a sterilizing immunity in the spleen in our i.p. model is currently in progress.

The great majority of vaccination studies (Al-Mariri, 2009; Leclerq *et al.*, 2002; Pasquevich *et al.*, 2009) used to analyze the isotypes induced by their vaccine candidate and discussed the interest of IFN-γ/IL-12 dependent isotypes in the control of *Brucella* infection. In contrast our results suggest that the generation of Th1-dependent isotypes is not a critical parameter in vaccination.

While IgG has long been taken as surrogate marker for memory, some studies have brought evidence for a long-lived IgM memory B cell population (reviewed by (Racine & Winslow, 2009), (Dogan *et al.*, 2009; Pape *et al.*, 2011; Racine *et al.*, 2011; Tomayko *et al.*, 2010)). Two types of IgM can be found in circulation in normal mice. Natural IgM, secreted mainly by $CD5^{+}$ B-1a cells in the apparent absence of antigen stimulation, constitutes most of the circulating IgM and tends to be polyreactive to both foreign antigen and self-components. In contrast, immune (or antigen-induced) IgM is mostly produced by conventional B-2 cells following antigen or pathogen encounter (Baumgarth *et al.*, 1999). Nevertheless other particular B cell subsets can perform this task, as it has been demonstrated during the chronic intracellular bacterial infection caused by *Ehrlichia muris,* where bone marrow plasmablasts are responsible for the production of immune IgM mediating long-term protection (Racine *et al.*, 2011). In addition, some studies have demonstrated that B-1b cells, which are the main B cell subpopulation in pleural and peritoneal cavities (Baumgarth, 2011), also contribute to the production of immune IgM in response to T-independent antigens (Alugupalli *et al.*, 2003; Alugupalli *et al.*, 2004; Haas *et al.*, 2005). In agreement, we demonstrate that the protective humoral response was also induced by immunization with HK bacteria, suggesting that *Brucella* PAMPs are implicated in the activation of B cells and that PRRs recognizing these PAMPs can act by a MyD88-independent signaling pathway. We could hypothesize that B1-b cells are activated following i.p. inoculation of *B. melitensis* and generate a long-term protection to *Brucella* infection, as it has been demonstrated for *Borrelia hermsii*, another bacteria causing bacteremia associated with relapsing fever (Alugupalli *et al.*, 2003; Alugupalli *et al.*, 2004). Note that persistent infection does not appear to be required for the maintenance of the long-term

Ag-specific IgM responses since the antibiotic treatment of infected mice still allows antibody-mediated protection.

3.1.2 CD4$^+$ but not CD8$^+$ T cells are activated to produce IFN-γ during secondary infection

Whereas both CD4$^+$ and CD8$^+$ T cells produce IFN-γ during the first week of a primary infection, only CD4$^+$ T cells are activated and produce large quantities of IFN-γ early after the challenge. The activation of these cells is strictly dependent on MyD88/IL-12p35 signaling pathways and only occurs in mice immunized with live bacteria. Strikingly, in contrast with the primary immune response, CD8$^+$ T cells are not able to compensate the lack of IFN-γ producing CD4$^+$ T cells in MHC-II$^{-/-}$ mice, suggesting that despite their activation in these deficient mice, CD8$^+$ T cells do probably not generate a memory response. This is in agreement with the recent demonstration of low quality responding CD8$^+$ T cells made by Durward *et al.* (Durward *et al.*, 2012) and described above (paragraph 2.1.2 of this Discussion section).

3.1.3 IFN-γ production correlates with bacterial clearance but does it make it the mediator of protection?

During the secondary immune response, early production of IFN-γ in the peritoneal cavity correlates with enhanced bacterial clearance in the spleen of immunized mice when compared to primo-infected mice. However, while IFN-γ has been clearly identified as a crucial cytokine for controlling and containing *Brucella* infection during a primary response (Murphy *et al.*, 2001b), our study does not formally prove that early expression of IFN-γ producing CD4$^+$ T cells in the peritoneal cavity is the mediator of protection.

In the *Mycobacterium tuberculosis* model of infection, it has recently been shown that despite an essential role during a primary infection, IFN-γ is not a required protective mediator of CD4$^+$ T cell defense upon a secondary infection (Gallegos *et al.*, 2011). In agreement several studies have presented evidence suggesting that memory CD4$^+$ T cells can protect against tuberculosis in the absence of IFN-γ ((Caruso *et al.*, 1999; Cowley &

Elkins, 2003; Kagina *et al.*, 2010; Majlessi *et al.*, 2006; Mittrucker *et al.*, 2007; Scanga *et al.*, 2000) and reviewed by (Goldsack & Kirman, 2007))

In the *Brucella* model of infection, it is thus important to consider whether the production of IFN-γ is relevant to the enhanced bacterial reduction observed in the spleen of immunized mice upon a secondary infection. Interestingly, oral immunization of IFN-$\gamma^{-/-}$ BALB/c mice with the attenuated strain *ΔznuA* of *B. melitensis* (*znuA* gene is involved in zinc transport) conferred significant protection in lungs and spleen against nasal *B. melitensis* challenge. Authors proposed that additional cellular mechanisms are activated by oral immunization, possibly via Th17 cells (Clapp *et al.*, 2011). As IFN-$\gamma^{-/-}$ mice do not survive to primary infection by virulent strains of *Brucella* (Brandao *et al.*, 2012; Murphy *et al.*, 2001b), we are not able to consider this question with genetically deficient mice. However it could be more interesting to address this issue by blocking IFN-γ production during the secondary immune response. In addition, passive transfer experiments of $CD4^+$ T cells from mice previously immunized with *Brucella* could also bring consistent answers, although not proving that IFN-γ is the key mediator of protection, since it is possible that memory $CD4^+$ T cells provide protection independently of IFN-γ. The only transfer experiment performed in this way did not come to such a conclusion, presumably because of technical problems.

A recent work of Pasquevich *et al.* (Pasquevich *et al.*, 2011) has demonstrated that vaccine-induced IL-17 is critical against an oral delivery challenge with virulent *B. abortus* 2308. Indeed IL-17A neutralization during the challenge period abrogates U-Omp19 oral vaccine efficacy, without affecting the bacterial burden in PBS-immunized mice. Clapp *et al.* also reported that *in vivo* neutralization of IL-17A in IFN-$\gamma^{-/-}$ mice abrogated protection at 4 weeks post-challenge (Clapp *et al.*, 2011). These results, suggesting a potential protective role of IL-17 producing T cells in the secondary response that is not revealed in the primary response, have also been observed by Khader *et al.* in the *M. tuberculosis* model of infection (Khader *et al.*, 2007). Although the results of Pasquevich *et al.* can be explained by the delivery route of the vaccine (i.e. mucosal immunity induced by the oral route), it indicates that memory $CD4^+$ T cells may play additional roles in functional protection against *Brucella* infection. In the present case IL-17 could promote rapid recruitment of immune cells including IFN-γ-producing T cells to the gut during the recall response. Interestingly, in our work we were not able to detect any IL-17-producing $CD4^+$ T cells in the peritoneal cavity early after challenge (data not shown).

3.1.4 IL-4 receptor signaling pathways negatively impact on the sterilizing immunity in the spleen after challenge without affecting primary response to Brucella infection in BALB/c mice

In BALB/c mice, IL-4R deficiency allows the recruitment of a greater frequency of IFN-γ producing cells early after a primary infection without improving bacterial clearance. This argues for a necessary but not sufficient role of IFN-γ in controlling *Brucella* infection.

In contrast, during a secondary response, protective immunity is enhanced in $STAT6^{-/-}$ BALB/c mice, showing the negative impact of Th2 response on vaccination protocols. Surprisingly this better protection was not associated with a higher frequency of IFN-γ producing cells at 12h post challenge in the peritoneal cavity. In agreement with the idea of an IFN-γ-independent $CD4^+$ T cell response, this unexpected data could nevertheless results from a different kinetic of IFN-γ production in $STAT6^{-/-}$ mice. The implication of IL-4 and/or IL-13 on the humoral and cellular secondary response against *Brucella* requires further study.

3.2 On differences between live and HK immunization

Our results revealed a clear dichotomy between live and HK immunization. They indicate that HK bacteria induce an incomplete or a wrong activation of the immune system, leading to the absence of cognate IFN-γ-producing $CD4^+$ (Th1) cells able to mount a protective immune response upon challenge. However, HK *Brucella* induce a sufficient T-independent B cell response, as demonstrated by early antibody-mediated protection in the blood of HK-immunized mice.

These results are in agreement with the studies of Zhan and Cheers that reported a differential activation of $CD4^+$ T cells by *Brucella* infection or immunization with antigenic extracts (Zhan *et al.*, 1993; Zhan *et al.*, 1995). They demonstrated that IFN-γ-producing T cells are generated only by immunization with live organisms (Zhan *et al.*, 1993) and that immunization with non-living vaccines (i.e. soluble *Brucella* proteins in the study of Zhan *et al.*) not only induces a weak immune response but also fails to induce a desirable, protective immune response (Zhan *et al.*, 1995).

3.3 Manipulation of the model: on the need for antibiotic treatment prior to the challenge

Mackaness has demonstrated in the 60s that mice infected with *B. abortus* were able to eliminate, for a given time, unrelated intracellular pathogens such as *L. monocytogenes*. This non-specific immunity was mediated by activated macrophages (Mackaness, 1964). Other studies have shown that the lack of pathogen clearance may impair long-term memory development through the repeated stimulation of antigen-specific T cells (Brooks *et al.*, 2005; Fuller & Zajac, 2003; Jelley-Gibbs *et al.*, 2005; Wherry *et al.*, 2004). Together these data prompted us to cure mice by antibiotic treatment prior to the challenge in order to reliably identify effectors of immune memory.

It is important to note that two studies have reported an impact of antibiotic treatment on the development of antibody response during murine brucellosis (Bowden *et al.*, 1999; Domingo *et al.*, 1995). However, as shown in this work, antibiotic-treated mice remained able to mount a potent and protective antibody response.

Nevertheless, it should be of great interest to perform experiments on secondary immune response without antibiotic treatment in order to measure the impact of the persistence of the vaccinal strain on the newly infecting challenge strain. Histological analysis could reveal a distinct niche for the two strains, the former being protected in special structures from immune effectors re-activated by the latter. Some preliminary experiments allowing the differentiation of the splenic CFUs from both strains following challenge infection seem to be in line with this hypothesis.

4 Limitations of our model: organ, timing, inoculation route and mice

In a general way, one major thing that we could point out in our experiments is the focus on the spleen. Indeed several studies have shown that *Brucella* is able to persist for a long time in mice. Persistence of bacteria is also reported in the tail, feet and the urogenital and submandibular areas (Durward *et al.*, 2012; Rajashekara *et al.*, 2005). Following infection with a high dose of *B. melitensis* (10^7 CFU, in BALB/c mice) the spleen is chronically infected, with an undulation of bacteria above and below the level of detection over time that could suggest similarities to human brucellosis (Durward *et al.*, 2012). However some studies revealed significant differences in the control and clearance of *Brucella* from different tissues (Pei *et al.*, 2012). Future studies should ideally investigate the control of the infection in other organs (liver, lymph nodes, etc.).

Likewise, due to the chronicity of *Brucella* infection, bacterial burden analysis in genetically deficient mice during primary infection should be extended beyond 28 days. Indeed it has already been demonstrated that some differences in the implication of lymphocytes subsets could occur later in the course of the infection (Freches *et al.*, submitted Eur. J. Immunol., (Rafiei *et al.*, 2006))

In addition, the intraperitoneal (i.p.) route of inoculation used in this work is clearly not a physiological route for *Brucella* infection. Since the mucosal/respiratory system is the primary portal of entry for human infection reported in many laboratory incidents (Ashford *et al.*, 2004) and relevant to biothreats (Pappas *et al.*, 2006a), the study of pathogenesis and immune responses resulting from aerosol exposure should be of great interest. As already suggested by Pasquevich *et al*. (Pasquevich *et al.*, 2011), we could expect some differences in immune effectors activated for the control of *Brucella* infection.

Finally, results obtained from genetically deficient mice have to be interpreted cautiously since other functions than the one targeted can be affected by the deficiency (Goldszmid *et al.*, 2007; Kim *et al.*, 2008; Locksley *et al.*, 1993). Moreover recent studies bring evidence that environmental and dietary differences can shape the gut microbiota of mice and strongly modify the immune response (Gill & Finlay, 2011). Even in the same animal facility, moving young mice from one room to another can shift the microbial populations that dominate the gut (Friswell *et al.*, 2010), underlining the importance of

breeding conditions. In agreement, Ubeda *et al.* have recently shown that disparities observed in the colonizing microbiota of different MyD88- and TLR-deficient mouse colonies correlated with membership in a specific mouse litter and not with impaired innate immune signalling (Ubeda *et al.*, 2012).

In consequence, all mice used for the present study were bred in the same animal facilities for a minimum of 1 year and thus displayed similar hygienic status.

5 Final conclusion

After *B. melitensis* intraperitoneal (i.p.) inoculation of the mouse, bacteria transit by the blood to reach several organs of the reticulo-endothelial system and cause a systemic infection (**figure 19.A**). The naive murine immune system fails to neutralize *B. melitensis* in the blood because of the particular properties of the outer membrane that confer to these bacteria a strong resistance to various bactericidal proteins (lysozyme, complement, defensins) (Eisenschenk et al., 1999; Freer et al., 1996; Martinez de Tejada et al., 1995) and natural antibodies. This escape mechanism allows *B. melitensis* to persist undetected several weeks in the blood. Bacteria disseminate extracellularly during the first 24 hours then infect erythrocytes where they do not replicate (**figure 19.A**) (this work; Results section, Part II). This unexpected niche could be used as a reservoir for *Brucella* and provide a shield against antibiotic treatments and immune system. Moreover erythrocytes are an appetizing niche, plenty of iron that is known to be required for full virulence of *Brucella* (Ojeda et al., 2012; Paulley et al., 2007).

However *B. melitensis* does not remain much longer undetectable by the immune system. Five days after the entry of bacteria, infected cells in the spleen (macrophages and dendritic cells) are found clustered in granulomas (Copin et al., 2012) and several cells from the immune system already secrete IFN-γ, i.e. NK cells, $CD4^+$ and $CD8^+$ T cells (**figure 19.B**) (this work; Results section, Part I (Vitry et al., 2012)). This pro-inflammatory cytokine induces the production of nitric oxide by inflammatory dendritic cells that will help to control the infection (Copin et al., 2007). At 12 days post infection, only $CD4^+$ T cells are able to sustain IFN-γ production (**figure 19.B**). MHC-II-dependent antigen presentation to $CD4^+$ cells and the IFN-γ-mediated Th1 response appear to play a central role in the control of *B. melitensis* infection, whereas the implication of MHC-I-dependent antigen presentation to $CD8^+$ T cells seems unnecessary (shaded in Figure 19). Although humoral immunity develops, as demonstrated by the production of specific antibodies (this work; Results section, Part II), this response is not helpful for the elimination of *B. melitensis* (shaded in Figure 19) (this work; Results section, Part I (Vitry et al., 2012)). Moreover the regulatory role played by B cells (Goenka et al., 2011; Goenka et al., 2012) is not beneficial for the host resistance.

Upon re-infection, the cognate experimented immune system of the mouse is able to mount a stronger specific response against *B. melitensis*. First of all, specific circulating antibodies secreted by B cells reduce the dissemination of bacteria by the blood stream (eliminating 99,99% of blood bacteria), probably before their entry into erythrocytes (**figure 19.C**). Specific IgM appeared sufficient to carry out this task since the absence of isotypic switch in AID deficient mice did not affect this step of control. The presence of specific antibodies is also critical for the late complete elimination of *B. melitensis* in the spleen (**figure 19.D**) (this work; Results section, Part III). Secondly, "ready to act" effector memory $CD4^+$ T cells secreting large amounts of IFN-γ in the peritoneal cavity early after re-infection coordinate bacterial clearance and provide a long-term protection in the spleen (**figure 19.E**) (this work; Results section, Part III). $CD8^+$ T cells are not activated to produce IFN-γ (shaded in Figure 19), even in the absence of MHC-II-mediated antigen presentation. In conclusion, in a secondary infection, both humoral and cell-mediated immune responses work together to provide an efficient protective immune response and eliminate *B. melitensis*. The use of genetically deficient mice showed that humoral immunity able to restrict *Brucella* dissemination in the bloodstream does not require AID-mediated class switch or MyD88/IL-12p35 signaling pathways. This humoral response is induced by both live or HK immunization. In contrast IFN-γ producing T cells are strictly dependent on MyD88/IL-12p35 signaling pathways and are not induced by killed bacteria.

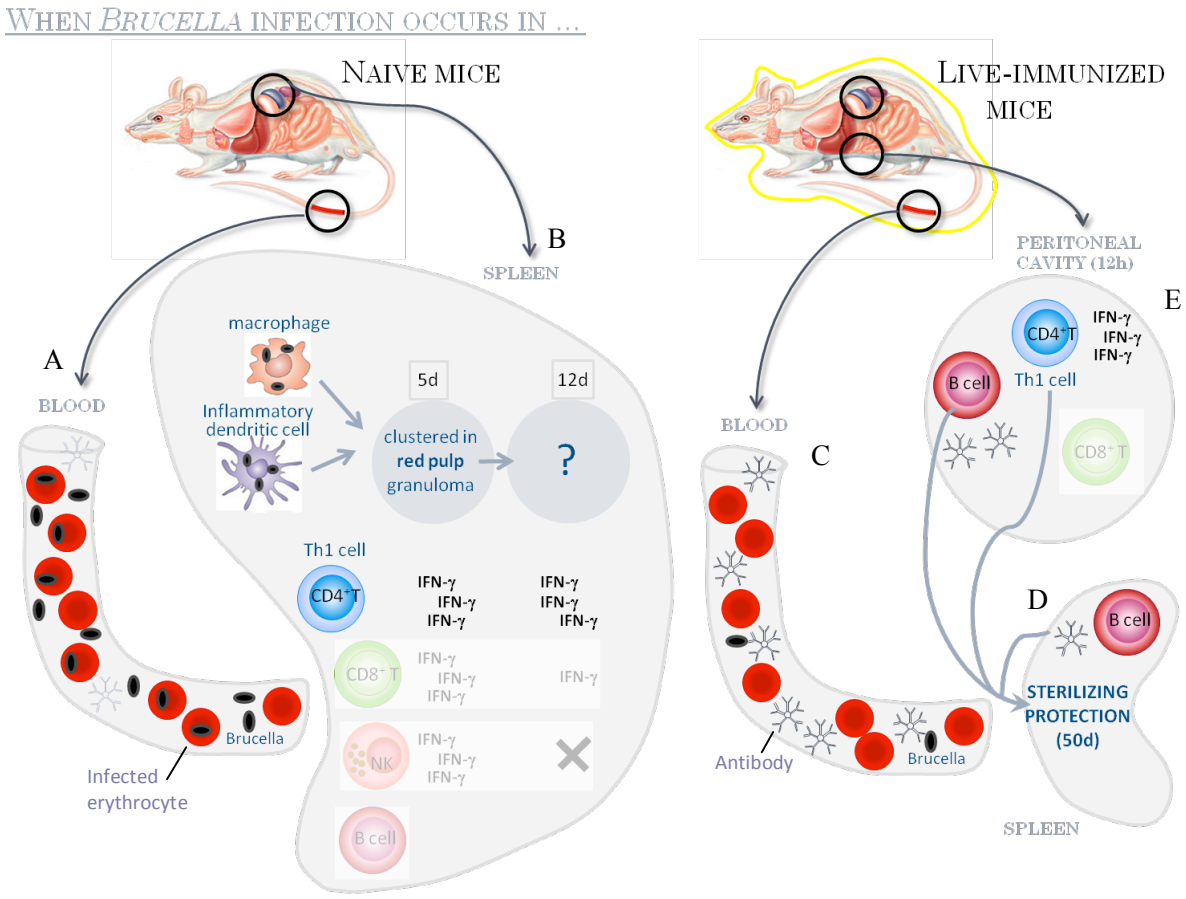

Figure 19. Model of the dynamic of the immune response developed in naive (primary response) or immunized mice (secondary response) upon infection with *Brucella melitensis*.

By providing new insights on the lymphocyte subsets coordinating bacterial clearance during primary infection and on the effectors providing a protective immune memory upon a secondary infection, this study could help to define a rational strategy for the generation of new vaccines against animal and human brucellosis. The development of a protective vaccine should require a vaccination protocol that favors both humoral and cellular immunity. MyD88/IL-12 signaling pathways and MHC-II dependent presentation to $CD4^+$ T cells should be predominantly targeted. Killed vaccines should be avoided since functional $CD4^+$ Th1 cells only developed following immunization with live bacteria. However, live-attenuated strains of *Brucella* used for vaccination in animals retain generally unacceptable levels of virulence. A safe human vaccine against brucellosis could use gamma-irradiated *Brucella*: inactivated but yet metabolically active. Some studies have already successfully investigated this way (Magnani *et al.*, 2009).

Together our results contribute to a better understanding of immunological mechanisms activated during murine brucellosis. They provide new keys for vaccine development and revealed a novel probable escape mechanism of *Brucella* early after the infection, i.e. the erythrocytes. This latter finding demonstrates that *Brucella* has more tricks contributing to its pathogenicity than expected and opens new exciting areas of research.

Appendix

1 Réplication et persistance de *Brucella* dans la rate de souris C57BL/6 ou BALB/c génétiquement déficientes pour des composants de la réponse immunitaire.

[1] (↑) Susceptible par rapport aux souris sauvages (WT) ; (↓) résistante par rapport aux souris WT ; (↔) pas de différence significative avec les souris WT ; ND : non déterminé ; (✝) décès des souris déficientes. Seules les différences significatives sont prises en compte, sauf mention (ns), non significatif.
Le surlignage jaune indique les données obtenues durant cette thèse et publiées.
Adapté d'après (Grillo *et al.*, 2012)

Souche souris	Déficience	Fonctions effectrices affectées	Conditions expérimentales	Impact de la déficience sur les CFUs[1]		Références
				< 14 jours	> 15 jours	
C57BL/6	**IFN-γ**	Production d'IFN-γ Réponse cellulaire et humorale Th1	i.p. $5x10^3$ CFU of *B. abortus* S2308	↑	↑ ✝	(Murphy *et al.*, 2001b)
			i.p. $5x10^4$ CFU of *B. abortus* S2308	↔	↑	(Goenka et al., 2011)
			i.p. $5x10^4$ CFU of *B. abortus* S2308	↑	ND	(Skyberg et al., 2011)
			i.p. $1x10^6$ CFU of *B. abortus* S2308	↑	↑ ✝	(Brandao et al., 2012)
			i.p. $4x10^4$ CFU of *B. melitensis* 16M	↑	ND	(Copin et al., 2007)
			i.p. $1x10^8$ CFU of *B. melitensis* 16M	↑	ND	(Copin et al., 2012)
			i.p. mixed infection 1:1 $5x10^5$ CFU of *B. abortus* S2308 WT/virB	↑	↑	(Rolan & Tsolis, 2007)

C57BL/6	**IRF-1**	Induction d'IL-12p40 et iNOS, production d'IFN-γ et TNF-α. Cellules NK et T CD8$^+$	i.p. 5x10^5 CFU of *B. abortus* S2308	↑	✝	(Ko et al., 2002a; Ko et al., 2002b)
			i.p. 1x10^6 CFU of *B. abortus* S2308, S19 or *B. melitensis* 16M	↑	✝	(Arenas-Gamboa *et al.*, 2012)
	IRF-2	Induction d'IL-12p40 Cellules NK	i.p. 5x10^5 CFU of *B. abortus* S2308	↓	↔	(Ko et al., 2002b)
	ICSBP	Induction d'IL-12p40 Production de ROI	i.p. 5x10^5 CFU of *B. abortus* S2308	↑	↑	(Ko et al., 2002b)
	IL-4	Réponse Th2	**i.p. 4x10^4 CFU of *B. melitensis* 16M**	↔	↓	(Vitry *et al.*, 2012)
	IL-12p35	Production d'IL-12p35 Réponse Th1	i.p. 1x10^8 CFU of *B. melitensis* 16M	↑	ND	(Copin et al., 2012)
			i.p. 4x10^4 CFU of *B. melitensis* 16M	↑	↑	(Vitry *et al.*, 2012)
	IL-12p40	Production d'IL-12p40 Réponse Th1 et Th17	i.p. 5x10^5 CFU of *B. abortus* S2308	↑	↑	(Ko et al., 2002b)
			i.p. 4x10^4 CFU of *B. melitensis* 16M	↑	↑	(Vitry *et al.*, 2012)
	IL-12/β2-microglobuline	Production d'IL-12 Cellules NKT et T CD8$^+$ Réponse IgG	i.p. 1x10^6 CFU of *B. abortus* S2308	↑	↑ ✝	(Brandao et al., 2012)
	IL-17Rα	Recrutement et activation des neutrophiles Réponse Th17	i.p. 5x10^4 CFU of *B. abortus* S2308	↔	ND	(Skyberg et al., 2011)
			i.p. 4x10^4 CFU of *B. melitensis* 16M	↔	↔	(Vitry *et al.*, 2012)
	IL-21R	Réponse des lymphocytes T, B et des cellules NK. Réponse Th17	**i.p. 4x10^4 CFU of *B. melitensis* 16M**	↔	↔	(Vitry *et al.*, 2012)
	IL-22	Réponse Th17	**i.p. 4x10^4 CFU of *B. melitensis* 16M**	↔	↔	(Vitry *et al.*, 2012)

C57BL/6	**TNF-αR**	Réponse au TNF-α Production d'IL-12 et NO	i.v. 5x10^5 CFU of *B. abortus* S19	↑	ND	(Zhan & Cheers, 1998)
	NOS2	Induction d'iNOS	i.p. 5x10^5 CFU of *B. abortus* S2308	↑	↑	(Ko et al., 2002b)
			i.p. 4x10^4 CFU of *B. melitensis* 16M	↑	ND	(Copin et al., 2007)
	Gp91^{phox}	Production de ROI	i.p. 5x10^5 CFU of *B. abortus* S2308	↑	↑	(Ko et al., 2002b)
	Perforine (Pfp)	Cytotoxicité dépendante de Pfp	i.p. 5x10^3 CFU of *B. abortus* S2308	↑	↔	(Murphy *et al.*, 2001b)
	RAG-1	Cellules B et T	i.n. 1x10^4 CFU of *B. melitensis* 16M	↑	↑	(Izadjoo et al., 2000)
			i.p. 1x10^8 CFU of *B. melitensis* 16M	↑	ND	(Copin et al., 2012)
			i.p. mixed infection 1:1 5x10^5 CFU of *B. abortus* S2308 WT/virB	↔	↔	(Rolan & Tsolis, 2007)
			i.p. 4x10^4 CFU of *B. melitensis* 16M	**↑**	**↑**	(Vitry *et al.*, 2012)
	MuMT (Igh6)	Cellules B et production d'anticorps	i.p. 5x10^4 CFU of *B. abortus* S2308	↓	↓	(Goenka et al., 2011)
			i.p. 5x10^5 CFU of *B. abortus* S2308	↔	↓	(Rolan & Tsolis, 2007)
			i.p. 4x10^4 CFU of *B. melitensis* 16M	**↓**	**↓**	(Vitry *et al.*, 2012)
	MHCII ou Aβ	Cellules T CD4$^+$	i.p. 5x10^4 CFU of *B. abortus* S2308	↔	↑ (ns)	(Goenka et al., 2011)
			i.v. 1x10^6 CFU of *B. abortus* S19	↓	↔	(Oliveira & Splitter, 1995)
			i.p. 4x10^4 CFU of *B. melitensis* 16M	**↑**	**↑**	(Vitry *et al.*, 2012)
	CD4	Cellules T CD4$^+$	i.p. mixed infection 1:1 5x10^5 CFU of *B. abortus* S2308 WT/virB	↔	↓	(Rolan & Tsolis, 2007)

C57BL/6	**β2-microglobuline**	Cellules NKT et T CD8$^+$ Réponse IgG	i.p. 5x10^3 CFU of *B. abortus* S2308	↑	↓ (ns)	(Murphy *et al.*, 2001b)
			i.p. 5x10^4 CFU of *B. abortus* S2308	↔	↓	(Goenka et al., 2011)
			i.p. mixed infection 1:1 5x10^5 CFU of *B. abortus* S2308 WT/virB	↔	↔	(Rolan & Tsolis, 2007)
			i.v. 1x10^6 CFU of *B. abortus* S19	↑	↑	(Oliveira & Splitter, 1995)
	TAP-1	Cellules T CD8$^+$	**i.p. 4x10^4 CFU of *B. melitensis* 16M**	**↑**	**↔**	(Vitry *et al.*, 2012)
	TCRγ	Cellules T γδ	i.p. 5x10^5 CFU of *B. abortus* S2308	↑	↔	(Skyberg et al., 2011)
	Genista	Leucocytes polymorphonucléaires	i.p. 1x10^6 CFU of *B. abortus* S2308	↑	↓	(Barquero-Calvo *et al.*, 2013)
	MyD88	Réponse pro-inflammatoire Réponse Th1	i.p. 4x10^4 CFU of *B. melitensis* 16M	↑	↑	(Copin et al., 2007)
			i.p. 1x10^8 CFU of *B. melitensis* 16M	↑	ND	(Copin et al., 2012)
			i.p. 1x10^6 CFU of *B. abortus* S2308	↑	↑	(Macedo et al., 2008)
			i.p. 1x10^6 CFU of *B. abortus* S19	↑	↑	(Weiss et al., 2005)
			i.n. 5x10^9 CFU of *B. melitensis* 16M	↔	↑	(Pei et al., 2012)
			i.p. 4x10^4 CFU of *B. melitensis* 16M	**↑**	**↑**	(Vitry *et al.*, 2012)
	TRIF	Réponse pro-inflammatoire	i.p. 4x10^4 CFU of *B. melitensis* 16M	↔	↔	(Copin et al., 2007)
	IRAK4	Réponse pro-inflammatoire	i.p. 1x10^6 CFU of *B. abortus* S2308	↑	↔	(Oliveira *et al.*, 2011a)
	TLR2	Détection des lipoprotéines et peptidoglycan	i.p. 1x10^5 CFU of *B. abortus* S2308	↔	↔	(Campos et al., 2004)
			i.p. 1x10^6 CFU of *B. abortus* S19	↔	↔	(Weiss et al., 2005)
			i.n. 5x10^9 CFU of *B. melitensis* 16M	↔	↔	(Pei et al., 2012)

C57BL/6	**TLR2/4**	Détection des lipoprotéines, peptidoglycan et LPS	i.p. $1x10^6$ CFU of *B. abortus* S19	↔	↔	(Weiss et al., 2005)
	TLR4	Détection du LPS	i.p. $1x10^6$ CFU of *B. abortus* S19	↔	↔	(Weiss et al., 2005)
			i.n. $5x10^9$ CFU of *B. melitensis* 16M	↔	↔	(Pei et al., 2012)
	TLR9	Reconnaissance motifs CpG	i.p. $1x10^6$ CFU of *B. abortus* S2308	↑	ND	(Macedo et al., 2008)
			i.p. $4x10^4$ CFU of *B. melitensis* 16M	↑	↑	(Copin et al., 2007)
	NLRC4	Détection de la flagelline et du système de sécrétion de type III, assemblage de l'inflammasome	i.p. $4x10^4$ CFU of *B. melitensis* 16M	ND	↑	(Terwagne *et al.*, 2012)
	Caspase-1	Réponse pro-inflammatoire	i.p. $4x10^4$ CFU of *B. melitensis* 16M	ND	↑	(Terwagne *et al.*, 2012)
BALB/c	**IFN-γ**	Production d'IFN-γ Réponse Th1	i.p. $5x10^3$ CFU of *B. abortus* S2308	↔	↑✝	(Murphy *et al.*, 2001b)
	IL-4R	Réponse Th2	**i.p. $4x10^4$ CFU of *B. melitensis* 16M**	**↔**	**↓**	(Vitry *et al.*, 2012)
	IL-12p40	Production d'IL-12p40 Réponse Th1 et Th17	i.p. $1x10^8$ CFU of *B. melitensis* 16M	↑	↑	(Copin et al., 2012)
			i.p. $4x10^4$ CFU of *B. melitensis* 16M	**↑**	**↑**	(Vitry *et al.*, 2012)
	MuMT (jh)	Cellules B et production d'anticorps	i.p. $5x10^4$ CFU of *B. abortus* S2308	↓	↓	(Goenka et al., 2011)
	TLR2	Détection des lipoprotéines et peptidoglycan	i.p. $4x10^4$ CFU of *B. melitensis* 16M	↔	↔	(Copin et al., 2007)
			i.n. $2x10^4$ CFU of *B. abortus* 2308	↔	↑	(Surendran et al., 2012)
	TLR2/4	Détection des lipoprotéines, peptidoglycan et LPS	i.p. $4x10^4$ CFU of *B. melitensis* 16M	↑	↔	(Copin et al., 2007)

BALB/c	**TLR4**	Détection du LPS	i.p. $4x10^4$ CFU of *B. melitensis* 16M	↑	↔	(Copin et al., 2007)
			i.n. $2x10^4$ CFU of *B. abortus* 2308	↔	↔	(Surendran et al., 2012)
	TLR9	Reconnaissance motifs CpG	i.n. $2x10^4$ CFU of *B. abortus* 2308	↔	↔	(Surendran et al., 2012)

2 Additional results on genetically deficient mice tested during this thesis

The following results are not subjected to publication and are shown for guidance. They will not be discussed here for several reasons: they have not always been replicated (see column « number of experiments »), the results varied from one experiment to another, discordant data resulting from a bad choice of control mice (not originated from the same animal facilities than knocked-out mice), etc.

Mice were evaluated for their resistance to *B. melitensis* by counting CFU in the spleen in the context of a primo-infection ($4x10^4$ CFU) or a secondary infection with a low-dose (LD : 10^5 CFU) or a high dose (HD) ($5x10^7$ CFU) of *B. melitensis*. When it is mentioned in the table, CFU were counted in the blood (primo-blood) following an infection with $2x10^6$ CFU of mCherry *B. melitensis*. The trend of results is given in comparison with wild-type mice.

For more details on protocols of infection, see the Material and Methods section from manuscripts included in the Result section of this thesis.

WT : Wild-Type, p.i. : post infection, p. Ch. : post challenge, HD : High dose, LD : Low dose

C57BL/6 mice

Mouse strain	Phenotypic defect	Infection type	Trend of results	Number of experiments
CD4	Affects $CD4^+$ T cells	Primo	No difference at 28 days p.i. Bad WT control mice : not the same animal facility than knock-out mice	2
MHCII	Affects $CD4^+$ T cells	Primo - blood	No difference at 3h, 24h and 72h p.i.	3
		Second HD	No protection at 1 and 6 days p. Ch. but high variability from one experiment to another	3
CD8	Affects $CD8^+$ T cells	Primo	No difference at 28 days p.i. Bad WT control mice : not the same animal facility than knock-out mice	2
		Second LD	Protection at 2 days p. Ch.	1

TAP-1	Affects $CD8^+$ T cells	Primo - blood	High variability but TAP-1-/- mice tend to be more resistant at 3h, 24h and 72h p.i. Bad WT control mice : not the same animal facility than knock-out mice	2
		Second LD	Poor clearing rate at 50 days p. Ch.	1
		Second HD	Intermediate protection at 1 and 6 days p. Ch.	1
RAG-1	Affects B and T cells	Second HD	No protection at 1 and 6 days p. Ch.	2
RAGγc	Affects B, T and NK cells	Primo - blood	No difference at 3h, 24h and 72h p.i. Bad WT control mice : not the same animal facility than knock-out mice	1
CD28	Affects T cells costimulation	Primo	More susceptible at 12 (+0,4 log***) and 28 (+0,6 log***) days p.i. Reproducible	2
		Second LD	Protection at 2 days but variable clearing rate at 50 days p. Ch.	3
CD40	Affects T cell-dependent B cells costimulation	Primo	Slightly more resistant at 28 days p.i. Reproducible	2
		Second LD	No protection at 2 days and variable clearing rate at 50 days p. Ch.	2
SLAT	Affects Th1 and Th2 inflammatory responses	Primo	No difference at 5, 12 and 28 days p.i.	1
MyD88	Affects inflammatory response	Primo-blood	More resistant at 3h, 24h and 72h p.i.	2
		Second LD	Protection at 2 days p. Ch.	1
		Second HD	Protection at 1 day p. Ch.	1
TRIF	Affects inflammatory response	Primo	More susceptible at 28 days p.i.	1
		Second LD	Protection at 2 days but poor clearing rate at 50 days p. Ch.	3
		Second HD	Protection at 1 and 6 days p. Ch.	1
TLR2/4	Affects LPS and lipoproteins detection	Second LD	Protection at 2 days p. Ch.	2
TLR2/4/9	Affects LPS and lipoproteins detection and CpG motifs recognition	Second LD	Poor clearing at 50 days p. Ch.	1
TLR9	Affects CpG motifs recognition	Second LD	Protection at 2 days p. Ch.	2
IL-12p35	Affects Th1 response	Second HD	No protection at 1 and 6 days p. Ch. but high variability from one experiment to another	2
iNOS	Affects	Primo	Slightly more susceptible at 5, 28 and 50 days p.i.	2

	respiratory burst in phagocytes	Second LD	Bad WT control mice : not the same animal facility than knock-out mice Protection at 2 days but poor clearing rate at 50 days p. Ch.	3
TNF-α	Affects proinflammatory response	Primo	More susceptible at 12 days p.i.	1
		Second LD	Protection at 2 days p. Ch.	4
MuMT	Affects B cells	Primo-blood	No difference at 3h, 24h and 72h p.i. Bad WT control mice : not the same animal facility than knock-out mice	3
		Second LD	No protection at 2 days	3
		Second HD	No protection at 6 days p. Ch.	2
AID	Absence of class switching in B cells	Primo	Slightly more susceptible at 5 and 28 days p.i. Bad WT control mice : not the same animal facility than knock-out mice	1
		Second LD	Protection at 2 days p. Ch.	2
γδ TCR	Absence of Tγδ cells	Primo	No difference at 5 and 28 days p.i.	2
		Second LD	Protection at 2 days and variable clearing rate at 50 days p. Ch.	2
CD1d	Affects NKT cells activation	Second LD	Variable protection at 2 days and poor clearing rate at 50 days p. Ch.	2
CCL3	Affects inflammatory response	Primo	No difference at 5 and 28 days p.i.	1
CCR2	Affects DC migration and localization	Primo	More susceptible at 5, 12 and 28 days p.i. Bad WT control mice : not the same animal facility than knock-out mice	1
		Second LD	Protection at 2 days but poor clearing rate at 50 days p. Ch.	1
CX3CR1	Affects monocytes trafficking	Primo	No difference at 5, 12 and 28 days p.i.	1
IRF3	Affects transcription of type I IFN	Primo	No difference at 5 and 28 days p.i.	1
IRF7	Affects transcription of type I IFN	Primo	No difference at 5 and 28 days p.i.	1

BALB/c mice

Mouse strain	Phenotypic defect	Infection type	Trend of results	Number of experiments
IL-4R	Affects Th2 response	Second LD	Intermediate clearing rate at 50 days p. Ch.	1
STAT6	Affects Th2 response	Primo	No difference at 5, 12 and 28 days p.i.	7
IL-12p40	Affects Th1 and Th17 responses	Second LD	No clearing and high CFU counts at 50 days p. Ch.	1

3 In situ microscopy analysis reveals local innate immune response developed around *Brucella* infected cells in resistant and susceptible mice

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In Situ Microscopy Analysis Reveals Local Innate Immune Response Developed around *Brucella* Infected Cells in Resistant and Susceptible Mice

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Abstract

Brucella are facultative intracellular bacteria that chronically infect humans and animals causing brucellosis. *Brucella* are able to invade and replicate in a broad range of cell lines *in vitro*, however the cells supporting bacterial growth *in vivo* are largely unknown. In order to identify these, we used a *Brucella melitensis* strain stably expressing mCherry fluorescent protein to determine the phenotype of infected cells in spleen and liver, two major sites of *B. melitensis* growth in mice. In both tissues, the majority of primary infected cells expressed the F4/80 myeloid marker. The peak of infection correlated with granuloma development. These structures were mainly composed of $CD11b^+$ $F4/80^+$ $MHC\text{-}II^+$ cells expressing iNOS/NOS2 enzyme. A fraction of these cells also expressed CD11c marker and appeared similar to inflammatory dendritic cells (DCs). Analysis of genetically deficient mice revealed that differentiation of $iNOS^+$ inflammatory DC, granuloma formation and control of bacterial growth were deeply affected by the absence of MyD88, IL-12p35 and IFN-γ molecules. During chronic phase of infection in susceptible mice, we identified a particular subset of DC expressing both CD11c and CD205, serving as a reservoir for the bacteria. Taken together, our results describe the cellular nature of immune effectors involved during *Brucella* infection and reveal a previously unappreciated role for DC subsets, both as effectors and reservoir cells, in the pathogenesis of brucellosis.

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Introduction

Brucella (α-proteobacteria) are facultative intracellular Gram-negative coccobacilli that infect humans as well as domestic (goat, sheep, swine, etc.) and wild type mammals. Animal infection leads to abortion and infertility with dramatic economic costs. Brucellosis is mainly transmitted to humans through the ingestion of raw milk or non-pasteurized cheese contaminated with *Brucella*. It is characterized by undulant fever which, if left untreated, can result in chronic disease with serious clinical manifestations, such as orchitis, osteoarthritis, spondylitis, endocarditis, and several neurological disorders [1–5]. Human brucellosis remains a significant public health concern in areas of the world where *Brucella* infections are endemic in food animals. Indeed, brucellosis has been described as being the most common zoonotic disease worldwide with more than 500,000 new human cases annually [4].

Brucella are highly infectious via oral and aerosol routes and difficult to treat with antibiotics. No safe or effective vaccine is available to prevent human infection. These characteristics justify the classification of *Brucella* strains as a category B pathogens, which represent a risk for use as bioweapons [6],[7].

B. melitensis is the most frequent cause of human brucellosis [1,8]. Recently, bioluminescent *B. melitensis* has been used to visualize the dynamic of bacterial dissemination following intraperitoneal (i.p.) inoculation in mice [9]. Results confirmed that this model parallels human infection and identified major sites of bacterial growth, such as the spleen and the liver, during the early chronic phase of infection. However, cells supporting bacterial growth *in vivo* in these organs and serving as reservoir are unknown.

Despite recent progress in mouse models of brucellosis, much remains unknown regarding cellular components of the innate and adaptive immune responses induced by *B. melitensis* infection. We

Author Summary

Brucella are facultative intracellular bacteria chronically infecting humans and animals causing brucellosis, one of the most common zoonotic disease worldwide which can result in infertility and chronic debilitating disease. The cells supporting *Brucella* growth *in vivo* remain largely unknown. In order to identify these, we constructed a *Brucella melitensis* strain expressing a fluorescent protein that allowed us to characterize infected cells by microscopy of the spleen and liver from infected mice. In both tissues, the majority of primary infected cells were cells from the macrophage lineage. The peak of infection correlated with granuloma development. These structures contained the majority of bacteria and were mainly composed of cells expressing CD11b, F4/80, MHC-II, which are specific of activated monocytes/macrophages. A fraction of granuloma cells also expressed CD11c and were similar to inflammatory dendritic cells (DCs). During the chronic phase of infection in susceptible mice, we identified a particular subset of DC expressing CD205 and serving as a reservoir for the bacteria. Overall, our results describe the nature of immune cells infected by *Brucella in vivo* and reveal an unappreciated role for DC subsets, both as effectors and reservoir cells. These results could help develop new therapeutic strategies to control *Brucella* infection.

and others have shown that IFN-γ-producing $CD4^+$ T cells [10–14] and inducible Nitric Oxide Synthase (iNOS/NOS2)-producing inflammatory dendritic cells (iNOS-DC) [10] are major components of protective immune response against *B. melitensis*. Activation of these cells involves Toll Like Receptor (TLR) 4 and TLR9 coupled to MyD88 adaptor protein [10]. However, there is little understanding on where these cells are localized *in situ* so they can initiate cellular interactions to control infection. Several experimental models of infection by intracellular bacteria such as *Mycobacteria tuberculosis* [15] and *Listeria monocytogenes* [9,16] have illustrated the importance of the granulomatous lesion in limiting both tissue damage and bacterial dissemination. The granulomatous lesion is an organized and dynamic structure implicating activated monocytes surrounded by T cells and granulocytes. Its formation involves an orchestrated production of chemokines and cytokines and the upregulation of their cognate receptors along with the expression of addressins, selectins and integrins. Altogether, these elements coordinate the recruitment, migration and retention of cells to and within the granuloma. Chronic granulomatous inflammation has been reported in spleen and liver from natural hosts, human and mice infected by *Brucella* bacteria [17]. However, the importance of granulomas in the control of *Brucella* growth, their cellular composition and the signalling pathways implicated in their formation are largely unknown.

To address these issues, we developed a *B. melitensis* strain stably expressing mCherry fluorescent protein (mCherry-Br). This novel tool has allowed us to determine the phenotype of *Brucella*-targeted cells and characterize the composition and the dynamic of granuloma in the spleens and livers of infected mice. We observed the formation of $iNOS^+$ granuloma structures surrounding infected cells and identified iNOS-DC ($CD11b^+$ $CD11c^+$ Ly-$6G^-$ MHC-II^+ $iNOS^+$) and activated monocytes ($CD11b^+$ $CD11c^-$ Ly-$6G^-$ MHC-II^+ $iNOS^+$) as the major cell types constituting granulomatous lesions in both tissues. In addition, we observed that alteration of the MyD88/IL-12/IFN-γ axis deeply affects the cellular composition of granulomas, reducing their ability to control bacterial dissemination and leading to replication and persistence of the pathogen in distinct cellular niches.

Results

Construction of mCherry stably expressing *Brucella melitensis* 16M

In order to detect infected cells on tissue sections by fluorescent microscopy, we constructed a constitutively fluorescent strain of *B. melitensis*. The mCherry protein, a previously described rapidly maturing variant of the red fluorescent protein DsRed [18], was cloned into the suicide vector *pKSoriT-bla-kan* downstream of a strong *Brucella* spp. promoter P*sojA* [19,20]. The final construct was transformed into *Escherichia coli* strain S17-1, and introduced into *B. melitensis* strain by conjugation (see Material and Method for details).

Flow cytometry (Figure S1.A) and fluorescent microscopy (Figure S1.B) were used to validate mCherry-expressing *Brucella* (mCherry-Br) strain *in vitro*. The comparison of wild type and mCherry-Br strains growth curve revealed that mCherry gene expression does not affect ability of *B. melitensis* to replicate *in vitro* (data not shown). In addition, following i.p. inoculation of 4×10^4 CFU, we showed that mCherry-Br was able to infect chronically resistant C57BL/6 mice (Figure S1.C). At 5 days post infection (p.i.), bacterial burden in spleen from mCherry-Br infected mice was reduced by approximately half-a-log when compared to wild type bacteria-infected mice. However, at 25 and 42 days p.i., bacterial counts from both groups were similar, suggesting that bacterial virulence is moderately and transiently affected by the insertion of mCherry tracer.

Detection of mCherry-expressing *Brucella* in tissues from infected mice

In order to characterize the spatio-temporal behavior of *Brucella* in spleen and liver of infected animals, we used immunofluorescence microscopy techniques. At low dose of infection (10^4 to 10^6 CFU) in C57BL/6 and BALB/c wild type mice, growth of *B. melitensis* peaked at 5 days p.i. with modest CFU counts (between 10^5–10^6 CFU/g of spleen [10]) rendering its visualization on tissue sections very difficult. Our preliminary studies demonstrated that 10^6 CFU/g of tissue was the limit of sensitivity of our technique, with less than 0.1 infected cells by observation surface (200×230 µm) in tissue sections of 5 µm in thickness and using a 63× objective (data not shown). Therefore, in order to maximize our chances of visualizing the dynamic behavior of mCherry-Br *in vivo* several days after inoculation, we injected 10^8 CFU of bacteria in wild type mice. With this dose, no increase in mortality of mCherry-Br infected wild type C57BL/6 and BALB/c mice was observed (data not shown). Importantly, when compared to the routinely used low bacterial inoculum, a high infectious dose (10^8 CFU) showed similar impact on the composition of recruited spleen cell populations involved in innate inflammatory responses to *Brucella*. Overall, a high bacterial inoculum only shorten the kinetic of cell recruitment (Figure S2).

First analyses revealed that ten minutes after i.p. inoculation, bacteria were massively present in the spleen and the liver, with an average of 10^6 CFU/g (Figure S3.A). Both organs maintained a level of 10^6–10^8 CFU/g during the 5 first days p.i. (Figure S3.A), allowing the analysis of mCherry-Br infected cells *in situ* during this time (Figure S3.B). Infected susceptible BALB/c mice displayed significantly higher count of bacteria in the spleen at 3 and 5 days p.i. when compared to C57BL/6 mice. However, at day 12, spleens and livers from wild type mice had less than 10^5–10^6 CFU/g and bacteria became undetectable by microscopy

analysis (data not shown). Consequently, we limited our analysis to 120 h p.i. in wild type mice.

First *Brucella*-infected splenocytes are mainly F4/80$^+$ red pulp macrophages and MOMA-1$^+$ metallophilic marginal zone macrophages

The functions of the spleen are centered on systemic blood circulation. As such, it lacks afferent lymphatic vessels. It is comprised of two functionally and morphologically distinct compartments, namely the red pulp (r.p.) and the white pulp (w.p.). The r.p. is a blood filter rich in F4/80$^+$ macrophages that removes foreign material as well as damaged and effete erythrocytes. The spleen is also the largest secondary lymphoid organ of the body that initiates immune responses to blood-borne antigens. This function is usually associated to the w.p. compartment. This latter is centered on the central arteriole within the T cell rich area surrounded by B lymphocyte-associated follicles. At the interface between the r.p. and w.p., the marginal zone (m.z.) is a unique region of the spleen. Considered to be a separate compartment rather than part of the w.p., it is designed to screen systemic blood circulation for antigens and pathogens and plays an important role in antigen processing. It also contains several populations of specialized macrophages, such as marginal zone-associated metallophilic macrophages that can be identified with anti-MOMA-1 antibody (for a review see [21]).

Following i.p. inoculation of 10^8 CFU in resistant wild type C57BL/6 mice, mCherry-Br was detectable in the spleen by 10 minutes p.i. (Figure S3.A). Importantly, at this time, our observation showed that mCherry-Br already localized in the r.p. and the m.z. of infected spleens (data not shown). Then, bacteria increased exponentially during the first 24 h (Figure S3.A) and the number of intracellular bacteria peaked at 120 h p.i. with an average of 8–10 bacteria per cell (Figure 1.A). Importantly, infected cells were mainly located in m.z. and r.p. during the whole kinetic of infection (Figure 1.B–C and Figure 2.A) while infected cells were rarely observed in splenic w.p. area (w.p.).

Further semi-quantitative analyses of infected cell phenotypes in C57BL/6 mice (Figure 2.B and Figure 3.A–B) showed that first infected cells in spleen were mainly r.p. macrophages with a F4/80$^+$ CD11b$^-$ phenotype (~80%) and MOMA-1$^+$ m.z. metallophilic macrophages (~20%). Between 3 and 6 h after inoculation, the frequency of infected MOMA-1$^+$ cells decreased strongly and CD11c$^+$ dendritic cells progressively became infected (~30% of total infected cells at 6 h) (Figure 3.A).

Using a home-made rabbit polyclonal antibody specific of *B. melitensis* (*Brucella*-Ag), we showed that mCherry-Br fluorescence and *Brucella*-Ag staining were generally overlapping (Figure S4), although there were some instances where *Brucella*-Ag staining could be detected in the absence of appreciable mCherry-Br fluorescence, possibly as a consequence of bacterial death or trafficking of antigens away from the bacteria. These results confirm that the majority of *Brucella*-infected cells can be detected *in situ* using mCherry-Br.

Confocal analysis in Z-stack demonstrated that bacteria localized inside F4/80$^+$ r.p. macrophages (Figure S5.A–B). Importantly, this was also true for mCherry-expressing VirB-defective *Brucella* mutant (Figure S5.C). The VirB mutant displays a strongly reduced ability to infect cells *in vitro* [22] though it is able to persist several days *in vivo* and colonize mice similarly to wild type bacteria [23]. Therefore, it has been hypothesized that *in vivo* persistence could be due to extracellular replication [24]. However, our observations are not in favor of this hypothesis since VirB mutant and wild type bacteria both localized within cells of the same phenotype in infected spleen at least in the first 72 h p.i. (data not shown). As expected, VirB mutant is no longer detectable *in situ* at 120 h p.i.

Control of *Brucella* infection in the spleen is correlated to the formation of F4/80$^+$ MHC-II$^+$ iNOS$^+$ granulomas

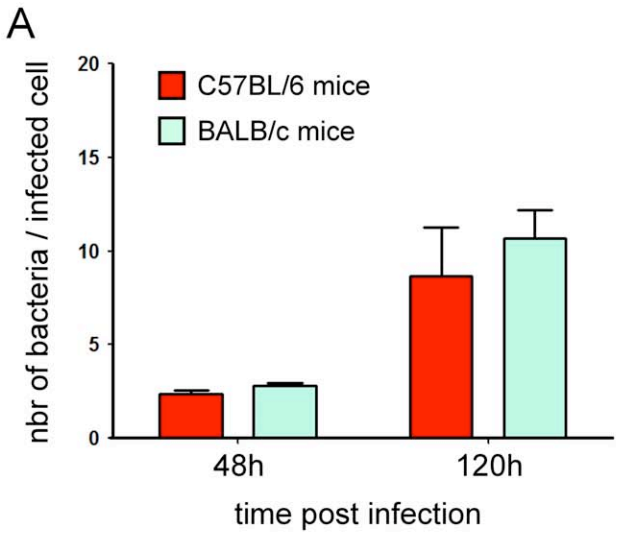
Our analysis revealed that the phenotype of infected spleen cells presented a drastic evolution between 24 h and 120 h p.i. Indeed, at 24 h p.i., about 80% of infected cells were CD11b$^-$ F4/80$^+$ r.p. macrophages (Figure 3.A–B), a fraction of which also expressed MHC-II$^+$ molecules (Figure 3.B and Figure 4.A). Between 24 h and 72 h, the percentage of CD11b$^+$ Ly-6G$^-$-infected monocytes increased progressively (Figure 3.B and Figure S6). At 120 h, these monocytes constituted ~30–40% of the infected cell population (Figure 3.B) and were mainly found clustered in granulomas (data not Shown).

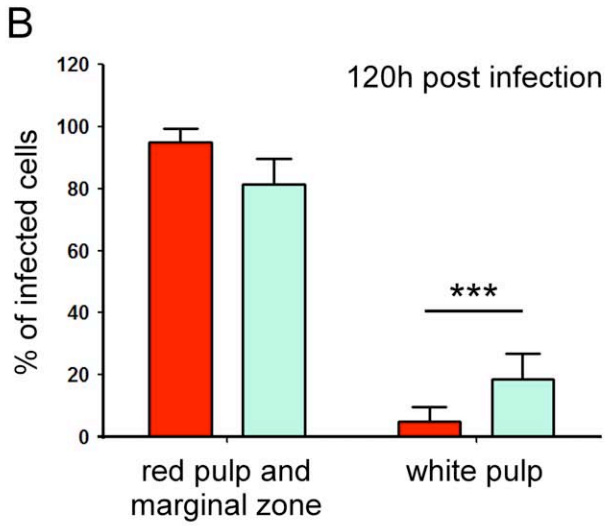
Granulomas were constituted of CD11b$^+$ F4/80$^+$ Ly-6G$^-$ MHC-II$^+$ cells surrounded by CD90$^+$ cells (T cells) and Ly-6G$^+$ cells (granulocytes) (Figure 3.A–B, Figure 4.B, Figure 5.A). A fraction of CD11b$^+$ F4/80$^+$ MHC-II$^+$ cells was also CD11c$^+$ (Figure 5.A), suggesting that these cells were inflammatory DC. In previous reports, these inflammatory DC were detected by flow cytometry and identified as the main iNOS-producing cells during *Brucella* infection [10]. In agreement with this finding, we observed *in situ* that CD11b$^+$ and CD11c$^+$ cells located in granulomas were iNOS$^+$ (Figure 5.B) and co-localized with mCherry-Br signal. Importantly, B cells (B220$^+$ cells), T cells (CD90.2$^+$ cells) or granulocytes (Ly-6G$^+$ cells) seemed very rarely infected during the course of infection in the spleen (Figure 3.A–B and data not shown). Of note, a weak expression of CD90.2 was detected on a fraction of highly infected cells within granulomas (Figure 3.A). These cells displayed a cellular morphology (large cells with uncondensed nuclei) distinct from T lymphocytes and co-localized with CD11b and F4/80 (data not shown), suggesting that they were not lymphocytes but rather myeloid cells.

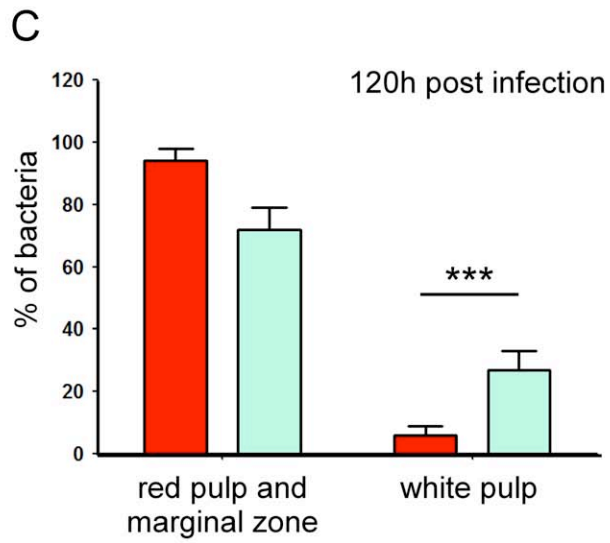
In summary, we concluded that infection seems largely limited to a fraction of the myeloid cell compartment in the spleen. Since the establishment of *Brucella* infection at day 5 in the spleen correlated with monocytes recruitment and formation of granulomas, we hypothesize that these latter are involved in bacterial containment and/or elimination as these structures contain most of the iNOS-producing cells. Granulomas were also detected in r.p. area of spleen from C57BL/6 mice infected for 5 days with 4×10^4 CFU (Figure S7.A). As expected, their numbers were reduced when compared to mice infected with 10^8 CFU. Surprisingly, at 12 days p.i. with both doses, granulomas were mostly located in w.p. area, specifically in T cells zones (Figure S7.A–B). Unfortunately, at this time point, we were not able to determine the presence of *Brucella* since the bacterial load was under our limit of detection. However, the similarity of the phenotype of these structures (CD11b$^+$ and F4/80$^+$, Figure S7.A and S7.B) with r.p. granulomas suggests that they may surround rare infected cells.

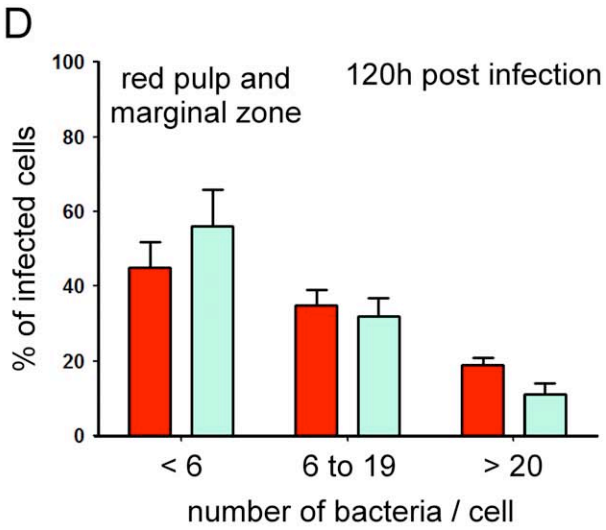
Brucella infects similar cell phenotypes in resistant C57BL/6 and susceptible BALB/c mice

It has been frequently reported that BALB/c mice display higher bacterial loads per organs when compared to C57BL/6 mice during *Brucella* infection [10,25]. The reasons for this susceptibility are unclear but it has been associated with lower frequency of IFN-γ-secreting CD4$^+$ cells and iNOS-producing cells in these mice [10]. In accordance with CFU counts measured in spleens (Figure S3.A), the average number of bacteria per cell observed by microscopy analysis in BALB/c and C57BL/6 mice


A
C57BL/6 mice
BALB/c mice
nbr of bacteria / infected cell
20
15
10
5
0
48h
120h
time post infection


B
120h post infection
% of infected cells
120
100
80
60
40
20
0
***
red pulp and marginal zone
white pulp


C
120h post infection
% of bacteria
120
100
80
60
40
20
0
***
red pulp and marginal zone
white pulp


D
red pulp and marginal zone
120h post infection
% of infected cells
100
80
60
40
20
0
< 6
6 to 19
> 20
number of bacteria / cell

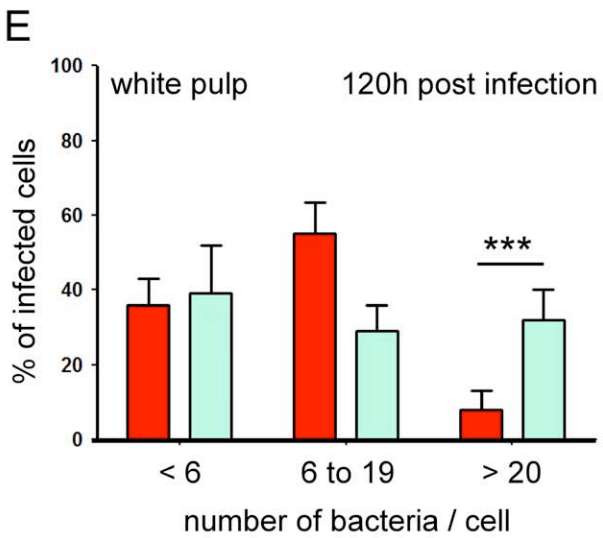

E
white pulp
120h post infection
% of infected cells
100
80
60
40
20
0
***
< 6
6 to 19
> 20
number of bacteria / cell

Figure 1. Spatial distribution of infected cells in spleen of C57BL/6 and BALB/c mice. Wild-type C57BL/6 and BALB/c mice were injected i.p. with PBS or 10^8 CFU of mCherry-Br. Mice were sacrificed at selected times and spleens were collected and examined by immunohistofluorescence. **A**, The average number of bacteria per infected spleen cells at 48 h and 120 h p.i.. **B**, **C**, The percentage of infected cells and bacteria localized in the r.p. and m.z., or w.p. at 120 h p.i.. **D**–**E**, The comparative analysis of the bacterial load per cell present in the r.p. and m.z., or w.p.. For each conditions, a minimum of 200 cells has been examined. The bars are the mean±SD from at least 3 spleen sections per spleen from 6 mice.
doi:10.1371/journal.ppat.1002575.g001

was similar at 48 h, though this number was slightly higher at 120 h in BALB/c mice (Figure 1.A). To determine whether enhanced susceptibility of BALB/c mice could be associated with the persistence of bacteria in distinct cell reservoirs, we further examined the phenotype of infected cells in both mouse strains at that time point. As observed for C57BL/6 mice, the large majority of infected cells were mainly located in splenic r.p. and m.z. in BALB/c mice (Figure 1.B–C) and semi-quantitative analyses of infected cell phenotypes (Figure 3.C) showed a global similarity between C57BL/6 and BALB/c mice. However, the frequency of infected cells located in w.p. was higher in BALB/c mice when compared to C57BL/6 mice (Figure 1.B–C). Further comparative analysis (Figure 1.D–E) of the spatial distribution of infected spleen cells showed that highly infected cells (>20 bacteria/cell) were more frequent in BALB/c infected mice and located mainly in w.p. areas.

In conclusion, infected cell phenotypes are relatively similar in both mouse strains but higher colonization of w.p. cells in infected BALB/c mice seems to explain for their higher CFU counts. These cells frequently expressed MOMA-1 or CD11c markers but were negative for CD11b, F4/80, Ly-6G and CD90.2 (data not shown).

Brucella infection induces splenic dendritic cell migration and maturation

Brucella have frequently been described as silent or stealthy pathogens, able to actively escape the immune response through their ability to grow furtively within cells [26]. This hypothesis is mainly based on the fact that *Brucella*'s lipopolysaccharide (LPS) is a weak activator of macrophages and DC by comparison to conventional LPS from *Escherichia coli* [27]. In our study, we observed that $CD11c^+$ DC represent 10–30% of spleen cells infected by mCherry-Br during the first 12 h of infection (Figure 2.B and Figure 3.A). Therefore, in our experimental conditions (10^8 CFU i.p.), we investigated the impact of *Brucella* infection on DC activation *in vivo* in C57BL/6 mice. Imaging

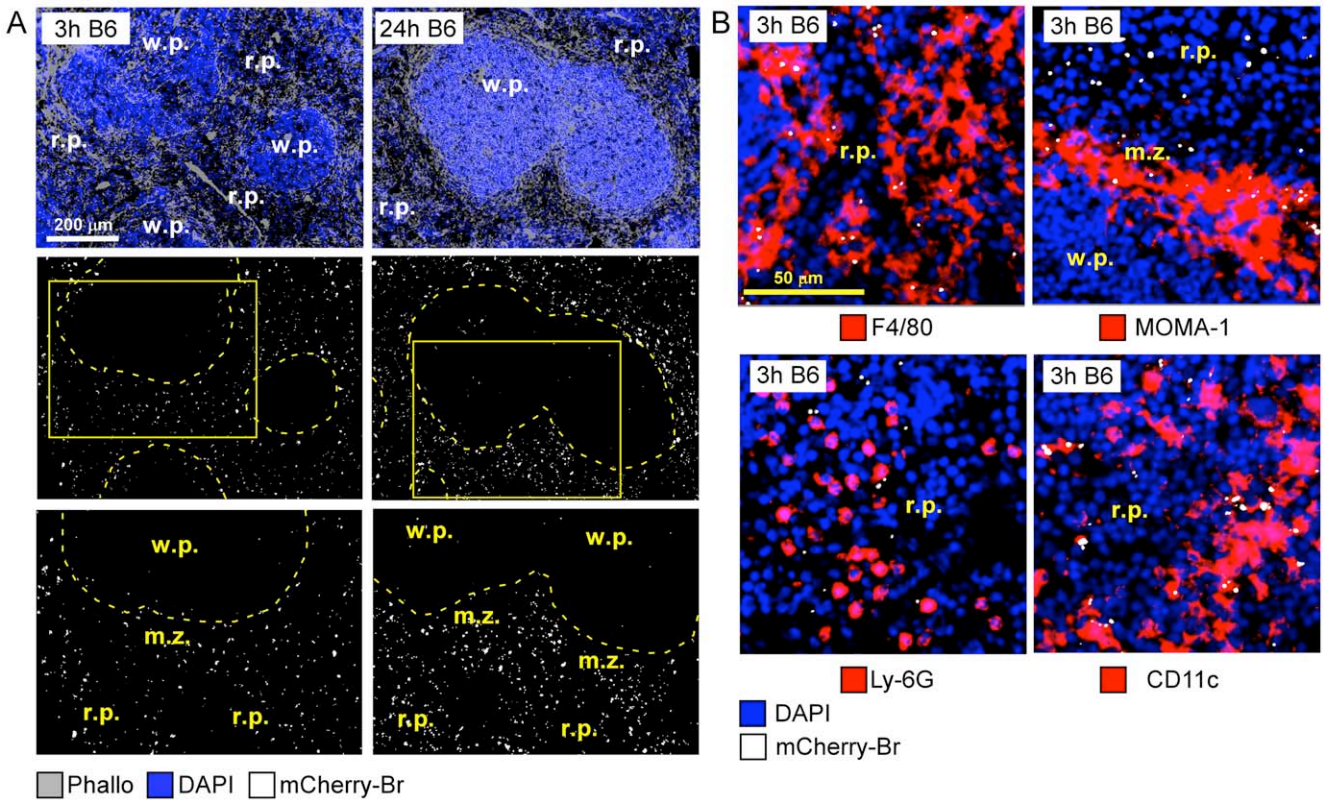


Figure 2. Red pulp localization of *B. melitensis* at the onset of infection. Wild-type C57BL/6 mice were injected i.p. with PBS or 10^8 CFU of mCherry-Br. Mice were sacrificed at selected times and spleens were collected and examined by immunohistofluorescence. **A**, Positioning of mCherry-Br in the spleen of mice at 3 and 24 h p.i. The inset areas in the middle panels are shown in the bottom panels. **B**, Immunoflurescence analysis of F4/80, MOMA-1, Ly-6G, and CD11c expressing cells and mCherry-Br in the spleen of mice at 3 h p.i. Panels are color-coded with the text for the antigen or mCherry-Br examined. Scale bar = 200 and 50 µm, as indicated. r.p.: red pulp; w.p.: white pulp; m.z.: marginal zone. Data are representative of at least 3 independent experiments.
doi:10.1371/journal.ppat.1002575.g002

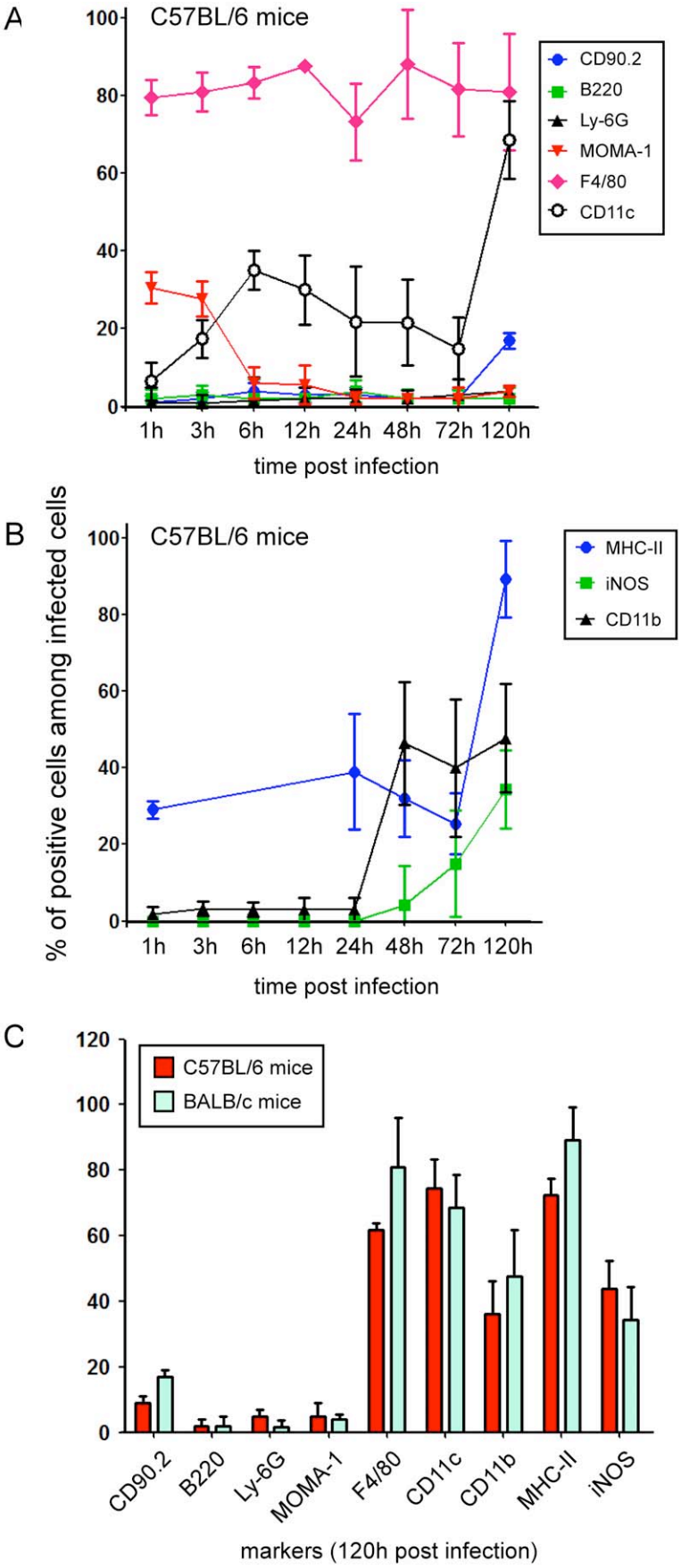

A
C57BL/6 mice
CD90.2
B220
Ly-6G
MOMA-1
F4/80
CD11c
time post infection
B
C57BL/6 mice
MHC-II
iNOS
CD11b
% of positive cells among infected cells
time post infection
C
C57BL/6 mice
BALB/c mice
CD90.2
B220
Ly-6G
MOMA-1
F4/80
CD11c
CD11b
MHC-II
iNOS
markers (120h post infection)

Figure 3. Phenotypical characterization of *B. melitensis* infected cells in the spleen. Wild-type C57BL/6 and BALB/c mice were injected i.p. with PBS or 10^8 CFU of mCherry-Br. Mice were sacrificed at selected times and spleens were collected and examined by immunohistofluorescence. Data indicate the percentage of mCherry-Br that colocalizes with (**A**) CD90.2-, B220-, Ly6G-, MOMA-1-, F4/80-, CD11c-, (**B**) MHCII-, iNOS-, CD11b-expressing cells at the selected time in C57BL/7 mice. (**C**) Comparison at 120 h p.i. between C57BL/6 and BALB/c mice. The bars are the mean±SD from at least 3 spleen sections per spleen from 5 mice.
doi:10.1371/journal.ppat.1002575.g003

analysis (Figure S8) of spleen sections revealed that 24 h post inoculation, $CD11c^{high}$ DCs relocated massively to w.p. T cell area.

Flow cytometry analyses demonstrated that DC migration observed *in situ* at 24 h p.i. was associated with their maturation as shown by cell surface up-regulation of MHC-II and CD86 co-stimulatory molecule expression on $CD11c^{high}$ spleen cells (Figure S9.A). Although we observed maturation of all splenic DC subsets ($CD8^-$ and $CD8^+$) (data not shown), we also found that direct infection of DC was not required to trigger the phenomenon since DC observed by microscopy in w.p. T cell area at 24 h p.i. were not associated with mCherry-Br signal (data not shown). A lower dose of bacteria (4×10^4 CFU) was able to induce DC maturation but the peak of maturation was reduced and displaced to 48 h p.i. (data not shown). Importantly, administration of heat-killed (HK) *Brucella* was also able to induce a dose-dependent DC maturation (Figure S9.B), suggesting that *Brucella*'s pathogen-associated molecular patterns (PAMPs) were sufficient to trigger this process. By comparison, HK *Escherichia coli* induced DC maturation at a ten-fold lower dose (Figure S9.B). Finally, using genetically deficient C57BL/6 mice, we investigated the role of MyD88 and TRIF adaptor molecules in DC maturation process *in vivo* and found that MyD88, but not TRIF, was important to *Brucella*-induced DC maturation (Figure S9.C).

Granuloma formation is observed in *Brucella*-infected livers

Since the liver constitutes another important site of bacterial growth, we investigated the phenotype of infected cells in this organ in order to identify the common characteristics of infected cells in immunological and non-immunological sites. At 10 min p.i., mCherry-Br signal was already detectable in liver sections

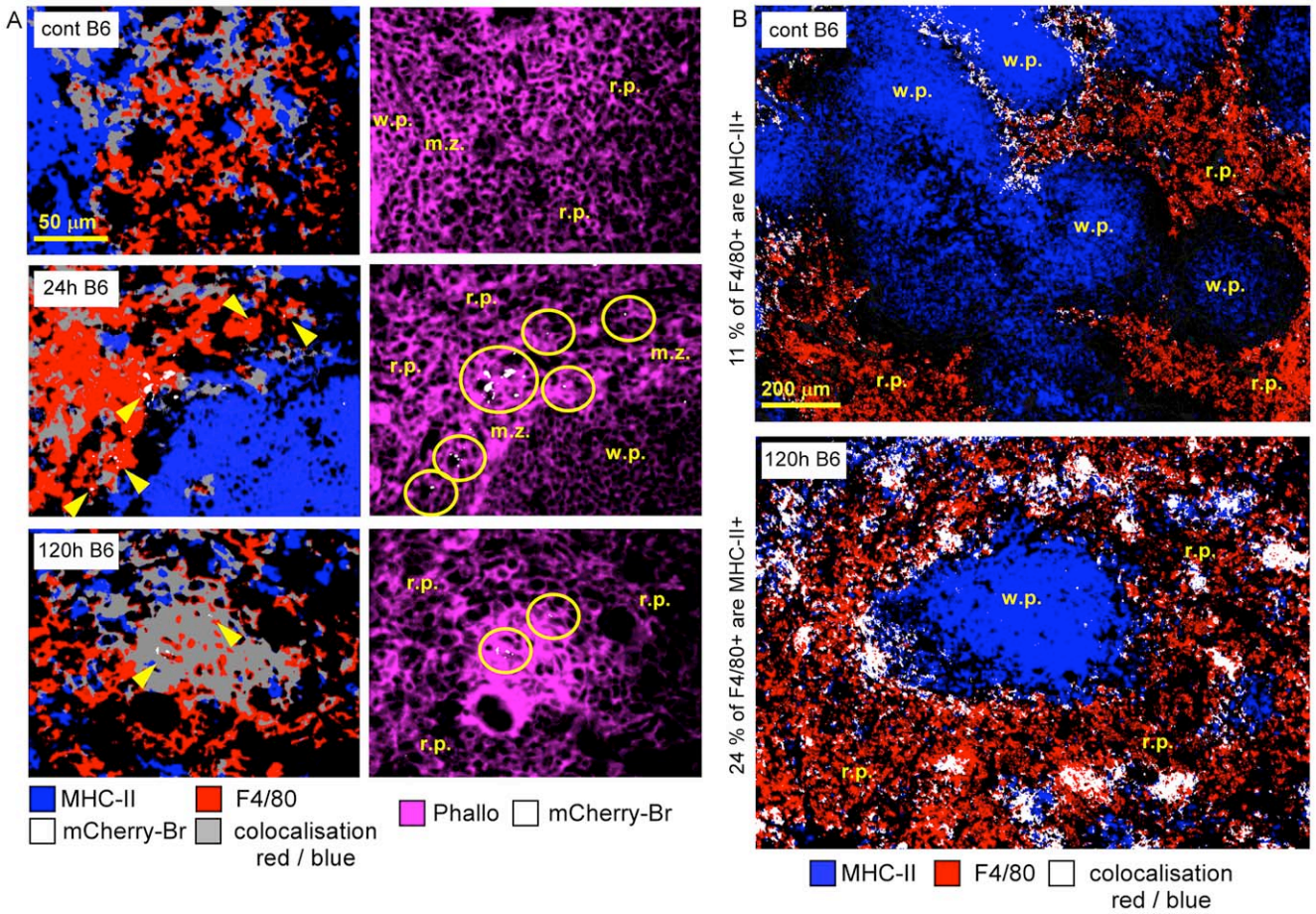


Figure 4. Characterization of $F4/80^+$ infected cells in the spleen. Wild-type C57BL/6 mice were injected i.p. with PBS or 10^8 CFU of mCherry-Br. Mice were sacrificed at selected times and spleens were collected and examined by immunohistofluorescence. **A**, Immunoflurescence analyses of F4/80, MHC-II, and actin expression in spleen of mice at 24 h and 120 h after mCherry-Br infection. Yellow circles and arrowheads indicate the presence of the bacterium. Panels are color-coded with the text for the antigen or mCherry-Br examined as well as the colocalization. **B**, Colocalization analysis of F4/80− and MHCII-expressing cells in spleen of mice before and 120 h after mCherry-Br infection. Scale bar = 50 and 200 µm, as indicated. r.p.: red pulp; w.p.: white pulp, m.z.: marginal zone. Data are representative of at least 3 independent experiments.
doi:10.1371/journal.ppat.1002575.g004

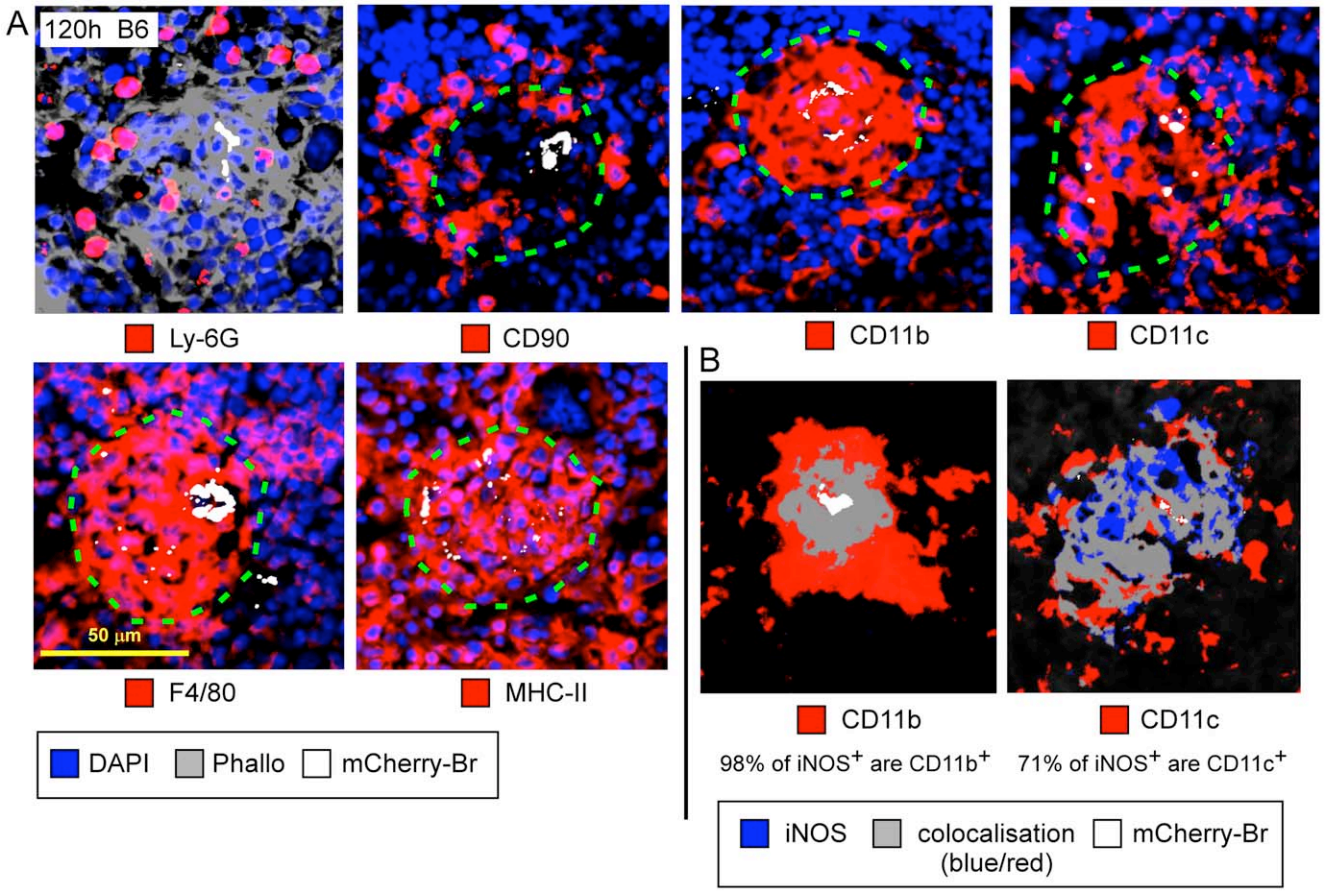


Figure 5. Phenotypical description of *B. melitensis* infected granulomas in the spleen. Wild-type C57BL/6 mice were injected i.p. with PBS or 10^8 CFU of mCherry-Br. Mice were sacrificed at 120 h p.i. and spleens were collected and examined by immunohistofluorescence. **A**, Immunofluorescence analysis of Ly-6G, CD90.2, CD11b, CD11c, F4/80, and MHC-II expressing cells and mCherry-Br. **B**, Percentage of $iNOS^+$ cells that colocalize with CD11b-, and CD11c-expressing cells. Numbers in B indicate the percentage of colocalizing cells in the upper panel. Images represent a single granuloma. Panels are color-coded with the text for the antigen or mCherry-Br examined as well as the colocalization. Scale bar = 50 µm, as indicated. Data are representative of at least 3 independent experiments.
doi:10.1371/journal.ppat.1002575.g005

from infected C57BL/6 and BALB/c wild type mice and co-localized strictly with $F4/80^+$ $MHC\text{-}II^+$ Kupffer cells (data not shown). At 120 h post-inoculation, microscopy analyses revealed that, as observed in the spleen, the liver also displayed numerous granulomas composed of $F4/80^+$ $MHC\text{-}II^+$ $CD11b^+$ cells (Figure S10). Granulomas frequently surrounded portal space and harbored the majority of infected cells (data not shown). As it was the case in the spleen, iNOS expression was mainly associated with a fraction of granuloma cells expressing CD11c, suggesting that activated monocytes and inflammatory DC were also major populations of these structures. Importantly, we never observed bacteria inside hepatocytes or $Ly\text{-}6G^+$ granulocytes suggesting that, like in the spleen, infection is limited to a specific fraction of the myeloid cell compartment.

Formation of granuloma is partially independent on adaptive immunity

As granulomas seem to be the main organized cellular structures containing *Brucella* in the spleen and the liver, we tried to define the role of T and B lymphocytes in their formation. We infected RAG deficient C57BL/6 mice with 10^8 CFU of mCherry-Br. $RAG^{-/-}$ mice displayed higher CFU counts in the spleen, but not in the liver, at 120 h p.i. (Figure S11). At the same time, microscopy analysis of tissue sections showed that absence of T and B lymphocytes did not impair $F4/80^+$ $CD11b^+$ $CD11c^+$ $iNOS^+$ granuloma formation in either tissues (Figure 6). Flow cytometry analyses confirmed that recruitment of iNOS-DC was not compromised in the spleen of $RAG^{-/-}$ infected mice (Figure S12). However, in $RAG^{-/-}$ infected spleens, granulomas seemed less organized and less dense. In addition, higher numbers of $iNOS^+$ cells and infected cells were observed outside of granulomas and frequency of $F4/80^-$ infected cells was higher when compared to wild type mice (Figure 6). These latter observations could be correlated with the presence of $Ly\text{-}6G^+$ infected cells in the spleen of $RAG^{-/-}$ mice (data not shown).

MyD88, IL-12 and IFNγ are required for granuloma formation

In order to gain insight into immune mechanisms controlling bacterial growth in the spleen, we used a genetic approach to investigate the impact of MyD88, IL-12p35 and IFN-γ deficiencies on the phenotype of infected cells with a particular focus on the ability of the immune response to establish granulomas. These molecules have been described as key elements of Th1-driven immune response controlling *Brucella* growth *in vivo* [10],[14,28],[12]. In agreement with previous published results, all

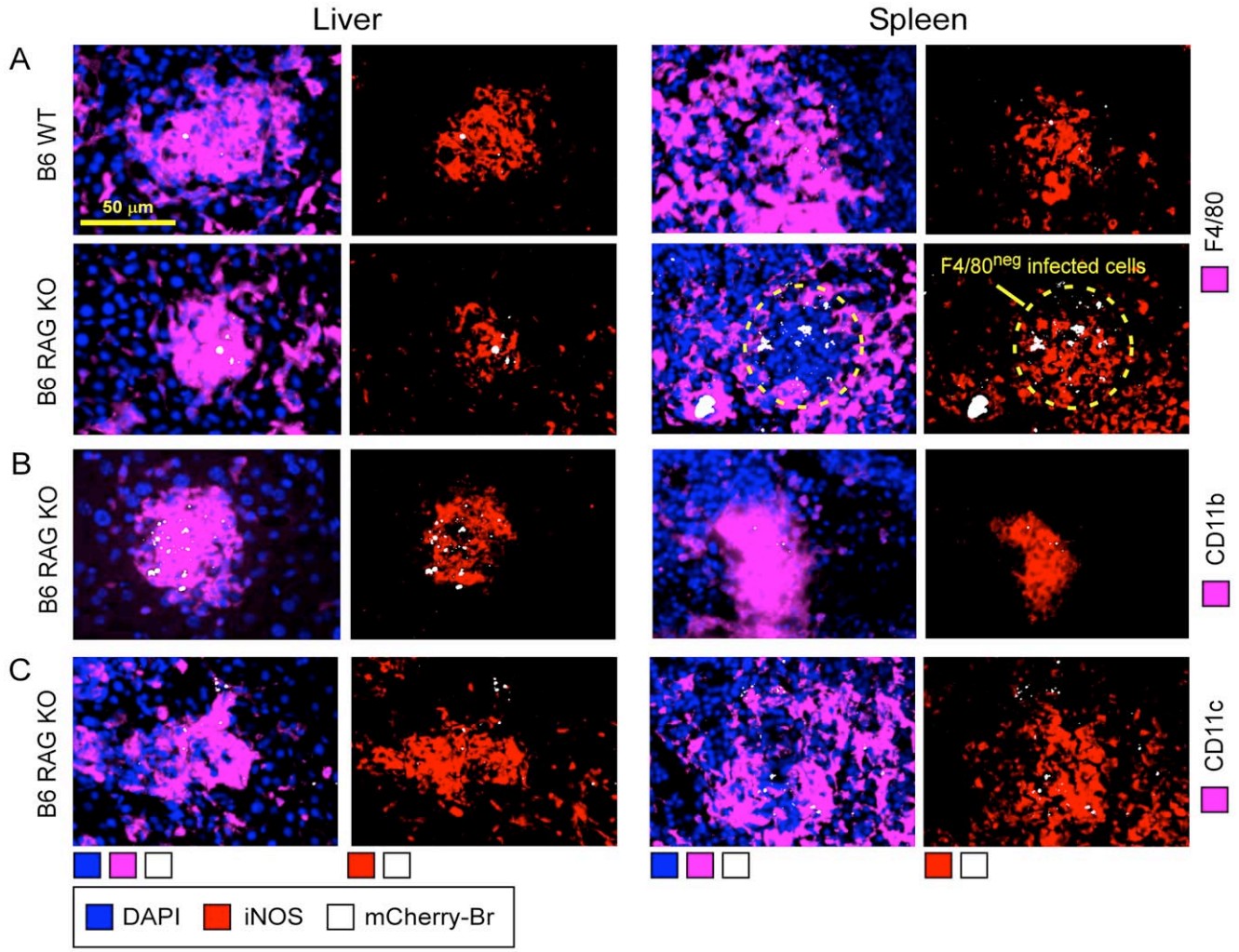


Figure 6. Phenotypical analysis of granuloma in the liver and spleen of wild type and RAG-deficient infected mice. Localization by immunohistofluorescence of (**A**) F4/80, (**B**) CD11b, (**C**) CD11c and iNOS expressing cells in liver and spleen from 120 h mCherry-Br infected wild-type and RAG$^{-/-}$ C57BL/6. Mice were injected i.p. with 10^8 CFU of mCherry-Br. Images represent a single granuloma. Panels are color-coded with the text for the antigen or mCherry-Br examined. Scale bar = 50 μm, as indicated. Data are representative of at least 3 independent experiments.
doi:10.1371/journal.ppat.1002575.g006

deficient mice displayed higher CFU counts in spleen and liver at 120 h p.i. (Figure S11). As expected, frequencies of IFN-γ^+ cells and iNOS-DC in infected spleen were drastically reduced in all Th1 deficient mice when compared to infected wild type mice (data not shown and Figure S12).

Immunofluorescence analysis of liver and spleen sections from 120 h-infected mice showed that all three deficient mouse strains displayed dense aggregates of CD11b$^+$ cells surrounding infected cells and resembling granulomas (Figure 7 for spleen and liver from wild type and MyD88$^{-/-}$ mice and data not shown for IL-12$^{-/-}$ and IFN-$\gamma^{-/-}$ mice). Whereas iNOS staining was strongly reduced in all three deficient mouse strains (Figure 7 and data not shown), CD90.2$^+$ T cells recruitment around infected cells was normal (data not shown). Moreover, granuloma-like structures in all deficient mouse strains frequently contained higher numbers of infected Ly-6G$^+$ cells but reduced density of F4/80$^+$ and CD11c$^+$ cells (Figure 8 for liver granulomas and data not shown for spleen granulomas). These "unconventional" granulomas, characterized by an inverted ratio of granulocytes versus monocytes/DC, failed to control bacterial growth as demonstrated by an increased mCherry-Br signal in deficient mice, with higher numbers of infected cells outside of granuloma like structures. These "unconventional" granulomas were also detected in infected wild type mice but at a low frequency.

Major cellular reservoir for *Brucella* in spleens of chronically infected susceptible mice localizes in white pulp and expresses MOMA-1 or CD11c and CD205 markers

One of our initial objectives was to characterize the phenotype of *Brucella* reservoir cell types in chronically infected mice. Unfortunately, the detection threshold of mCherry-Br signal *in situ* required approximately 10^6 CFU/g of tissue (Figure S3.A and B). Wild type mice initially infected with 10^8 bacteria displayed CFU counts in spleen and liver inferior to this threshold at 12 days p.i. and MyD88$^{-/-}$, IL-12$^{-/-}$ or IFN-$\gamma^{-/-}$ mice showed high mortality rate at the same time point. Thus, in order to increase the lifespan of genetically deficient mice, we tested an inoculum dose of 10^6 CFU and compared bacterial loads in spleen and liver at 5, 12 and 30 days p.i. (Figure S13). Only IL-12p40$^{-/-}$ BALB/c mice displayed CFU counts superior to the detection threshold in

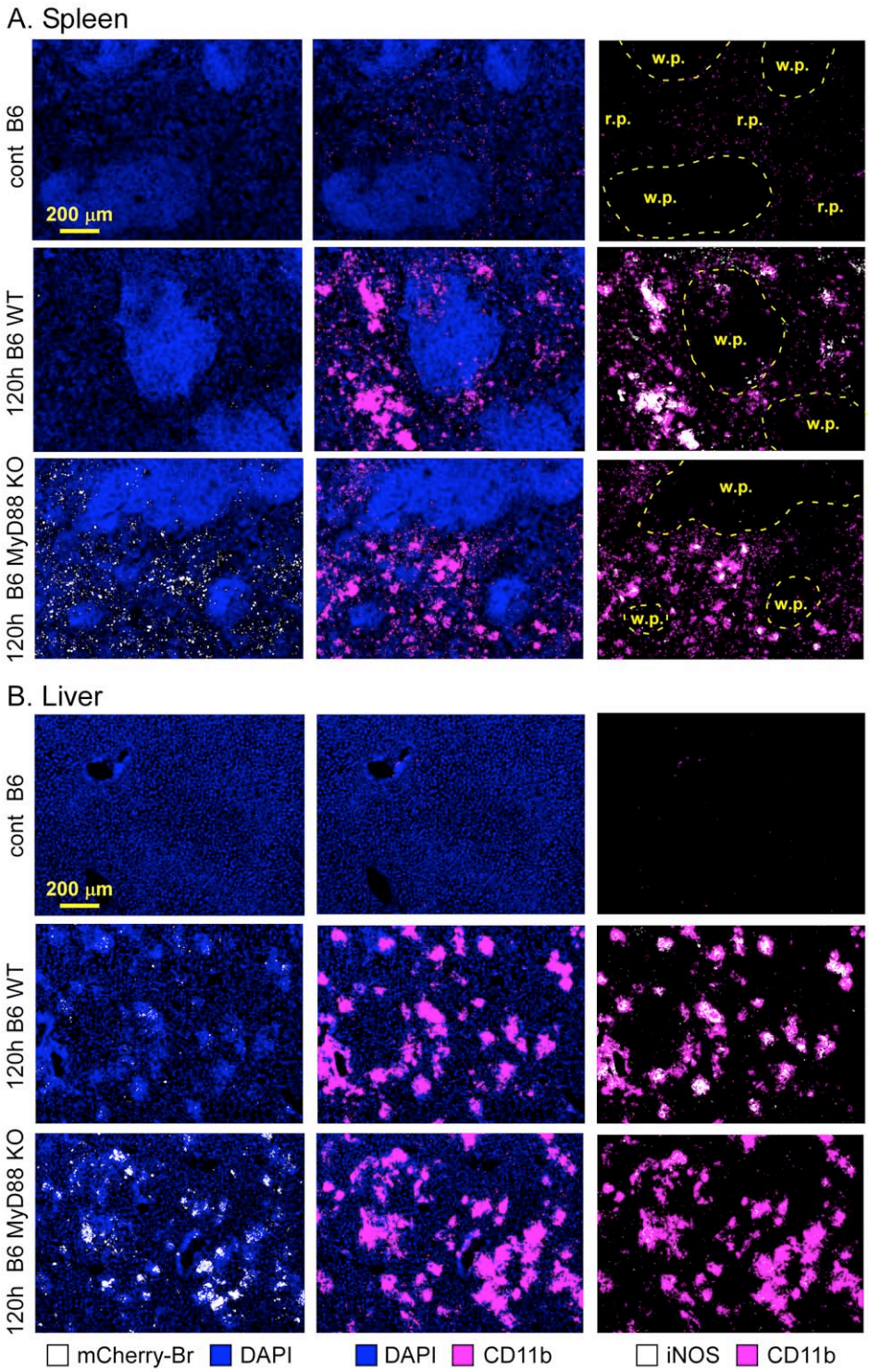
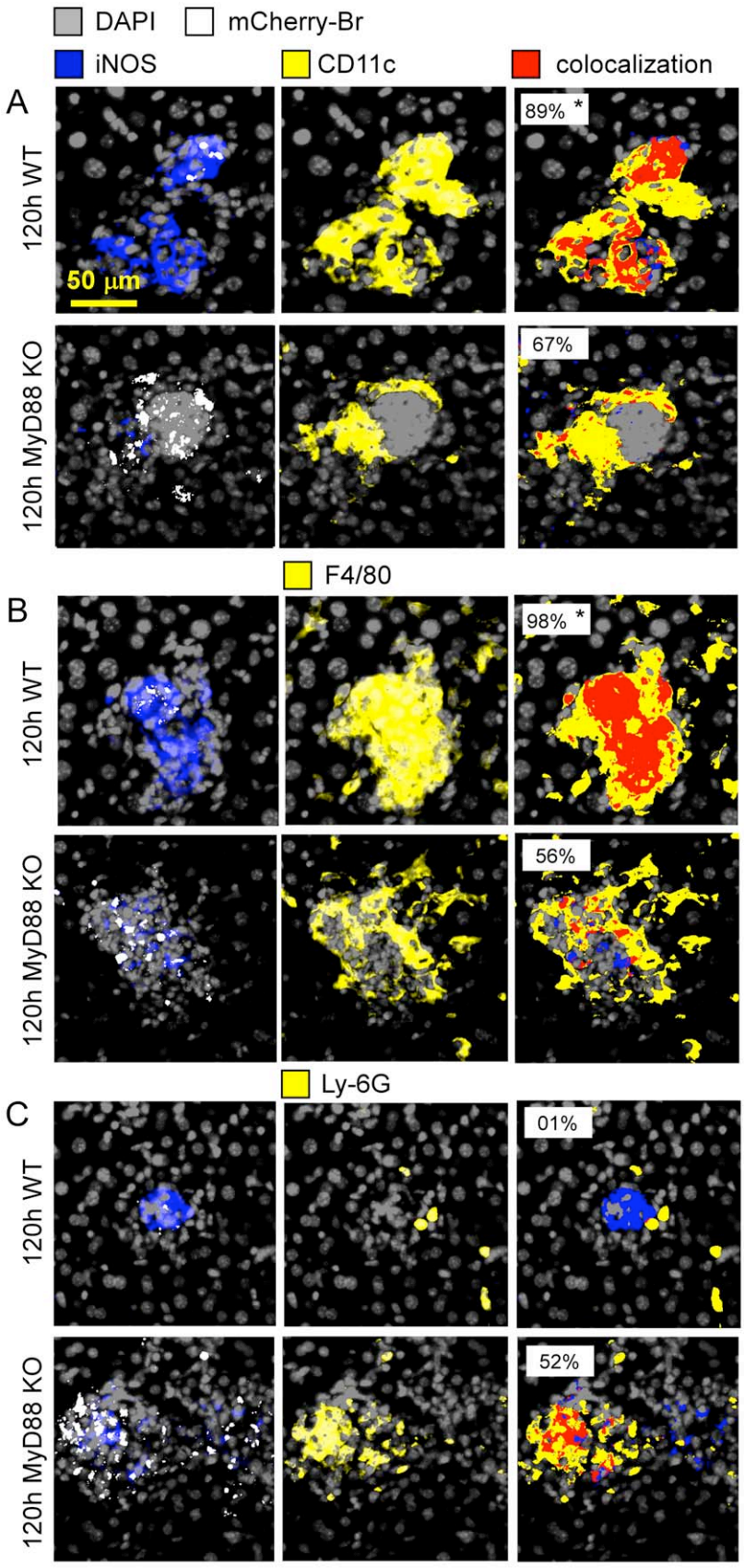


Figure 7. CD11b^{+} granulomas in the spleen and liver of wild type and MyD88-deficient infected mice. Mice were injected i.p. with PBS (control) or 10^8 CFU of mCherry-Br. The figure presents the localization by immunohistofluorescence of CD11b^{hi} and iNOS expressing cells in spleen (**A**) and liver (**B**) from control and 120 h mCherry-Br infected wild-type and MyD88$^{-/-}$ C57BL/6. Panels are color-coded with the text for the antigen or mCherry-Br examined. Scale bar = 200 μm, as indicated. r.p.: red pulp; w.p.: white pulp. Data are representative of at least 3 independent experiments.
doi:10.1371/journal.ppat.1002575.g007

spleens at 12 and 30 days p.i. Interestingly, microscopy analysis of tissue sections from spleens from infected IL-12$^{-/-}$ BALB/c mice (Figure S14.B) showed that mCherry-Br signal, initially located in r.p. at 12 h p.i., progressively relocated in w.p. at 5, 12 and 30 days p.i. This preferential localization in w.p. was also observed in infected IL-12$^{-/-}$ C57BL/6 at 5 days p.i. (Figure S14.C),



*: number indicate the % of iNOS signal that colocalize with CD11c, F4/80 and Ly-6G, respectively

Figure 8. Phenotypical analysis of granuloma in the liver of wild type and MyD88-deficient infected mice. Colocalization by immunohistofluorescence of CD11c (**A**), F4/80 (**B**), Ly-6G (**C**) and iNOS expressing cells with mCherry-Br signal in liver from 120 h mCherry-Br infected wild-type and MyD88$^{-/-}$ C57BL/6. Mice were injected i.p. with 10^8 CFU of mCherry-Br. Panels are color-coded with the text for the antigen or mCherry-Br examined. Scale bar = 50 μm, as indicated. Data are representative of at least 3 independent experiments.
doi:10.1371/journal.ppat.1002575.g008

demonstrating that the BALB/c background is dispensable to observe this phenomenon. Surprisingly, the phenotype of *Brucella*-containing cells located in w.p. of 12 days-infected IL-12$^{-/-}$ BALB/c mice was strikingly distinct from r.p. infected cells previously described in wild type mice (Figure 9.A and 9.B). Indeed, these cells were highly infected (>20 bacteria/cell) and harbored two distinct cell surface marker combinations. When localized in close proximity of m.z. area, the infected cells expressed MOMA-1, a specific marker of metallophilic marginal zone macrophages, whereas when located deeply within the w.p., they expressed CD11c and DEC205/CD205 (Figure 9.B), both DC-specific molecules. Both types of cells were negative for iNOS, F4/80, CD11b, CD86, CD90.2 and B220 (data not shown) and a fraction of them expressed weakly MHC-II. Although the bacterial load was reduced, the phenotype and location of IL-12$^{-/-}$ infected cells was similar 30 days p.i., (data not shown). Interestingly, highly infected CD11c$^+$ cells located in the w.p. of wild-type BALB/c mice could also be observed 5 days p.i. but at a very low frequency (Figure 9.C and data not shown) suggesting that those cells are not limited to Il-12$^{-/-}$ BALB/c mice.

Given that these heavily infected w.p. cells were not found associated with CD11b$^+$ F4/80$^+$ iNOS$^+$ effector cells, we conclude that they may constitute an important reservoir for *B. melitensis* under permissive conditions such as absence of Th1 protective response.

Discussion

Bacteria of the genus *Brucella*, have been long studied as a relevant experimental model to analyze chronic infections of animals and humans due to the great impact they have on both husbandry practice and human health worldwide [29]. Their virulence and the chronicity of the ensuing disease rely on their ability to modulate both innate and adaptive immune responses and the physiology of the host's cells in which they reside, survive and multiply (reviewed in [30]).

The mouse is considered as a useful animal model to investigate the pathogenesis of brucellosis, to identify specific virulence factors of *Brucella* spp and to characterize the host immune response [29]. If classical studies [31,32], and more recently the use of genetically deficient mice, have been useful to uncover the great avenues of protective immune responses against *Brucella* infection [33], this approach appears insufficient to dissect immune effector mechanisms elaborated by the host to fight *B. melitensis*. Like *Mycobacterium tuberculosis* [34,35],[36], and in contrast to other intracellular bacteria such as *Listeria monocytogenes* [37] or *Legionella pneumophila* [38], *B. melitensis* does not irreversibly affect the health status of the majority of mice genetically deficient for key element of Th1 protective immune response (such as MyD88 [10],[39], IL-12p40 [11], IFN-γ [12] and iNOS [11]) during the first four weeks of infection. These observations suggest that immune effector mechanisms against *Brucella* are multiple and redundant, and necessitate new approaches to be further characterized. This is the perspective from which we decided to use *in situ* microscopy techniques for studying the local immune response developed around *B. melitensis* infected cells. Up to now, only gross morphometric and histopathologic analyses have been conducted [40–42] on spleen and liver, that are important sites for colonization and replication of *Brucella* in the mouse model [29]. Both organs develop the so called "histiocytic infiltrates and microgranulomas" [41,42]. Of importance, *Brucella* infection in mice results in lesions mimicking those described in chronic infections in humans which may also develop splenomegaly and hepatomegaly [17,43]. To our knowledge, our study and the recent work from Archambaud, C *et al.* [44] are the first *in vivo* investigations characterizing the *in vivo* phenotype of *Brucella*-infected cells. Archambaud, C *et al.* have focused their study on *Brucella abortus*-infected pulmonary tissues following intranasal inoculation. In contrast, we used an i.p. systemic model of infection in order to analyse the phenotype of *Brucella melitensis*-infected cells in spleen and liver. The i.p. route of infection is generally used to establish a persistent infection in the mouse because it leads to a rapid systemic distribution of *Brucella* sp. and to high bacterial loads in both spleen and liver [33,45].

Brucella has been initially described as an intracellular bacteria able to replicate in professional phagocytes such as macrophages [46], DC [47] and granulocytes as well as non-professional phagocytes [48], including epithelial, fibroblastic and trophoblastic cells, in the context of cell line cultures. However, the identity of *Brucella* reservoir cell types during the course of infection *in vivo* is largely uncharacterized. If macrophages [40] and trophoblastic cells [49] have been clearly associated with *Brucella* infection in the natural hosts, little has been demonstrated regarding the contribution of other potential cellular niches for *Brucella in vivo*.

In this study, using a *Brucella* strain stably expressing mCherry, we demonstrated for the first time that these bacteria present a very restrictive cellular tropism as the majority of infected cells in the spleen and the liver of resistant C57BL/6 wild type mice belongs to a specific fraction of the myeloid lineage. Granulocytes, B cells, T cells, fibroblasts and hepatocytes were never found significantly infected. This result may explain the frequent discrepancies between *in vitro* and *in vivo* attenuation of various *Brucella* mutants. In the physiological cell reservoir and in the complex microenvironment of the host, some of the "virulence factors" identified *in vitro* may have little or no relevance. A striking example is the mutation affecting the gene *virB* which encodes the type IV secretion system of *Brucella*. The *virB* mutant is unable to grow within host cells *in vitro* [22] though it can replicate at a similar level as the wild type strain during the first 5 days of infection *in vivo* [23]. In the present study, *virB* mutant and wild type bacteria were localized within the same cells in infected spleen at all time-points analyzed during the first three days of infection. This argues in favor of the intracellular localization of *virB* mutant *in vivo* and strongly suggests that the type IV secretion system of *Brucella* is tightly regulated during the infectious process. Since we showed that the nature of the infected cells varied in a time-dependent manner during the first days of infection, it is tempting to speculate that the type IV secretion system depends on the nature of the infected cells and/or its activation status.

Our study also brought to light the complexity and the dynamic of the cellular environment of the pathogen during the course of infection. In the spleen, m.z. (MOMA-1$^+$) and r.p. (F4/80$^+$) macrophages are the first infected cells, followed by DCs (CD11c$^+$) located in m.z. and r.p.. We hypothesized that infection-mediated inflammation is responsible of subsequent recruitment of CD11b$^+$ Ly-6G$^-$ monocytes. Our data suggest that these cells are rapidly

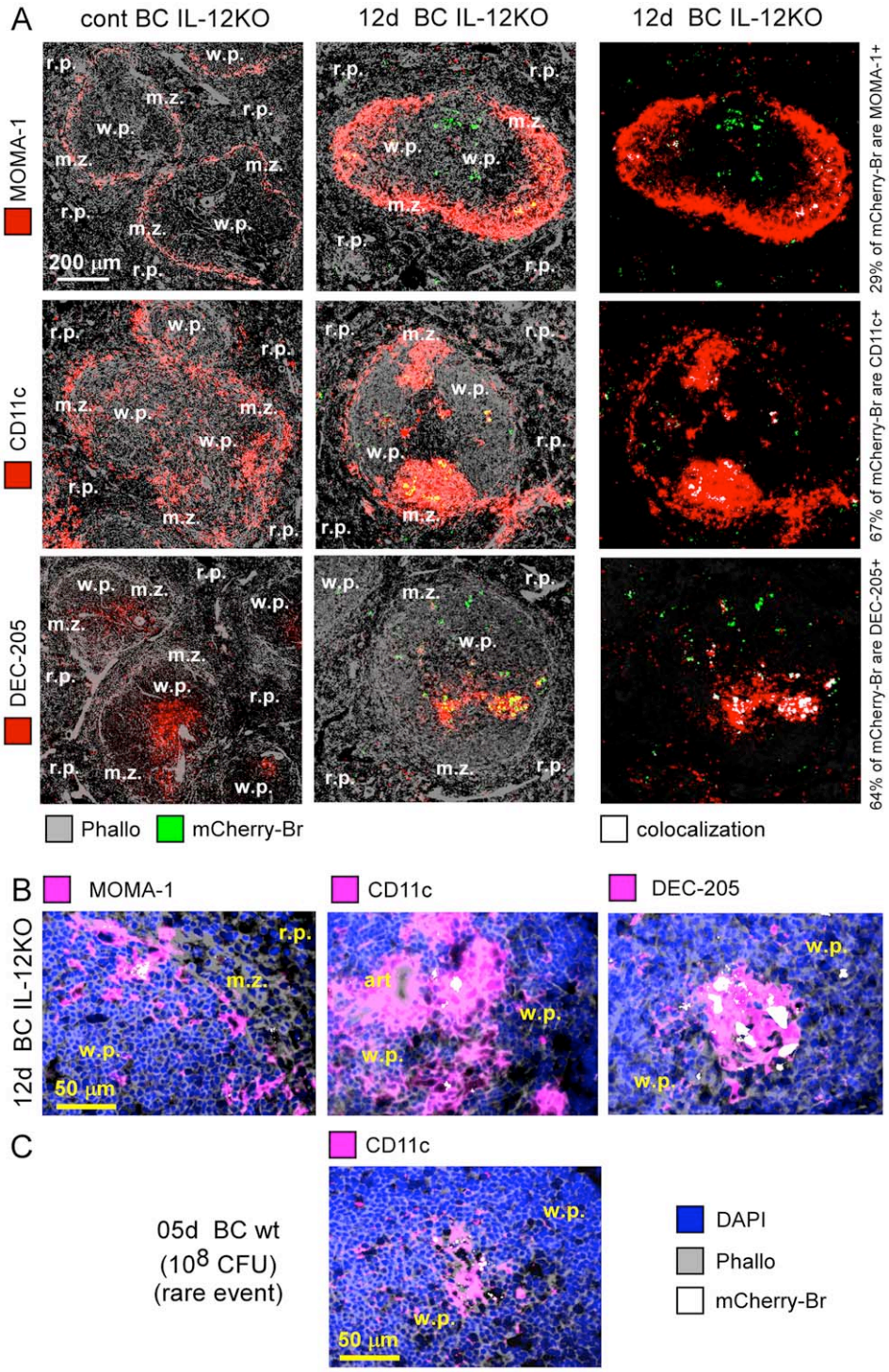


Figure 9. *B. melitenis* infects CD11c$^+$ CD205$^+$ or MOMA-1$^+$ cells in spleens of chronically infected Il-12-deficient BALB/c mice. Wild-type and Il-12p40$^{-/-}$ BALB/c mice were injected i.p. with PBS or mCherry-Br, as indicated. Mice were sacrificed 5 or 12 days p.i. and spleens were collected and examined by immunohistofluorescence. **A** & **B**, Colocalisation of mCherry-Br with MOMA-1, CD11c, CD205 expressing cells in the spleen of IL-12$^{-/-}$ BALB/c mice infected with 10^6 CFU 12 days p.i.. **C**, Higher magnification view of CD11c-expressing cells and mCherry-Br in w.p. of wild type BALB/c mice infected with 10^8 CFU 5 days p.i.. Panels are color-coded with the text for the antigen or mCherry-Br examined as well as the colocalization. Scale bar = 200 and 50 μm, as indicated. r.p.: red pulp; w.p.: white pulp, m.z.: marginal zone. Data are representative of at least 3 independent experiments.
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infected and progressively mature to form complex granulomas in r.p. composed of a mix of activated mature monocytes (CD11b$^+$ CD11c$^-$ F4/80$^+$ MHC-II$^+$) and inflammatory DC (CD11b$^+$ CD11c$^+$ F4/80$^+$ MHC-II$^+$) and surrounded by T cells (CD90.2$^+$) and granulocytes (CD11b$^+$ Ly-6G$^+$). This scenario yields major differences when compared with the observations by Archambaud

et al. [44] in the context of intra-nasal infection with *Brucella abortus*. Although macrophages are the first infected cells in spleen, liver, as shown in our study, and lungs [44], DC infection and granulomas formation are not observed in lungs but only in draining lymph node [44]. These discrepancies could be due to differences in dose of infection, route of inoculation and bacterial strain used in both studies.

It has been frequently reported that BALB/c mice display higher bacterial loads per organ when compared to C57BL/6 mice during *Brucella* infection [10,25]. The reasons of this susceptibility are unclear. In our experimental model, spleen and liver infected cells displayed a closely similar phenotype and localization. However, higher CFU counts observed in infected BALB/c mice was related to increase colonization of w.p. cells. These latter displayed high number of bacteria and expressed MOMA-1 or CD11c markers but were negative for CD11b, F4/80, Ly-6G and CD90.2,

Transient infection of $CD11c^+$ cells during the early stages of infection in the spleen leads us to characterize the impact of *Brucella* infection on the maturation of conventional DC. Flow cytometry and *in situ* staining showed that spleen infection with *B. melitensis* induced the massive migration of DC in the T cell area of w.p. as well as their maturation during the first 24 h of infection, followed later, by their gradual disappearance. The relationship between *Brucella* and DCs seems complex and necessitates further experiments to be elucidated. However, our results demonstrate clearly that the course of *Brucella* infection is associated to DCs activation in spleen. This maturation is dependent of MyD88 adapter molecule and does not require infection as heat killed *Brucella* induce a similar phenomenon. Importantly, microscopy analysis indicated that none of $CD11c^+$ mature cells migrating to T cell area were infected by *B. melitensis* (data not shown), suggesting that infection of DCs could impair their migration and maybe their maturation. Again, these results are in agreement with the stealthy strategy [30] attributed to *Brucella* and with *in vitro* studies [50],[51] demonstrating the ability of *Brucella* to regulate DC maturation.

Using genetically deficient mice, we partially clarified some requirements for granuloma formation during *Brucella* infection. Development of a Th1 response seems a critical step as $MyD88^{-/-}$, IL-$12p35^{-/-}$ and IFN-$\gamma^{-/-}$ mice displayed $iNOS^-$ altered granulomas, strongly enriched in granulocytes. In contrast, the analysis of $RAG^{-/-}$ infected mice demonstrated that granuloma formation at 5 days p.i. is, for a large part, independent of lymphocytes. We hypothesized, in accordance with the granuloma formation model proposed for *Listeria monocytogenes* infection [16], that IFN-γ is needed for maturation of monocytes in iNOS-DC and building of fully functional granuloma. As previously described [10], IFN-γ was produced by Natural Killer (NK) cells and T lymphocytes during *Brucella* infection. In $RAG^{-/-}$ mice, iNOS-producing DC (iNOS-DC) were detectable by flow cytometry and microscopy analysis suggesting that absence of IFN-γ-producing T lymphocyte might be compensated by IFN-γ-producing NK cells. This was confirmed by the absence of iNOS-DC in spleens of $RAG\gamma c^{-/-}$ (deficient for natural killer cells) infected mice (data not shown).

The presence of phenotypically similar granulomas in the infected spleen and liver support the hypothesis that granulomas constitute the "spearhead" of the immune response against *Brucella*. Correlation between high susceptibility of $MyD88^{-/-}$, IL-$12^{-/-}$ and IFN-$\gamma^{-/-}$ mice and strongly altered granuloma structures in these mice suggested a causal link between the presence of granulomas and *Brucella* growth control. However, the precise role of granuloma during *Brucella* infection remains to be clarified.

A prominent characteristic of *Brucella* is its capacity to persist in natural host for life (reviewed in [30]). This property is thought to be related to its capacity to (i) locate intracellularly and avoid the fusion of *Brucella*-containing vacuoles with host cell lysosomes (ii) limit or modulate the activation of innate and adaptive immune response (mainly due to poorly recognized PAMPs of its cell envelope and poorly characterized secreted effectors), (iii) prolong the lifespan of infected cells. The previously unappreciated restricted specificity of *Brucella* cell tropism that we observed *in vivo* allows us to hypothesize that the ability of *Brucella* to persist could also depend of its capacity to infect cell subsets particularly adapted to sustain its growth and persistence. In absence of Th1 response, granuloma formation was altered and the bacterial burden was significantly higher in spleens of IL-$12p40^{-/-}$ BALB/c mice, making possible microscopy observation *in situ* and characterization of infected cells during chronic phase of infection (12 and 30 days p.i.). Our analyses revealed that the main infected spleen cells at these time-points are located in the w.p. and express the following cell surface phenotype: $CD11b^-$ $CD11c^+$ $CD90^-$ $CD205^+$ $F4/80^-$ $Ly\text{-}6G^-$ $MHC\text{-}II^{low}$ $B220^-$ $iNOS^-$. High expression of CD11c and CD205 suggested that these cells are a particular subset of DC. This phenotype is also partially reminiscent of foamy macrophages that express high levels of CD11c, CD205 and low level of MHC-II [52] and constitute nutrient-rich reservoirs in lung granulomas of *M. tuberculosis*-infected mice [53]. Interestingly, these cells were also detected, albeit with a low frequency, five days post-infection in the w.p. of wild type BALB/c mice infected with 10^8 CFU. Previously, we demonstrated the reduced ability of BALB/c mice to mount Th1 [10] response in the context of *Brucella melitensis* infection. Thus, permissive environments, such as the absence of Th1 response, may drive the differentiation of macrophages toward a long-lived and anti-inflammatory phenotype allowing bacterial persistence. Identification of these cells as potential reservoir for *Brucella* in chronically infected mice could help to ameliorate therapeutic treatment of brucellosis.

In conclusion, this work dissected for the first time the nature of the effectors mechanisms developed *in vivo* by the immune system after *B. melitensis* systemic inoculation and described the phenotypic characteristics of infected cells during the initial and chronic steps of the infectious process. These results could help develop new strategies to control *Brucella* infection.

Materials and Methods

Ethics statement

The animal handling and procedures of this study were in accordance with the current European legislation (directive 86/609/EEC) and in agreement with the corresponding Belgian law "*Arrêté royal relatif à la protection des animaux d'expérience du 6 avril 2010 publié le 14 mai 2010*". The complete protocol was reviewed and approved by the Animal Welfare Committee of the Facultés Universitaires Notre-Dame de la Paix (FUNDP, Belgium)(Permit Number: 05-558).

Mice and reagents

Genetically deficient mice in C57BL/6 background: $MyD88^{-/-}$ [54] were obtained from Dr. S. Akira (Osaka University, Japan). $TRIF^{-/-}$ mice [55] were a kind gift from Dr. B. Beutler (The Scripps Research Institute, CA), IL-$12p35^{-/-}$ mice [56] from Dr. B. Ryffel (University of Orleans, France), IFN-$\gamma^{-/-}$ mice [57] from Dr. S. Magez (Vrije Universiteit Brussel, Belgium), $RAG1^{-/-}$ mice [58] from Dr. S. Goriely (Université Libre de Bruxelles, Belgium), $RAG\gamma c^{-/-}$ mice from Dr. Michel Y. Braun (Université

Libre de Bruxelles, Belgium). IL-12p40$^{-/-}$ BALB/c mice were obtained from Dr. V. Flamand (Université Libre de Bruxelles, Belgium). Wild type C57BL/6 mice and BALB/c mice purchased from Harlan (Bicester, UK) were used as control. All mice used in this study were bred in the animal facility of campus Gosselies from the Free University of Brussels (ULB, Belgium).

B. melitensis strain 16M (Biotype1, ATCC 23456) was isolated from an infected goat and grown in biosafety level III laboratory facility. Overnight culture grown with shaking at 37°C in 2YT media (Luria-Bertani broth with double quantity of yeast extract) to stationary phase was washed twice in PBS (3500×g, 10 min.) before use in mice inoculation.

Construction of a mCherry-producing *B. melitensis* strain

The mCherry protein, a previously described rapidly maturing variant of the red fluorescent protein DsRed [18], was cloned into the suicide vector pKSoriT-*bla-kan* downstream a strong *Brucella* spp. promoter [19,20], *nouvelle ref* Köhler S, Infect Immun. 1999) previously used to express constitutively the GFP. This promoter was called P*sojA* and described as controlling the expression of the protein translocase SecE (Köhler S.; personal communication).

The vector was constructed as follows: the mCherry coding sequence was amplified by PCR from pRSET-B-mCherry [18] with the mCherry-up and -down primers and ligated into pGemT-Easy (Promega) to generate pGEM-T-mCherry. The mCherry fragment was then excised from pGEM-T-mCherry by HindIII/XbaI double restriction and subsequently cloned into HindIII/XbaI-cut pKSoriT-*bla* (pBluescript II KS vector from Stratagene in which RP4 oriT was inserted in order to make this vector mobilizable [59]) to generate pKSoriT-*bla-mCherry*. Meanwhile, a 500 bp fragment upstream the coding sequence of secE was amplified from the *B. melitensis* genome by PCR with the P*sojA*-up and P*sojA*-down primers and ligated into pGemT-Easy (Promega) to generate pGEM-T-*SojA*. The P*sojA* fragment was then excised from pGEM-T-P*sojA* by NotI/XbaI double restriction and subsequently inserted into NotI/XbaI-cut pKSoriT-*bla-mCherry* to generate pKSoriT-*bla*-P*sojA-mCherry*. Finally, the aphA4 cassette (a promoterless kanamycin resistance gene [60] was excised from pUC4aphA4 with SalI, and subsequently cloned into the XhoI site of pKSoriT-*bla*-P*sojA-mCherry* to generate plasmid pKSoriT-*bla-kan*-P*sojA-mCherry*. This final construct was transformed into *E. coli* strain S17-1, and introduced into *B. melitensis* 16M NalR strain by conjugation. Clones that were kanamycin resistant and fluorescent were further checked by PCR, confirming the insertion of the plasmid at the targeted chromosomal P*sojA* promoter. Primers used in this study (Sequence (5′-3′)): mCherry-up (XbaI) tctagaatggtgagcaagggcgag, mCherry-down (HindIII) aagcttttacttgtacagctcgtcca, PsojA-up (NotI) gcggccgccttgactatggatgcccgtt, PsojA-down (XbaI) tctagactctgtctgatcaggcacaa.

Similar protocol has been used to construct mCherry-expressing ΔVirB *B. melitenis*. Construction of ΔVirB mutant has been previously described [61].

Mouse infection

Mice were injected intra-peritoneally (i.p.) with indicated dose of *B. melitensis* in 500 µl of PBS. Control animals were injected with the same volume of PBS. Infectious doses were validated by plating serial dilutions of inoculums. At selected time intervals, mice were sacrificed by cervical dislocation. Immediately after being killed, spleen and liver were collected for bacterial count and flow cytometry and microscopy analyses.

Bacterial count

For bacterial count, spleens and livers were recovered in PBS/0.1% X-100 triton (Sigma). We performed successive serial dilutions in PBS to get the most accurate bacterial count and we plated them onto 2YT media plates. CFU were counted after 3 days of culture at 37°C.

Cytofluorometry analysis

Spleen were harvested, cut in very small pieces and incubated with a cocktail of DNAse I fraction IX (Sigma-Aldrich Chimie SARL, Lyon, France) (100 µg/ml) and 1.6 mg/ml of collagenase (400 Mandl U/ml) at 37°C for 30 min. After washing, spleen cells were filtered and incubated in saturating doses of purified 2.4G2 (anti-mouse Fc receptor, ATCC) in 200 µl PBS 0.5% BSA 0.02% NaN3 (FACS buffer) for 10 minutes on ice to prevent antibody binding to Fc receptor. $3–5\times10^6$ cells were stained on ice with various fluorescent mAbs combinations in FACS buffer and further collected on a FACScalibur cytofluorometer (Becton Dickinson, BD). We purchased the following mAbs from BD Biosciences: Fluoresceine (FITC)-coupled 145-2C11 (anti-CD3ε), 53-8.7 (anti-CD8ε), M1/70 (anti-CD11b), GL-1 (anti-CD86), Phycoerythrin (PE)-coupled HL3 (anti-CD11c), RM4-5 (anti-CD4). Allophycocyanin (APC)-coupled M5/114.15.2 (anti-IA/IE). The cells were analyzed on a FACScalibur cytofluorometer. Cells were gated according to size and scatter to eliminate dead cells and debris from analysis.

Intracellular cytokine staining

Spleen cells were treated as previously described [62]. Spleen cells were incubated for 4 h in RPMI 1640 5% FCS with 1 µl/ml Golgi Plug (BD Pharmingen) at 37°C, 5% CO2. The cells were washed with FACS buffer and stained for cell surface markers before fixation in PBS/1% PFA for 15–20 min on ice. These cells were then permeabilized for 30 min using a saponin-based buffer (1× Perm/Wash, BD Pharmingen in FACS buffer) and stained with one or a combination of the following intracellular mAbs: allophycocyanin-coupled XMG1.2 (anti-IFN-γ; BD Biosciences), purified M-19 (rabbit polyclonal igG anti-NOS2; Santa Cruz Biotechnology) stained with Alexa Fluor 647 goat anti-rabbit (Molecular Probes). After final fixation in PBS/1% PFA, cells were analyzed on a FACScalibur cytofluorometer. No signal was detectable with control isotypes.

Immunofluorescence microscopy

Spleens and livers were fixed for 6 h at 4°C in 2% paraformaldehyde (pH 7.4), washed in PBS, incubated overnight at 4°C in a 20% PBS-sucrose solution under agitation, and washed again in PBS. Tissues were embedded in the Tissue-Tek OCT compound (Sakura), frozen, in liquid nitrogen, and cryostat sections (5 µm) were prepared. Tissues sections were rehydrated in PBS, then incubated successively in a PBS solution containing 1% blocking reagent (Boeringer) (PBS-BR 1%) and in PBS-BR 1% containing any of the following mAbs or reagents: DAPI nucleic acid stain, Alexa Fluor 350 or 488 phalloidin (Molecular Probes), purified 1A8 (anti-Ly-6G), or rabbit polyclonal antibodies anti-NOS2 (Calbiochem) (note that M-19 anti-NOS2; used for cytofluorometry analysis is not use for immunofluorescence microscopy), biotin-coupled HL3 (anti-CD11c, BD Biosciences), NLDC-145 (anti-DEC205/CD205, BMA Biomedical AG), MOMA-1 (anti Marginal Zone Macrophages, BMA Biomedicals), 53-2.1 (anti-CD90.2, BD Biosciences), RA3-6B2 (anti-CD45R/B220, BD Biosciences), Alexa Fluor 647-coupled BM8 (anti-F4/80, Abcam) M1/70 (anti-CD11b, BD Biosciences), M5/114.15.2

(anti-IA/IE, eBiosciences). Uncoupled 1A8 mAb and anti-NOS2 polyclonal antibodies were detected using biotin-coupled R67/1.30 (mouse anti-rat IgG2a, BD Biosciences) and Alexa Fluor 647-coupled goat anti-rabbit IgG (Molecular Probes) in PBS-BR 1%, respectively. Biotin-coupled mAbs were amplified using Alexa Fluor 350 or Alexa Fluor 647 Streptavidin (Molecular Probes) in PBS-BR 1%. Slides were mounted in Fluoro-Gel medium (Electron Microscopy Sciences, Hatfield, PA). Labeled tissues sections were visualized with an Axiovert M200 inverted microscope (Zeiss, Iena, Germany) equipped with high resolution monochrome camera (AxioCam HR, Zeiss). Images, 1384×1036 pixels (0.16 µm/pixel), were acquired sequentially for each fluorochrome with A-Plan 10×/0.25 N.A. and LD-Plan-NeoFluar 63×/0.75 N.A. dry objectives and recorded as eight bit grey levels *.zvi files. Colocalization between two stainings was analyzed using the AxioVision Colocalization module (Zeiss). Double positive pixels were rendered in white, gray or yellow as indicated in the Figures. Images were exported as TIFF files and figures prepared in Canvas 7 program.

For the estimation of the number of bacteria by cells or for the phenotype of infected cells, a minimum of 200 cells by condition were examined. These cells were counted in 6 mice minimum, in two independent experiments. When the number of bacteria by individual cell was too high to be determined, the number of bacteria was assumed to be of 20 or more (see Figure 1.E).

Confocal analysis

Confocal analysis were performed with LSM510 NLO multiphoton confocal microscope fitted on an Axiovert M200 inverted microscope equipped with C-Apochromat 40×/1.2 N.A. water immersion objectives (Zeiss). Optical sections of 1 µm thick, 568×568 pixels (0.1 µm/pixel), were collected sequentially for each fluorochrome and recorded as eight bit grey levels *.lsm files.

Statistical analysis

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

Supporting Information

Figure S1 Characterization of the mCherry-expressing fluorescent strain of *B. melitensis* 16M (mCherry-Br). A, Flow cytometry analysis of *B. melitensis* 16M and mCherry-Br. **B**, Infection of hamster fibroblastic cells BHK with *B. melitensis* 16M and mCherry-Br. The LPS of both bacteria is labelled in green and the mCherry-Br strain appears in red. **C**, Virulence curve of *B. melitensis* 16M and mCherry-Br. Groups of six mice were inoculated i.p. with 4×10^4 CFU of both *Brucella* strains. At the indicated times the CFU per spleen were calculated. Data are representative of at least 3 independent experiments.
(TIF)

Figure S2 Dynamic of dendritic cell, neutrophil and macrophage populations in spleen following low and high doses of bacteria. Wild-type C57BL/6 mice (5 per groups) were inoculated i.p. with PBS, 4×10^4, 10^7 CFU or 10^8 CFU of mCherry-Br, as indicated. At selected time, mice were sacrificed, spleens were collected and total spleen cells from each individual mouse were counted by Thoma cell. Cells were analyzed by flow cytometry, gated according to size and scatter to exclude dead cells and debris from analysis and then analyzed for CD11b, CD11c, F4/80, Ly-6C and Ly-6G expression, in order to obtain the frequencies of dendritic cell, neutrophil and macrophage subsets, as indicated at the top of each graph. The total number of each population by spleen was calculated and the data represent the median +/− SD of 5 mice. Data are representative of 2 independent experiments.
(TIF)

Figure S3 Time course of mCherry-Br infection and number of infected spleen cells by surface unit. A, Wild-type C57BL/6 and BALB/c mice (6 per groups) were inoculated i.p. with 10^8 CFU of mCherry-Br. At the indicated times, CFU per spleen were calculated. These results are representative of three independent experiments. **B**, Graphic representation of the number of splenic infected cells by surface unit of spleen section at 24 h and 120 h p.i. A unit surface is defined arbitrary as an area of 0,037 mm^2, corresponding to the tissue surface examined with 63× objective. The bars are the mean ± SD from at least 3 spleen sections per spleen from 5 mice.
(TIF)

Figure S4 Co-localization of *B. melitensis* and *Brucella* antigens *in vivo*. Colocalization analysis of F4/80 expressing cells, *Brucella* antigens and mCherry-Br before and 6 h after mCherry-Br infection in wild-type C57BL/6 mice. Mice were injected i.p. with PBS or 10^8 CFU of mCherry-Br. Numbers indicate the percentage of colocalizing cells in the upper panel. The inset areas in the middle-right panels are shown in the bottom panels. Panels are color-coded with the text for the antigen or mCherry-Br examined. Scale bar = 200 and 50 µm, as indicated. Data are representative of at least 3 independent experiments. r.p.: red pulp; w.p.: white pulp; m.z.: marginal zone.
(TIF)

Figure S5 *In vivo* intracellular localization of *Brucella* determined by *confocal* microscopy. Wild-type C57BL/6 mice were injected i.p. with PBS or 10^8 CFU of mCherry-Br. Mice were sacrificed at 1 day p.i. and the spleens were collected and examined by confocal microscopy. **A**, Representative confocal images of spleen section infected with mCherry-Br and labeled with antibodies against F4/80 (green) antigen. **B**, Deconvolution process of a spleen section infected with mCherry-Br and labeled with antibodies against F4/80 (green) antigen. **C**, Representative confocal images of spleen section infected with mCherry-expressing ΔvirB *B. melitensis* mutant and labeled with antibodies against F4/80 (green) antigen. m, micrometer.
(TIF)

Figure S6 *B. melitensis* infects LY-6G$^-$/CD11b$^+$ during the course of infection. Colocalization assay of CD11b, Ly-6G expressing cells and mCherry-Br, 72 h after infection in wild-type C57BL/6 mice. Mice were injected i.p. with PBS or 10^8 CFU of mCherry-Br. Numbers indicate the percentage of colocalizing cells in the juxtaposing left panel. Panels are color-coded with the text for the antigen or mCherry-Br examined. Scale bar = 50 µm, as indicated. Data are representative of at least 3 independent experiments. r.p.: red pulp; w.p.: white pulp; m.z.: marginal zone.
(TIF)

Figure S7 Granuloma formation in the spleen of mice infected by low and high doses of *B. melitensis*. Wild-type C57BL/6 mice (5 per groups) were inoculated i.p. with PBS, 4×10^4 or 10^8 CFU of mCherry-Br, as indicated. At selected time, mice were sacrificed, spleens were collected and examined by immunohistofluorescence. Panels are color-coded with the text for the antigen examined (**A**), CD11b and (**B**) F4/80. Scale

bar = 200 μm, as indicated. r.p.: red pulp; w.p.: white pulp; art.: central artery. Data are representative of 2 independent experiments.
(TIF)

Figure S8 *B. melitensis* infection induces the DC migration in the spleen during the course of infection. Positioning of DCs in the spleen of mice before and 12 or 24 h after mCherry-Br inoculation. Wild-type C57BL/6 mice were injected i.p. with PBS or 10^8 CFU of mCherry-Br. Mice were sacrificed at selected times and spleens were collected and examined by immunohistofluorescence. Panels are color-coded with the text for the antigen examined. Scale bar = 200 μm, as indicated. r.p.: red pulp; w.p.: white pulp; art.: central artery. Data are representative of at least 3 independent experiments.
(TIF)

Figure S9 *B. melitensis* infection induces the DC maturation. Wild type, MyD88$^{-/-}$ and TRIF$^{-/-}$ C57BL/6 mice were injected i.p. with, 10^8 CFU of live *B. melitenis* and various doses of heat killed (HK) *B. melitensis* and *Escherichia coli* O18:K1. Mice were sacrificed at selected times and spleens were collected and analyzed by flow cytometry. Cells were gated according to size and scatter to exclude dead cells and debris from analysis. **A**, Spleen cells from individual mice were first analyzed for Forward Size Scatter (FSC) and CD11c expression. CD11c^{hi} cells in each group were then analyzed for MHC-II and CD86 expression. Number indicates the percentage of positive cells per 10^6 spleen cells acquired for the specified marker. **B, C**, Comparative analysis of CD86 level on CD11c^{hi} cells. The data are the median. Data are representative of at least 3 independent experiments.
(TIF)

Figure S10 Phenotypical description of *Brucella* infected granuloma in the liver. Wild-type C57BL/6 mice were injected i.p. with PBS or 10^8 CFU of mCherry-Br. Mice were sacrificed at 5 days p.i. and livers were collected and examined by immunohistofluorescence. **A**, Immunofluorescence analysis of Ly-6G, CD90, CD11b, CD11c, F4/80, and MHC-II expressing cells and mCherry-Br. **B**, Percentage of iNOS$^+$ cells that colocalizes with CD11b-, and CD11c-expressing cells. Images represent a single granuloma. Numbers in Figure B indicate the percentage of colocalizing cells in the upper panel. Panels are color-coded with the text for the antigen or mCherry-Br examined as well as the colocalization. Scale bar = 50 μm, as indicated. Data are representative of at least 3 independent experiments.
(TIF)

Figure S11 Analysis of the bacterial load of the spleen and liver in wild type and genetically deficient mice. Wild-type, IFN-$\gamma^{-/-}$, IL-12p35$^{-/-}$, MyD88$^{-/-}$ and RAG$^{-/-}$ C57BL/6 mice and wild-type and IL-12p40$^{-/-}$ BALB/c mice (3–6 per group) were inoculated i.p. with 10^8 CFU. 120 h p.i., CFU per g of spleen (**A**) and liver (**B**) were calculated. Data are pooled from 3 independent experiments displaying similar CFU mean for wild type mice.
(TIF)

Figure S12 Flow cytometry analysis of iNOS-producing cells following *B. melitensis* infection. Wild-type, IFN-$\gamma^{-/-}$, IL-12p35$^{-/-}$, MyD88$^{-/-}$ and RAG$^{-/-}$ C57BL/6 mice and wild-type and IL-12p40$^{-/-}$ BALB/c mice (3–6 per group) were inoculated i.p. with 10^8 CFU of *B. melitensis*. Mice were sacrificed 5 days post-infection and spleen were collected and analyzed by flow cytometry. Cells were gated according to size and scatter to exclude dead cells and debris from analysis. **A**, Total pooled spleen cells were first analyzed for Forward Size Scatter (FSC) and iNOS expression. iNOS$^+$ cells in each group were then analyzed for CD11b and CD11c expression. Number indicates the percentage of cells in the selected quadrant. **B**, Number of CD11b$^+$ CD11c$^+$ iNOS$^+$ cells per 5×10^5 spleen cells acquired. Each data represents the value obtained from an individual spleen and the data are pooled from 4 independent experiments.
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Figure S13 Kinetic analysis of the bacterial load in the spleen and liver in wild type and genetically deficient mice. Wild-type, MyD88$^{-/-}$ and IL-12p35$^{-/-}$ C57BL/6 mice and wild-type and IL-12p40$^{-/-}$ BALB/c mice (4–8 per group) were inoculated i.p. with 10^6 CFU. At 5, 12 and 30 days p.i., CFU per g of spleen (**A**) and liver (**B**) were calculated. Data are representative from 2 independent experiments.
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Figure S14 Localization of *B. melitensis* during the course of infection in wild type and Il-12-deficient BALB/c mice. Wild-type and Il-12p40$^{-/-}$ BALB/c and Il-12p35$^{-/-}$ C57BL/6 mice were injected i.p. with PBS, 10^6 CFU or 10^8 CFU of mCherry-Br, as indicated. Mice were sacrificed at 12 h, 5 days, 12 days, and 30 days p.i., as indicated. Spleens were collected and examined by immunohistofluorescence. Positioning of mCherry-Br in the spleen of Wild-type (**A**), Il-12p40$^{-/-}$ BALB/c (**B**) and Il-12p35$^{-/-}$ C57BL/6 mice (**C**). Panels are color-coded with the text for the antigen or mCherry-Br examined. Scale bar = 200, as indicated. r.p.: red pulp; w.p.: white pulp. Data are representative of at least 3 independent experiments.
(TIF)

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Author Contributions

Conceived and designed the experiments: EM JJL. Performed the experiments: RC EM MAV DHM AM CDT. Analyzed the data: EM JJL RC. Contributed reagents/materials/analysis tools: EM JJL JMV SM SA BR YC. Wrote the paper: EM JJL.

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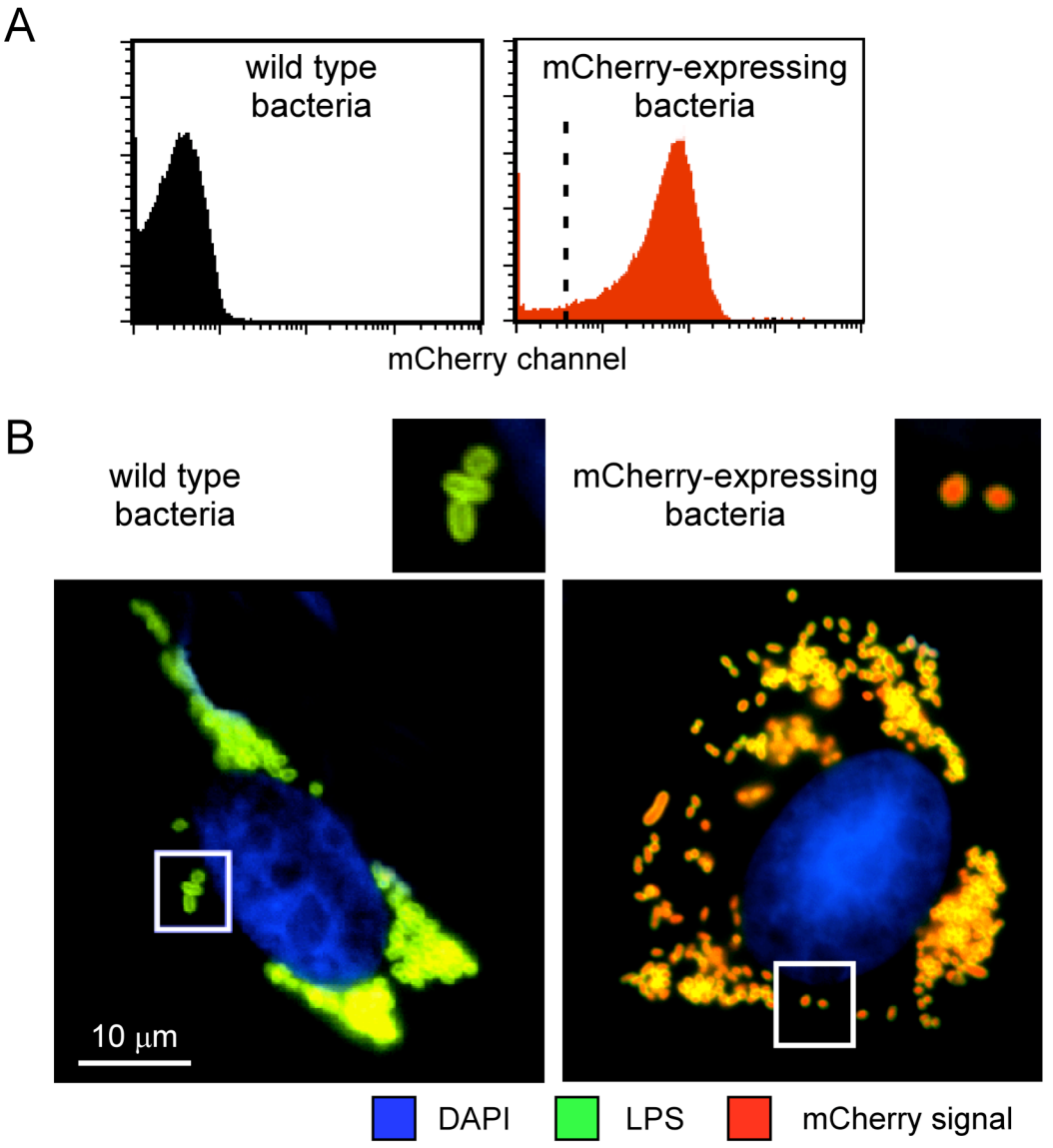
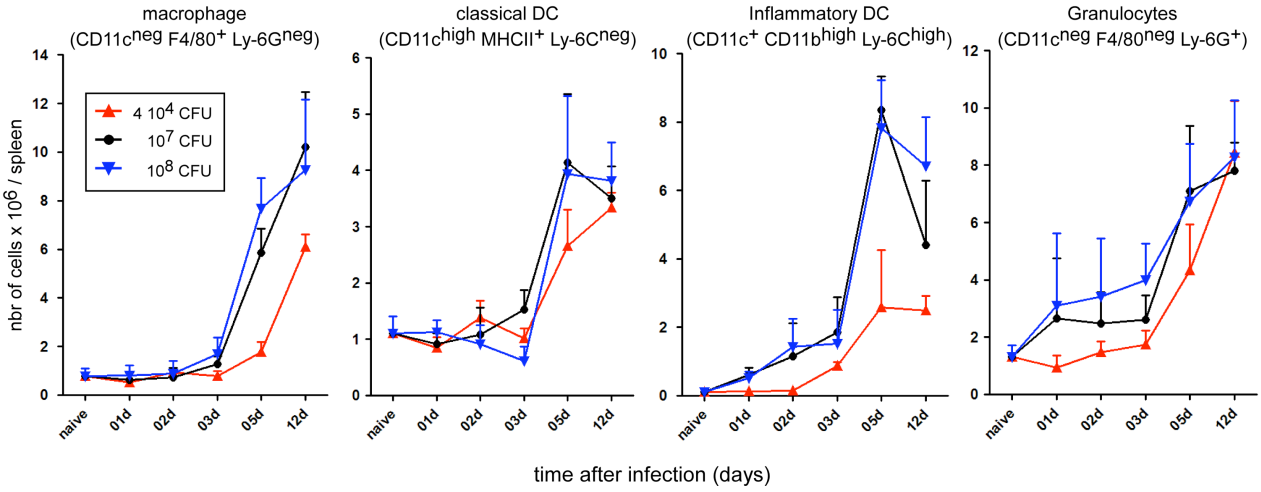
SUPPORTING INFORMATION



Copin *et al*, 2012 - **FIGURE S1**



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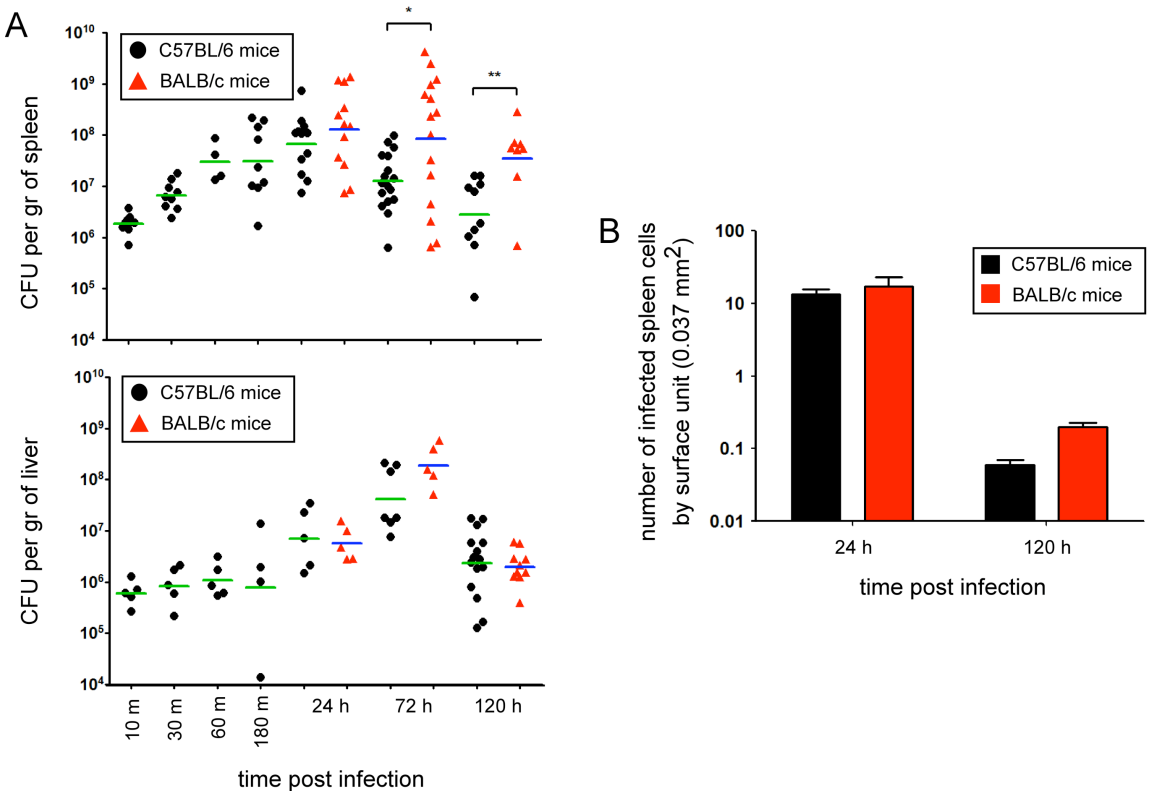

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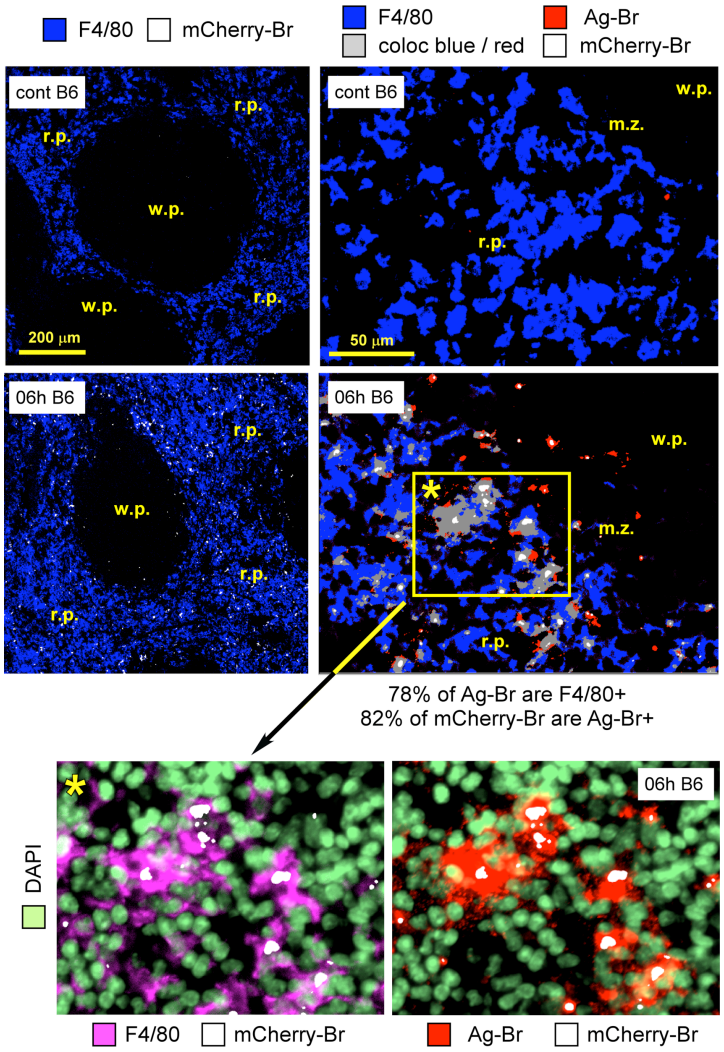

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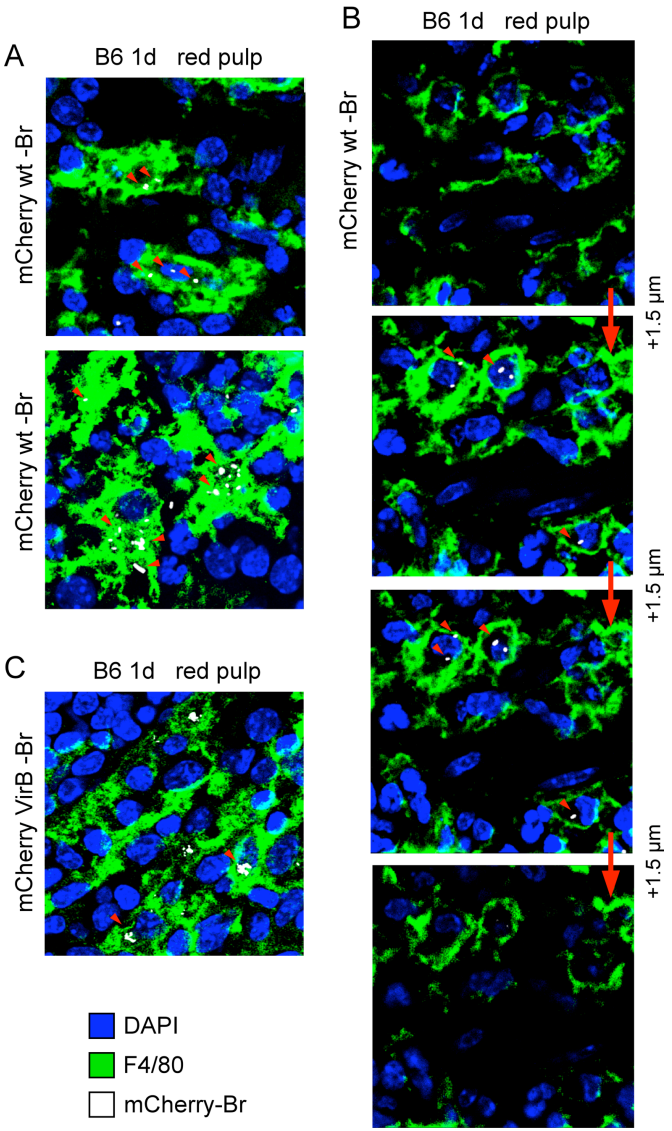

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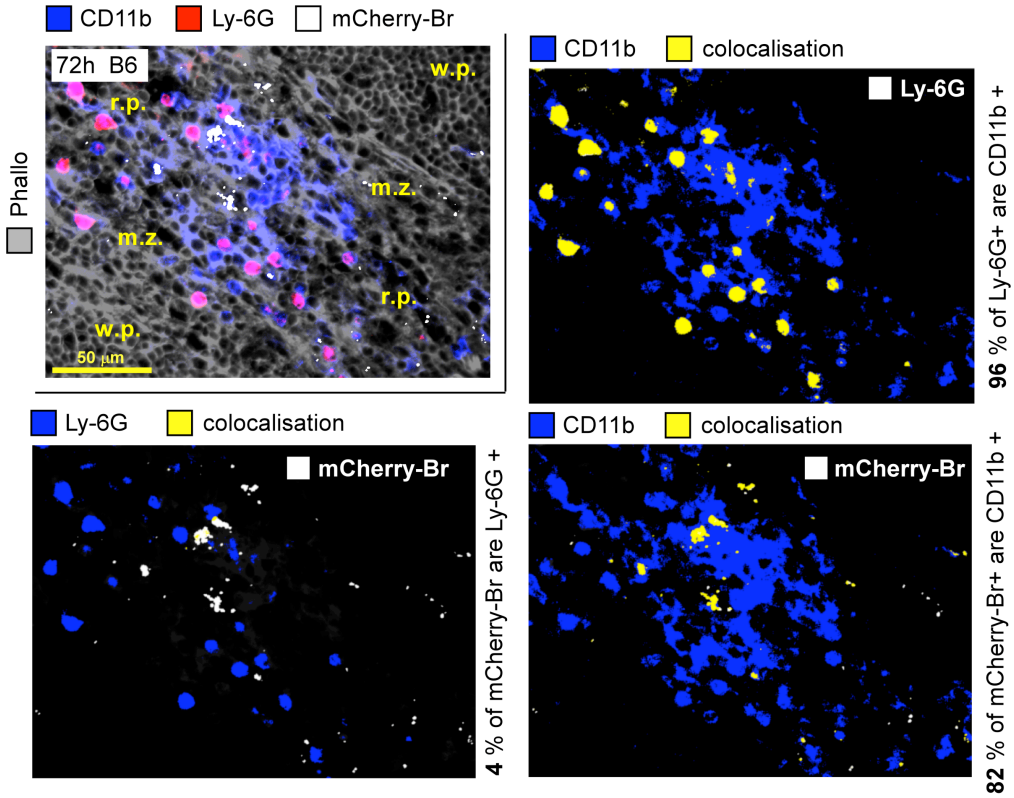

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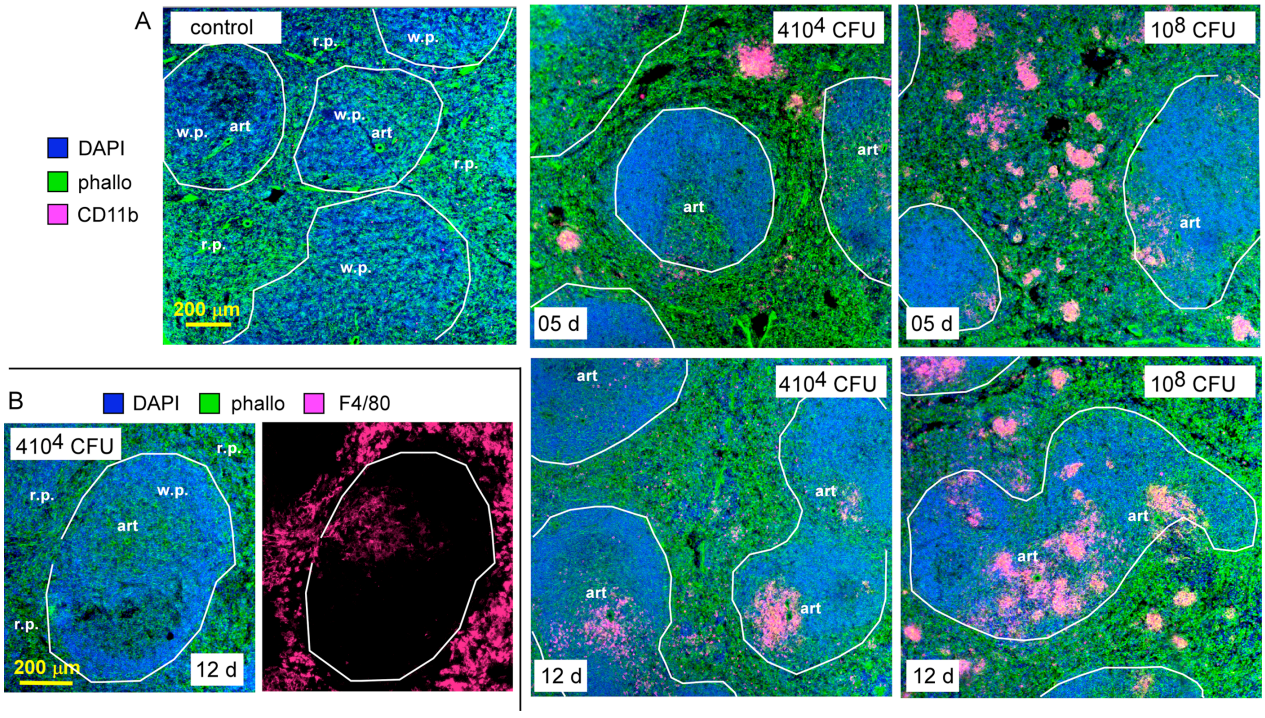

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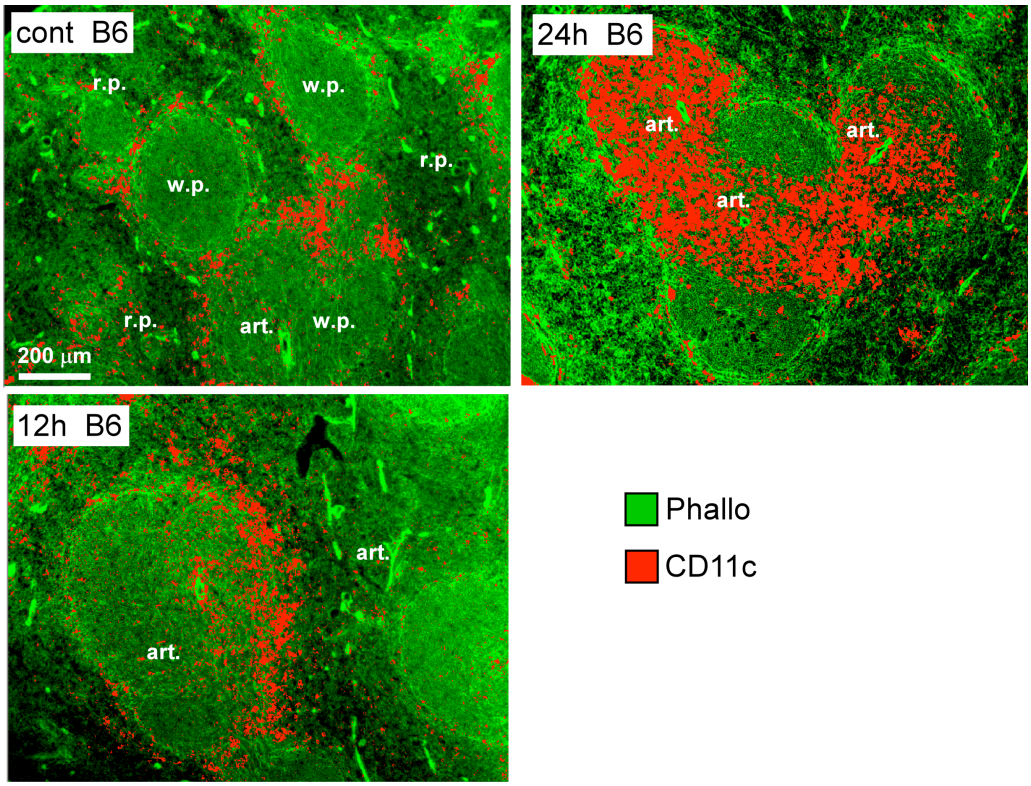

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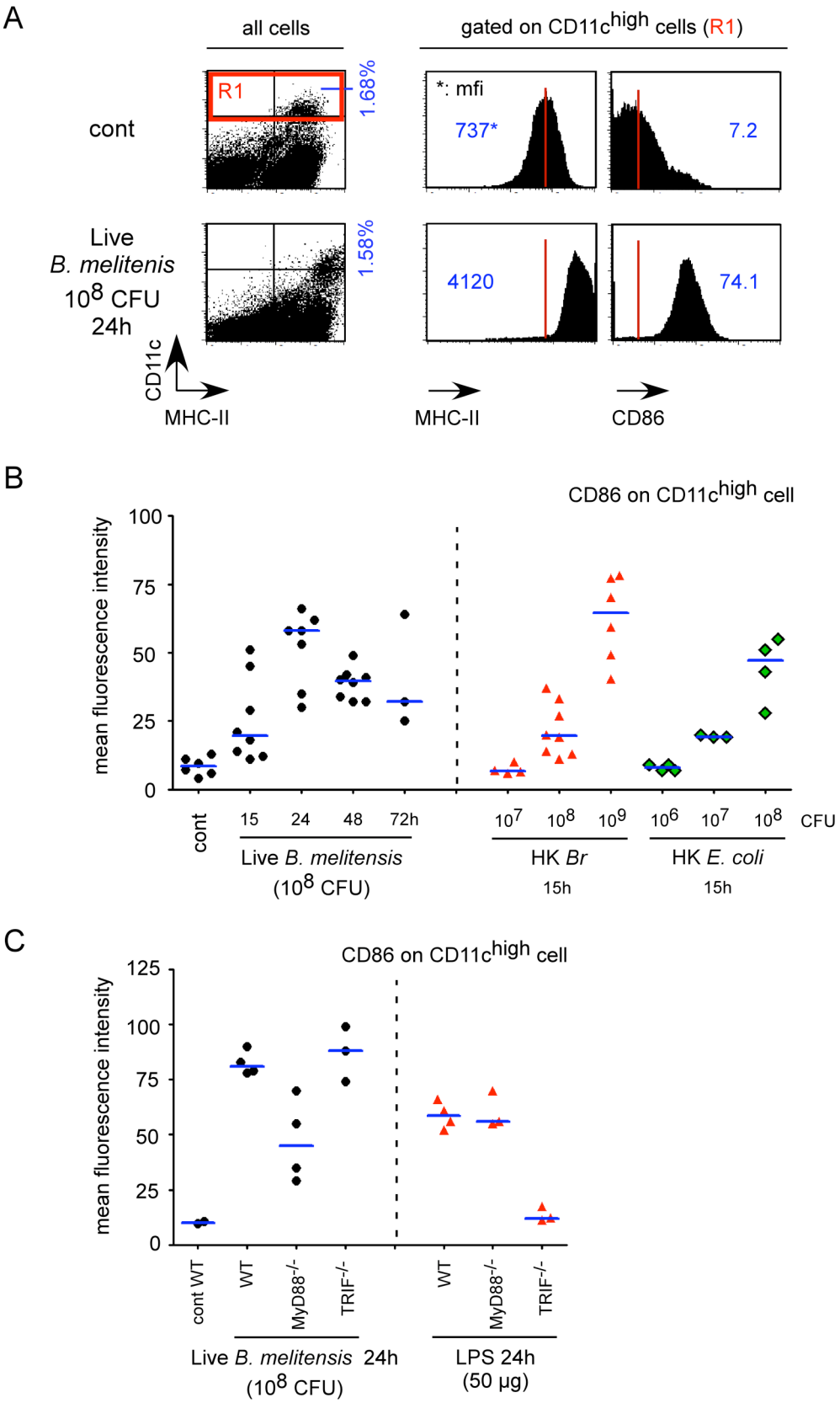
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Live
B. melitenis
10^8 CFU
24h
1.58%
4120
74.1
CD11c
MHC-II
MHC-II
CD86
B
CD86 on CD11c^high cell
mean fluorescence intensity
100
75
50
25
0
cont
15
24
48
72h
Live B. melitensis
(10^8 CFU)
10^7
10^8
10^9
HK Br
15h
10^6
10^7
10^8
CFU
HK E. coli
15h
C
CD86 on CD11c^high cell
mean fluorescence intensity
125
100
75
50
25
0
cont WT
WT
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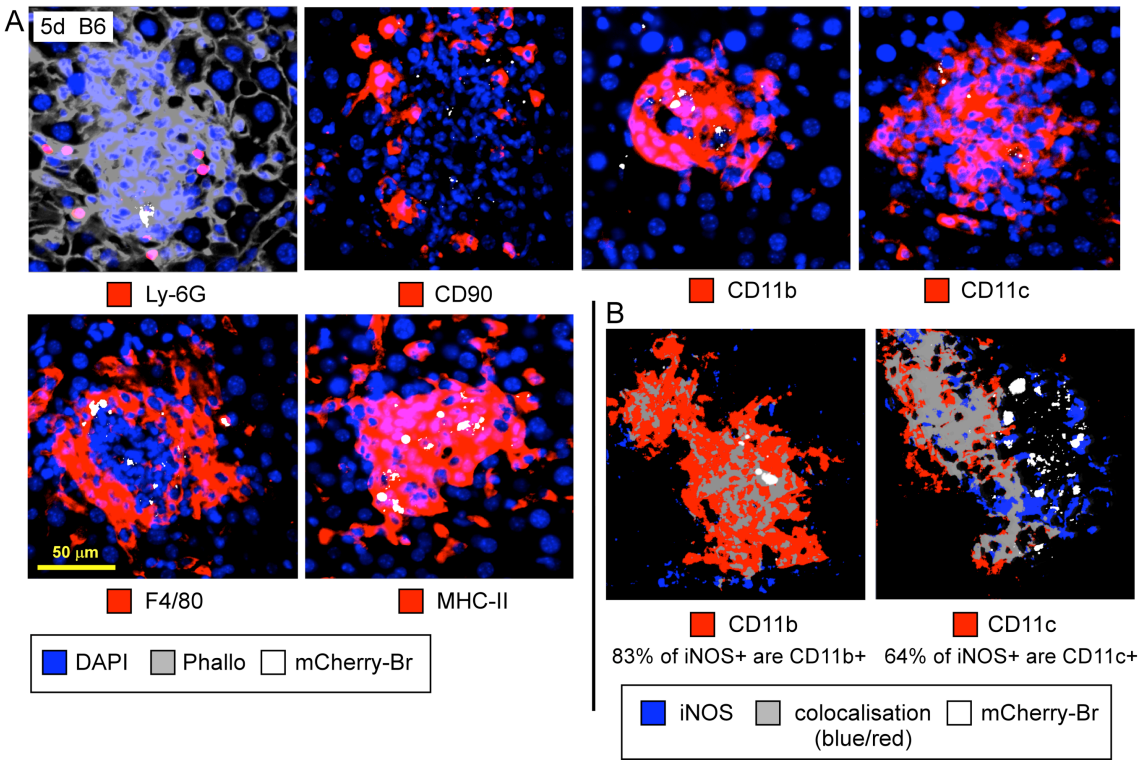

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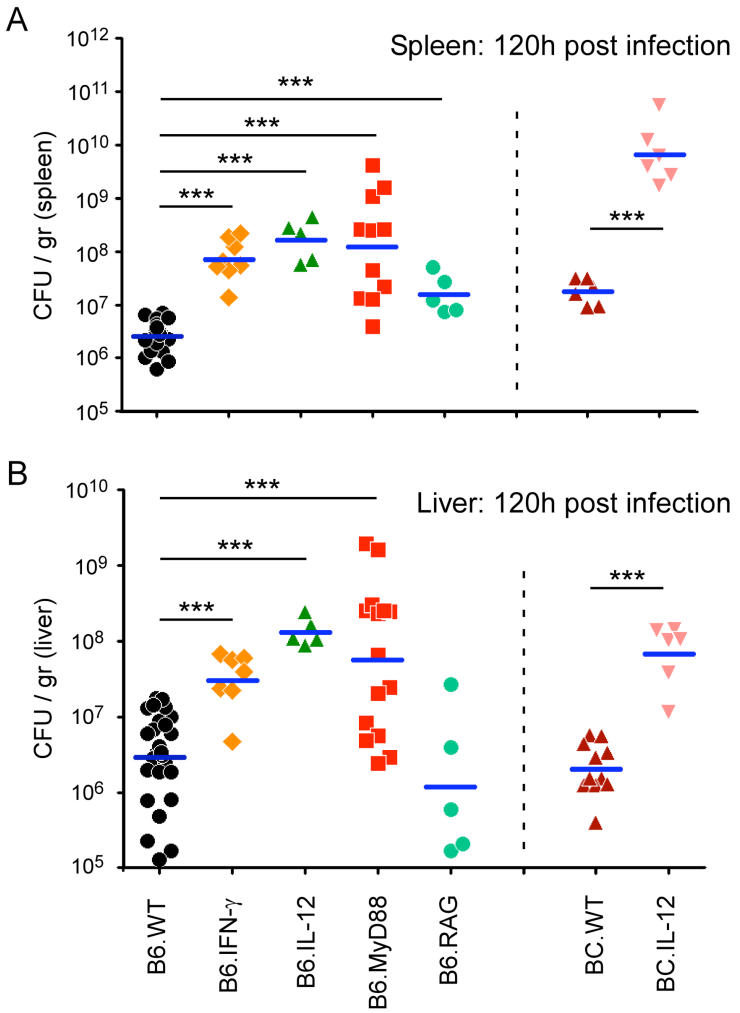

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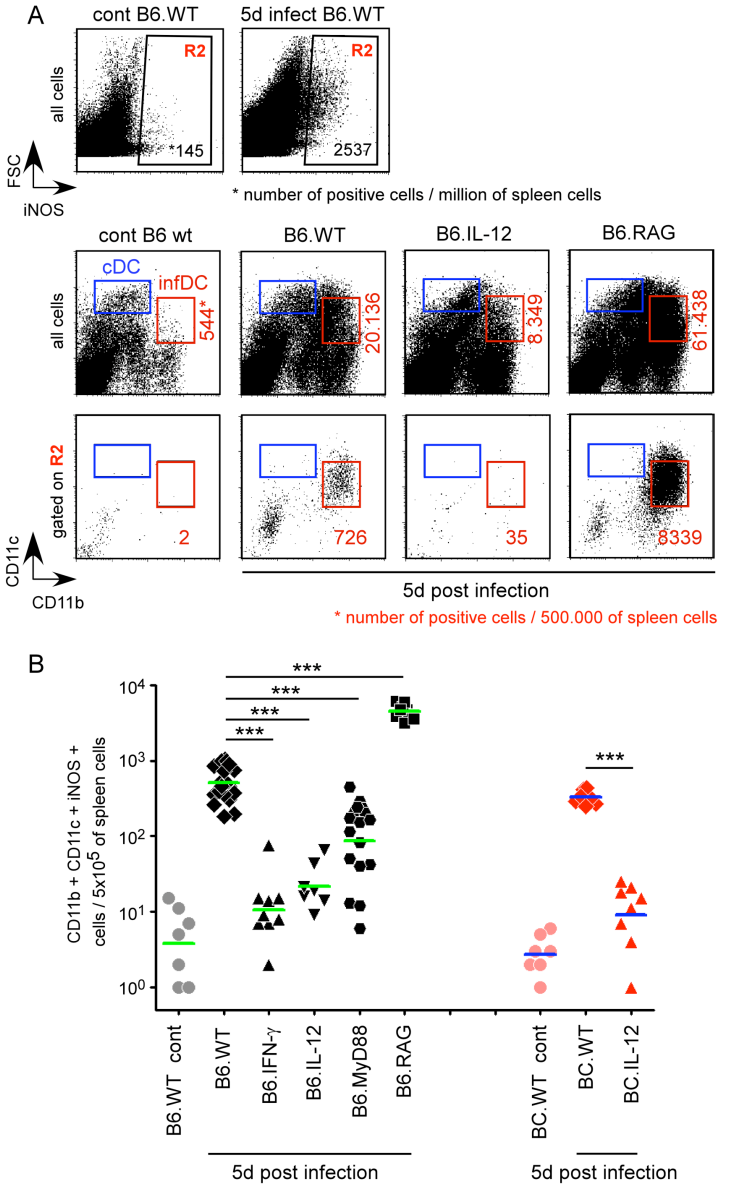

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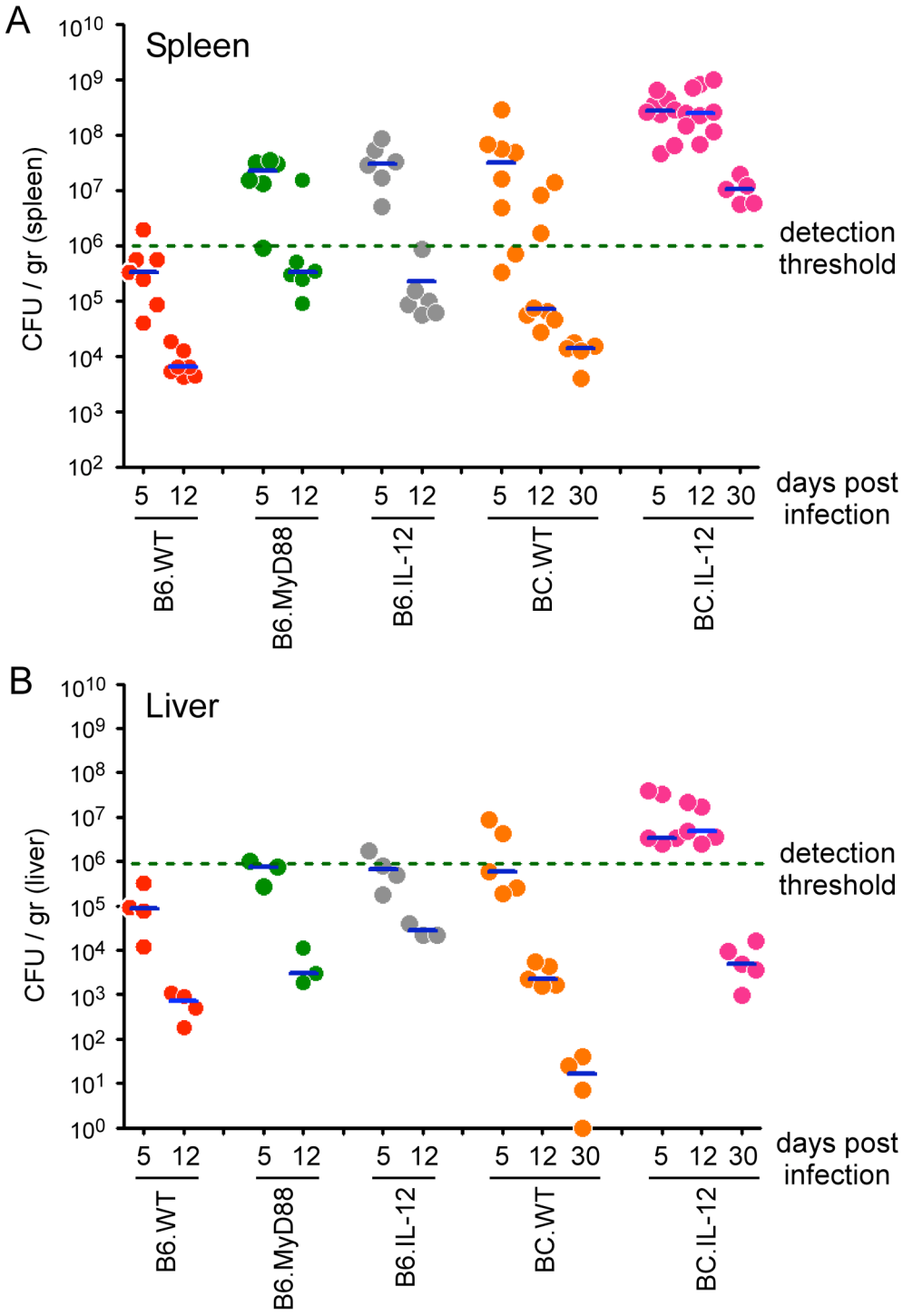

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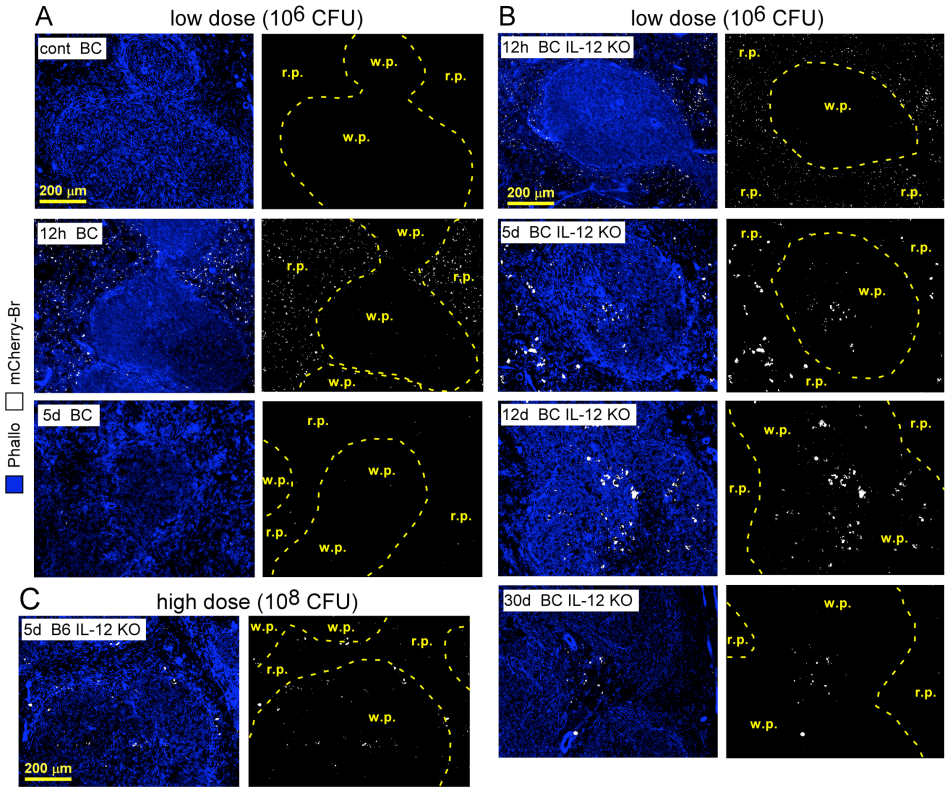

Copin *et al*, 2012 - **FIGURE S14**

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