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FACULTE DES SCIENCES

UNITE DE RECHERCHE EN BIOLOGIE MOLECULAIRE
URBM

Characterization of the relationship between *Brucella melitensis* and the innate immune response of the mouse

Caractérisation de la relation entre *Brucella melitensis* et le système immunitaire inné de la souris

Richard Copin

Dissertation présentée en vue de l'obtention du grade de Docteur en Sciences

Jury	Prof. Camille Locht *Institut Pasteur de Lille, France*
	Dr. Stefan Magez *Vrije Universiteit Brussel, Belgium*
	Prof. Ignacio Moriyón *Universidad de Navarra,Spain*
Co-Promoteur	Dr. Eric Muraille *Université libre de Bruxelles, Belgium*
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Rue de Bruxelles, 61
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La thèse… Il y a maintenant de cela 6 ans… Qui donc a commencé cette thèse et pourquoi l'a-t-il fait? Une chose est certaine : ce n'est pas le même homme qui la termine.

Tout a commencé par des rencontres. En fait, tout a commencé par une rencontre.

Cher Jean-Jacques, mes premiers remerciements vous sont adressés. Que puis-je ici écrire que je ne vous aie pas encore dit ou que vous ne sachiez pas déjà. Je pense être juste en disant que je vous dois mon passage à l'âge des Hommes. Je ne parle pas ici de l'âge adulte mais bien de celui de l'humanité. Beaucoup de choses m'étaient cachées lors de notre première rencontre et ce, par ma propre vision, ma simple perspective. Mais aujourd'hui, mon esprit est ouvert et j'ai la force de voir le monde et de m'y confronter. Vous êtes un homme de valeur et de cœur. Vous m'avez donné quelque chose que je ne pourrais jamais vous rendre. Dès lors, je ne peux vous proposer que mon amitié. Merci Jean-Jacques, sur ce que j'ai de plus cher, merci.

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"La virulence d'une bactérie est son immunité vis-à-vis de l'hôte, l'immunité de l'organisme est sa virulence pour la bactérie"

Jules Bordet.

Characterization of the relationship between *Brucella melitensis* and the innate immune response of the mouse.

By Richard Copin

Brucella melitensis is a facultative intracellular pathogen responsible for brucellosis, one of the world's most widespread zoonotic diseases causing abortion in domestic animals and a chronic granulomatous infection in humans. A key pathogenic trait of this organism is its ability to survive in immune cells such as macrophages and persist in tissues of the reticuloendothelial system, including the spleen and liver. The crucial component of immunity that results in survival of the host and thus maintenance of the chronic infective state is interferon-γ (IFN-γ), the cardinal mediator of the T helper 1 (T_H1) immune response. Production of IFN-γ results from the ability of Toll-like receptors to detect *Brucella* components and induce production of Interleukin- 12 (IL-12). One of the major roles of IFN-γ is the stimulation of *Brucella*-killing processes, such as the production of reactive nitrogen intermediates by inducible Nitric Oxide Synthase (iNOS/NOS2) in phagocytic cells. Despite progresses in experimental animal models of brucellosis, much remains unknown regarding the characterization of the effector mechanisms induced by *B. melitensis* infection. In particular, the nature and cell surface phenotype of innate immune effectors implicated in *Brucella* control are largely undetermined. By using a combined histologic and flow-cytometric approach, we thus decided to study the relationship between *B. melitensis* and the immune system of the mouse by focusing on the description of the nature and function of the immune effector mechanisms developed by the host.

In the current study, we report the identification of the iNOS-producing dendritic cells (inflammatory DC) as the main iNOS-producing cells recruited and activated in the spleen and peritoneal cavity of the mouse during the early stages of *B. melitensis* infection. We identify the crucial role of TLR4/9 and MyD88 dependent signaling cascade in the activation of these cells. By using immunohistofluorescence techniques, we demonstrate the importance of inflammatory DC in the development of granulomas structures in the spleen and liver of infected mice and we delineate the negative impact of MyD88 deficiency on the establishment of these structures. Additionally, we describe the phenotype of *B. melitensis* infected cells in spleen and liver during the five first days of infection. Finally, we show that *B. melitensis* induces the migration and maturation of DCs in the spleen, in a dose dependant manner.

In conclusion, this work dissects for the first time the nature of the effectors mechanisms developed by the immune system after *B. melitensis* systemic inoculation and describes the phenotypical characteristics of the infected cells during the initial steps of the infectious process.

ABBREVIATION

ABC	ATP-binding cassette
ActA	Actin assembly inducing protein A
Ag	Antigen
AIDS	Acquired immune deficiency syndrome
APC	Antigen-presenting cell
ATP	Adenosine tri-phosphate
BCR	B cell receptors
BCV	*Brucella*-containing vacuole
BPI	Bactericidal permeability increasing protein
***Bru*LPS**	*Brucella*-LPS
cAMP	Cyclic adenosine monophosphate
CCL2	CC-chemokine ligand 2
CCR	Chemokine receptor
cDC	Classical dendritic cell
CDP	Common DC precursor
CLR	C-type lectin
CpG	Cytosine-phosphate-guanosine
CR3	Complement receptor 3
CREB	cAMP response-element-binding protein
CTL	Cytolytic T lympocyte
DAI	DNA-dependant activator of interferon-regulatory factors
DAMP	Damage-associated molecular patterns
DC	Dendritic cell
DC-SIGN	DC-specific ICAM3-grabbing non-integrin
E-cadherin	Epithelial cadherin
ECM	Extracellular matrix
ER	Endoplasmic reticulum
ERES	Endoplasmic reticulum exit sites
ESTA-6	6-kd early-secreted antigenic target
FasL/R	Fas ligand/receptor
FcγR	Fcγ receptors
FLT3	FMS-related tyrosine kinase3
G-CSF	Granulocyte colony-stimulating factor

GM-CSF	Granulocyte–macrophage colony-stimulating factor
HIV	Human immunodeficiency virus
HSC	Haemotopoietic stem cells
iDC	Inflammatory dendritic cell
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
InlA	Internalin A
iNOS	Inducible nitric oxyde synthase
IRAK	IL-1 receptor-associated kinases
IRF	Interferon-regulatory factor
JNK	JUN N-terminal kinase
LAM	Lipoarabinomanan
LAMP	Lysosome–associated membrane proteins
LBP	Lipopolysaccharide-binding protein
LC	Langerhans cells
LLO	Listeriolysin O
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
MØ	Macrophages
M1MØ	Classically activated macrophages
M2MØs	Alternatively activated macrophage
MAL/TIRAP	Myd88-adaptor-like/TIR-associated protein
MALT	Mucosa-associated lymphoid tissue
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemoattractant proteins
M-CSF	Macrophage colony-stimulating factor
MDA5	Melanoma differentiation-associated gene 5
MDP	Macrophage / dendritic cell progenitor
MDSC	Myeloid-derived suppressor cells
MHC	Major histocompatibility complex
MMØ	Metallophilic macrophages
MMP	Matrix metalloproteinases
MMR	Macrophage mannose receptor
Myd88	Myeloid differentiation factor 88
MZ	Marginal zone
MZMØ	Phagocytic marginal zone macrophages
NET	Neutrophils extracellular traps
NK	Natural killer
NLR	Nod-like receptors

Nod	Nucleotide-binding oligomerization domain
NRAMP1	Natural resistance associated macrophage protein 1
OMP	Outer membrane protein
PAMP	Pathogen-associated molecular patterns
pDC	Plasmacytoid dentritic cells
phox	Phagocyte oxydase
PI_3P	Phosphatidylinositol-3-phosphate
PMN	Polymorphonuclear neutrophils
pre-DC	Precursors- dendritic cell
PRR	Pattern recognition receptors
RANKL	Receptor activator of nuclear factor κB ligand
RIG-I	Retinoic acid-inducible gene I
RLHs	RIG-I -like helicases
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SARM	Sterile alpha motif and armadillo motif domain-containing protein
SCID	Severe combined immunodeficient
SNARE	Soluble NSF-attachment protein receptor
SP41	41 kDa surface protein
SR	Scavenger receptor
T4SS	Type IV secretion system
TCR	T cell receptors
TGF	Transforming growth factor
T_H	T helper
TipDC	TNF-α and iNOS producing dendritic cell
TIR	Toll/Interleukin-1 receptor
TLR	Toll like receptors
TNF	Tumor necrosis factor
TRAF	Tumor receptor-associated factor
TRAM/TICAM-2	Toll-receptor-associated molecule also called TIR-domain-containing adaptor molecule 2
T_{reg}	T regulator
TRIF/TICAM-1	TIR domain-containing adaptor inducing IFN-β also called TIR-domain-containing adaptor molecule 1

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INTRODUCTION

1. Foreword: Host-Pathogen Relationship

In the middle of the 19th century, Louis Pasteur and Robert Koch reported the results of experiments that definitively demonstrate that our environment is inhabited by invisible organisms, some of which are the cause of animal and plant diseases. This was the beginning of one of the most important biological revolution i.e., we live in a symbiotic world made of organisms with which we continuously interact in a multitude of ways.

The life history of all organisms is defined by host-microbe interactions. Since immemorial time, this mutual existence determines the path followed by each other's evolution. In a constant dynamic environment, each partner dedicates its energy to promote its own selfish needs in order to perpetuate itself. In many cases, the relationship leads to a beneficial or synergistic existence for each partner, e.g. the human gut microbiome. However, sometimes, the interplay between a given viral, bacterial or parasitic organism with their respective host turns in conflict. In this last situation, the microorganism, termed pathogen, develops to the detriment of its host, which accordingly reacts by setting up mechanisms to control the harmful invasion. The nature of this confrontation is the main subject of bacterial pathogenesis.

In literature, bacterial pathogenesis is often referred to the chain of events leading to the development of a disease initially caused by a bacterial organism. However, this definition forgets that some infections result in a "stalemate" between the pathogen and its host, conducting to a chronic or a latent infection without manifestation of clinical symptoms. Hence, immunologists prefer to define bacterial pathogenesis like the discipline that translates the complexity of the language between a bacterial pathogen and the immune system of its host.

The central question of bacterial pathogenesis is to understand what determines the outcome of an infection. In other words, how the adaptation of a pathogen facing the robustness of the immune system alters the course of the immune response and what are the countermeasures employed by the immune system to subvert this pathogen "plasticity". A cardinal feature of host-pathogen relationship is, consequently, the adaptability of both partners. Hosts have developed effective immune mechanisms to control the continuous microbial threat of the environment and reciprocally, successful pathogens have worked out elaborated strategies to overcome immune responses generated by the host.

A host-pathogen relationship is thus a complex molecular conversation between two partners, which can result in acute disease, chronic infection, or to the death of either the pathogen or its host. The comprehension of the language used in this specific dialogue is a priority for developing new avenues for therapeutics. This being so, new investigations are required to improve our acquaintance of the nature of this confrontation.

This thesis intends to contribute to a better understanding of such interaction by focusing on the relationship between an intracellular bacterial pathogen of animals, *Brucella melitensis* and the immune response developed by the mouse during the early steps of the infection.

The introduction will first describe the key features of the immune system and will mostly emphasize the central position held by innate immune cells in the control of the immune response. Then, we will focus on virulent strategies used by successful intracellular bacteria to evade host immune defenses. And finally, we will introduce *Brucella melitensis* and describe the current knowledge about its relationship with the mice immune system.

2. The Immune System

The definition of the immune system is complex and in constant evolution. Firstly considered as fighting infection, it became obvious that this homeostatic system is involved in many other physiological programs such as body maintenance, embryogenesis, and aging processes. Recently, the discovery of a common chemical language between the nervous system and immune system, led some authors to assimilate the immune system to the sixth sense of the body. The present introduction will give to readers some clues to understand the role of the immune system in an infectious context. Readers are refereed to the following reviews for a more exhaustive description of the many functions of the immune system [Blalock, 2005] [Zhou *et al.*, 1995] [Tepass *et al.*, 1994].

2.1. The immune system and the pathogen: "The never ending story"

Since the first appearance of microorganisms on earth, 3.5 billion of years ago, immune mechanisms can be found at almost every level of the evolutionary tree of life. This highlights their importance in species survival that probably began during the earliest encounter between a primitive bacterium and a virus [Barrangou *et al.*, 2007; Brouns *et al.*, 2008].

In mammalians, the immune system comprises an intricate network of cellular and soluble components that contribute to eliminate microbial infections through different sequential stages of interactions. Originating from hematopoietic precursors, immune cells are found in body fluids as well as in many tissues and structure lymphoid organs implicated in the maturation of leukocytes and in the fine-tuning of the immune response. Functions and localization of lymphoid organs are described in **Table1**. Intrinsic to its role of defender, the immune system is able to recognize molecular structures demarcating infectious non-self, as well as normal self and abnormal self [Karre *et al.*, 1986; Janeway, 1989; Matzinger, 2002].

The immune system can be divided into two main interrelated subsystems: the immediate innate immune system (section 2.2) and the temporally delayed, adaptive immune system (section 2.3).

Table 1| Human Lymphoid Organs

Lymphoid Organs		Localization	Function
Primary	Bone Marrow	Soft tissue found in bone cavity	Site of hematopoiesis implicated in the maturation process of leukocytes, particularly B-lymphocytes
	Thymus	Two lobes that join in front of the trachea behind the breast bone	Site of maturation of T-lymphocytes
Secondary	Spleen	A fist-sized organ located in the abdominal cavity	Immunological filter of the blood. Involved in the shaping of the immune response. Site of senescent-red blood cells disposal
	Lymph Nodes	Small organ shaped like beans, which are located throughout the body and connected via lymphatic vessels	Immunological filter of the lymph. Implicated in the organization of the immune response
	Tonsils	Two oval masses in the back of the throat	Mostly composed of lymphoid tissue, tonsils and adenoids are thought to survey the upper mucosa of the body though their presence is not essential to maintain the healthy status of the organism
	Adenoids	Tissue island located at the back of the nasal passage	
	MALT*	Loosely organized lymphoid follicles found in various sites of the body such as the gastrointestinal tract, breast, lung, salivary glands, and eye	MALT and appendix are populated by diverse leukocytes, which are well situated to sample antigens passing through the mucosal epithelium
	Appendix	A small tube that is connected to the large intestine	

*MALT: Mucosa-Associated Lymphoid Tissue

2.2. Innate immune system

Until a decade ago, the innate immune system was solely viewed as a first barrier of defense that rapidly reacts with more or less success to the invading pathogen. This system is now appreciated for its complexity, its importance as first line of defense and its role in triggering and tuning adaptive immunity.

The term innate immunity encompasses the elements of the immune system, which provide non-specific and immediate host defense. These include the physical, chemical and microbiological barriers, and a broad range of cellular and humoral immune components (neutrophils, dendritic cells, complement cascade etc…). All these innate immune actors are unified by a common theme i.e., the pattern recognition receptors (PRRs). PRRs are germ line-encoded innate immune receptors that detect relatively invariant molecular pattern found in most microorganism of a given class (see chapter 4) [Janeway, 1989; Hoffmann *et al.*, 1999]. In addition, PRRs are also able to recognize damaged self-components released by non-physiological cell death [Kono and Rock, 2008]. Innate immunity is thus evolutionary programmed to detect invariant features of invading microbes or abnormal endogenous structures.

This system is constituted of functionally distinct but highly connected "modules" that provide different forms of protection against pathogens (phagocytosis, acute-phase response, granulomas formation, etc…). Under baseline conditions, these defense mechanisms are sufficient to prevent invasion by common environmental microbes. Importantly, the origin of innate immunity as a system pre-dates the separation of the plants and animals lineage[1] [Gura, 2001] [Ganz, 2003].

[1] One form of host defense found in plants and animals is the production of antimicrobial peptides. These are small proteins such as defensins that act by disrupting the cell membrane of bacteria and fungi and also the membrane envelope of viruses, although some of these peptides have been described to be able to cross microbial cell membranes and enter the cell.

2.3. Adaptive immune system

The adaptive immune system corresponds to an additional layer of complexity in immune defense, which emerged during jawless vertebrates evolution 500 million of years ago [Guo *et al.*, 2009]. Its response consists of antigen-specific reactions through T lymphocytes and B lymphocytes. It employs, clonally expressed, highly specific receptors (T cell receptors (TCRs), and B cell receptors (BCRs) or Immunoglobulins (Igs)), which are not encoded in the germ line but generated *de novo* in each maturing lymphocytes via somatic recombination. Genes encoding adaptive receptors are generated from the assembling of variable and constant fragments yielding a huge repertoire of realigned genes [Schatz *et al.*, 1992]. Additional mechanisms such as gene conversion and somatic hypermutation (for B cells) ultimately generate an "unlimited" range of receptors with the potential to recognize almost any antigenic structure [Schatz *et al.*, 1992].

Whereas the innate immune response is immediate and only discriminates classes of pathogens, the adaptive response is highly specific to invading intruders but takes several days to develop. Moreover, the adaptive response is endowed with the remarkable ability to remember, so that an eventual subsequent exposure to the pathogen leads to a more efficient and rapid response.

Until recently, these classical immune features conducted immunologists to consider immune system as two separate subsystems, the response of which succeed one another over the course of an encounter with pathogen.

This division of the immune system into two separate boxes is nevertheless over simplistic since in practice, the innate and the adaptive responses are so intricate that they cannot be dissociated. Immune response is now viewed as a global response integrating elements of both systems that work together from the first hours of the immune response, till its resolution.

2.4. Immunity: an integrated response

The dynamic interplay between both systems is best illustrated during the course of an infectious process |**Fig.1**. The invasion of the body by a microbe engages innate PRRs, which recognize the nature of the infectious agent and activate transduction cascades within the innate immune cell. This physical interaction allows the innate immunity to assess the degree of threat and decide whether to mount a response to that specific microbe or not [Medzhitov and Janeway, 2002]. If a response is launched, the innate immune system instructs the adaptive immune system about how to generate the appropriate response against the intruder [Iwasaki and Medzhitov]. This process is regulated by a complex set of cytokines[2] and an equally complex combination of molecules expressed on the surface of immune cells among which professional antigen-presenting cells (APCs) hold a central position. Antigens presented on the surface of APCs, activate adaptive cells (T cells), which undergo further maturation (see next section). In turns, these adaptive cells reinforce the optimal innate mechanisms implicated in the elimination of a targeted invader and induce an immunological memory through which the antigenic encounter can be remembered even for a lifetime [Lanzavecchia and Sallusto, 2000].

This intricate relationship of both elements of the immune system during an infectious process illustrates the fallacy of visualizing immunity as two separate systems |**Fig.1**.

[2] The term cytokine (Greek cyto-, cell; and -kinos, movement) encompasses a large and diverse family of polypeptide regulators (proteins, peptides, or glycoproteins) that are principally involved in cellular communication. Produced and secreted widely throughout the body by cells of diverse embryological origin, they have been classed as lymphokines, interleukins, and chemokines, based on their presumed function, cellular origin, or target of action.

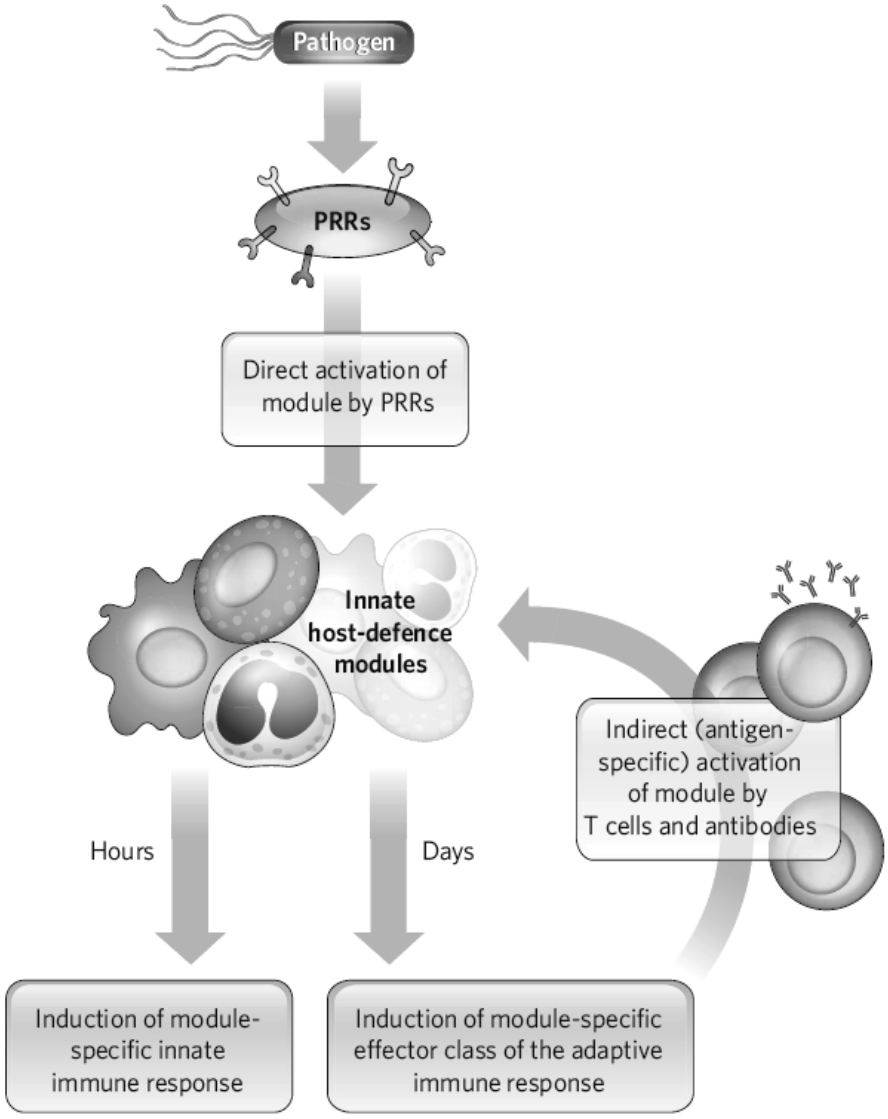


Figure 1| Intricate relationship between the elements of the immune system. Induction of the immune response begins by the engagement of innate immune cells, which decode the features of the pathogen and subsequently trigger host-defense modules to directly eradicate the microbial threat. In the meantime, the adaptive immune system is informed to mount a specific response involving a specific innate immune effector class that is often essential for pathogen elimination. PRRs, pattern recognition receptors. **[Medzhitov, 2007].**

2.5. Immunity: the hard choice of the right response

Macroorganisms are the targets of different classes of microorganisms (viruses, bacteria, protozoa, etc…), which have fundamentally different behaviors and virulence mechanisms. The challenge of the immune system is not to develop a response but to choose the right one that protects against a given infectious agent. Several types of response exist and not all of them are equally effective. Moreover, these responses can be detrimental by themselves by damaging the host tissues. The generation of the adapted class of immune response can thus be a matter of life and death.

T helper (T_H) lymphocytes express the co-receptor CD4 on the cell surface and occupy a central position in the establishment of all adaptive immune responses. These cells contribute to immune response because they help B cells to produce antibodies, promote the development of $CD8^+$ cytolytic T lympocytes (CTLs), activate innate immune cells, and counteract excessive immune responses. To ensure such pleiotropic roles, T_H cells undergo a further differentiation into several decisive types of effector cells: T_H1, T_H2, T_H17 and T regulators (T_{reg}) cells [Reinhardt *et al.*, 2006] |**Fig.2**. These different cells are characterized by the production of distinct array of cytokines [Seder and Paul, 1994; Glimcher and Murphy, 2000]. Interferon (IFN)-γ is the hallmark of T_H1 cells. This cytokine is mainly involved in the control of intracellular pathogen such as viruses and intracellular bacteria, by improving innate immune effector mechanisms. In contrast, parasites like helminthes induce the development of T_H2 cells producing cytokines such as interleukin (IL) -4, IL-5, and IL-10 that direct Ig-E and eosinophil mediated destruction of the pathogen [Mosmann and Coffman, 1989]. T_H17 cells produce IL-17, which activate non-hematopoietic cell types, including epithelial cells, that subsequently produce chemokines able to recruit neutrophils to the site of infection [Weaver *et al.*, 2007]. The T_H17 lineage is involved in protection against fungi and extracellular bacteria [LeibundGut-Landmann *et al.*, 2007]. Finally, T_{reg} cells control and counteract excessive immune responses by producing immune mediators such as IL-10 and transforming growth factor (TGF)-β [Belkaid *et al.*, 2002].

As quoted earlier, adaptive immune recognition is mediated by highly specific-antigen receptors. Because the specificity of each receptor is not directly linked to the nature of the antigen, T_H cells need supplementary instructions to mature into the optimal effector subset. Such instructions are provided by APCs through the surface expression of molecules and the cytokine milieu that they produced. These APCs can sense infection and translate the relevant features of pathogens into appropriate set of signals determining the choice of the effector response [Janeway, 1989]. The differentiation of adaptive cells into a specific effector-cell type leading to the elaboration of an effective immune response will thus only rely on the right information being given by the innate immune cells |**Fig.2**.

Innate immune cells are, consequently, at the crossroads of host-pathogen interaction.

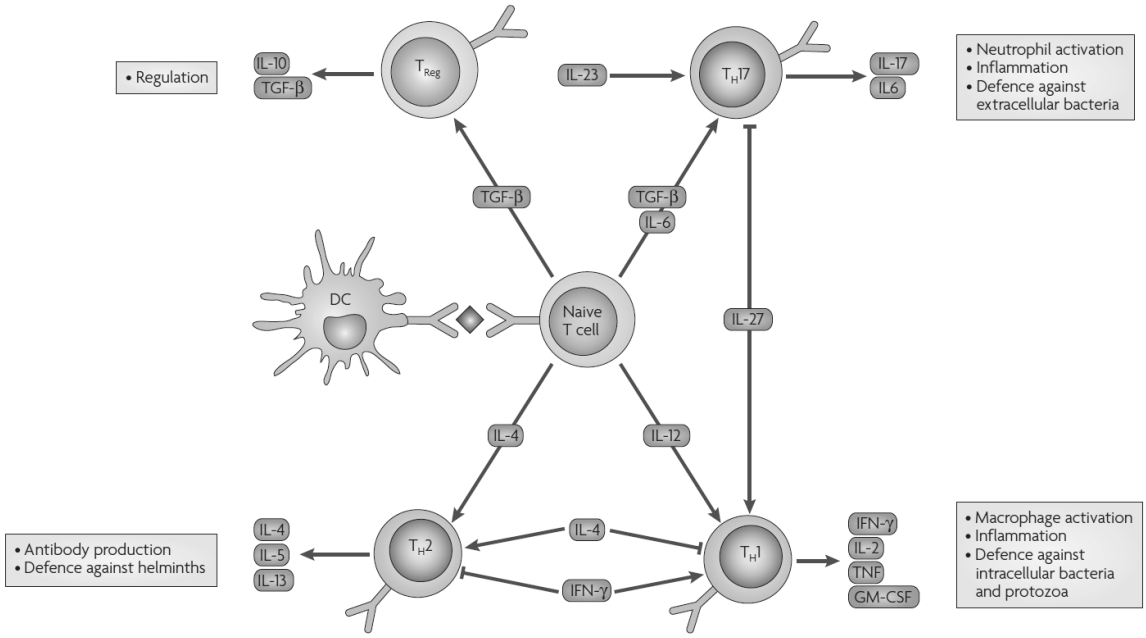


Figure |2 CD4$^+$ T_H-cell lineages. According to the information received from innate immunity, T_H cells differentiate into one of several effector-cell lineages (T_H1, T_H2, T_H17 and T_{Reg}). The subsequent mature T_H-cell produces specific cytokines, which induce particular innate immune mechanisms implicated in the fighting of specific classes of pathogens. IL, interleukin; IFN, interferon; DC, dendritic cell; GM-CSF, granulocyte–macrophage colony-stimulating factor; TNF, tumor necrosis factor; TGF, transforming growth factor. [Kaufmann, 2007]

Long considered as only implicated in the direct destruction of pathogens, innate immune cells are now viewed as the "immunological sensor" of the immune system, which alerts for potential dangerous situations, by decoding and integrating such signals. Essential in the shaping of the immune response, these pleiotropic competences are the hallmark of all innate immune cells though each subset can be specialized in a specific performance.

The next section describes the main features of the diverse innate immune cell subsets and highlights their potential roles during an infectious process.

3. Innate Immune Cells

Once across the passive barriers of the body, potential pathogens firstly encounter tissular macrophages (M∅) and blood neutrophils, which constitute the primary defense against bacterial invasion. In a second time, circulating monocytes as well as natural killer cells can be recruited and constitute essential players triggering specific antimicrobial modules. Dendritic cells [Cole *et al.*] are the main cells involved in the communication between innate and adaptive immune systems. Eosinophils, as well as basophils and mast cells form the principal weapons against multicellular parasites, such as helminthes.

3.1. Neutrophils

Neutrophils are the most abundant cells of the innate immunity implicated in the very early stages of infection. They represent about two-thirds of the peripheral blood leukocytes. Together with eosinophils, basophils, and mast cells, they constitute the class of granulocytes, so called because of the presence of densely stained granules within their cytoplasm. Of note, they are also named polymorphonuclear neutrophils (PMN) because of the typical shape of their nucleus.

Neutrophils are professional phagocytic cells able to recognize, ingest and destroy many pathogens without the aid of an adaptive response. They are short-lived cells with a half-life of just 6-10h, abundant in the blood but barely present in normal healthy tissue [Bainton *et al.*, 1971]. The development of neutrophils in the bone marrow depends on the production of the granulocyte colony-stimulating factor (G-CSF), which mobilizes and increases the proliferation of the granulocyte progenitor cells. In turn, this cytokine is regulated by IL-17 and IL-23, which are produced by γδ and αβ lymphocyte cell subsets and macrophages respectively [Stark *et al.*, 2005]. Mature neutrophils are mainly identified by the membrane expression of CD11b and Ly-6G markers.

3.1.1. Neutrophils: a born-killer

During an infectious process, the injured tissue is rapidly colonized by the recruitment of a large number of neutrophils, which crawl along the boundaries between endothelial cells, penetrate the tissue and move up a chemotactic gradient in an attempt to engulf and destroy invading microbes. For an elegant recent review on neutrophils, readers are referred to [Nathan, 2006]

Neutrophils may kill microorganisms through two distinct mechanisms: the respiratory burst and the degranulation response |**Fig.3**.

The respiratory burst is an abrupt, non-mitochondrial reduction of oxygen responsible for the production of highly reactive molecules known as reactive oxygen species (ROSs). These products include superoxyde ($O_2^{\cdot-}$), singlet oxygen ($^1O_2$), ozone (O_3), hydrogen peroxide (H_2O_2), and other reactive components. They are mainly produced by the phagocyte oxydase (phox) which is the principal ROS-producing enzyme expressed by neutrophils.

ROSs are essential for bacterial killing and are thought to function by oxidizing proteins, nucleic acids, and other molecules [Hampton *et al.*, 1998]. Among many other roles, ROSs can also contribute to the release of bactericidal neutrophil proteases from the granules matrix of neutrophils [Reeves *et al.*, 2002].

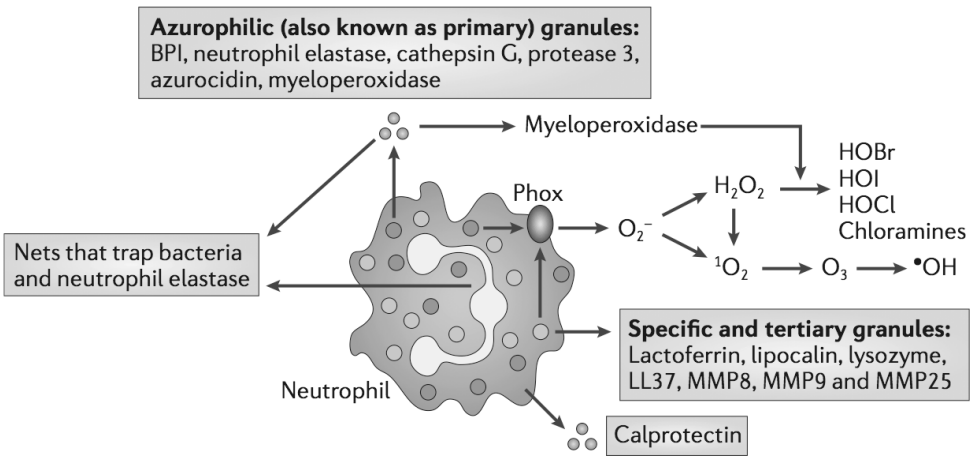


Figure 3| Bactericidal components of Neutrophils. Most of the neutrophil compartments are equipped with antimicrobial proprieties. It comprises azurophilic granules (also known as primary granules), specific granules (also known as secondary granules), tertiary granules, plasma membranes, the nucleus and the cytosol. BPI, bactericidal permeability increasing protein; H_2O_2, hydrogen peroxide; HOBr, hypobromous acid; HOCl, hypochlorous acid; HOI, hypoiodous acid; MMP, matrix metalloproteinase; $^1O_2$, singlet oxygen; O_2^-, superoxide; O_3, ozone; $^{\cdot}OH$, hydroxyl radical; phox, phagocyte oxidase [Nathan, 2006].

The degranulation response is the capacity of neutrophils to progressively discharge three distinct sets of granules, activate a killing cascade at the plasma membrane, extrude their nuclear proteins and chromatin and finally pour out their cytosol [Nathan, 2006], all of these actions having an antimicrobial effect. For instance, the granules content is composed of a broad range of bactericidal proteins such as myeloperoxydase, which converts H_2O_2 into much more powerful antiseptics (hypohalid acids) [Klebanoff, 2005] and α-defensins, which are small broad spectrum bactericidal polypeptides [Selsted and Ouellette, 2005]. The nuclei of stimulated neutrophils contribute to host defense by extruding their chromatin, which forms extracellular "NETs" (neutrophils extracellular traps) decorated with bactericidal proteins that capture and kill microorganisms [Brinkmann and Zychlinsky, 2007].

3.1.2. Neutrophil: an auxiliary nurse

Neutrophils are also implicated in the repair of damaged tissue [Lekstrom-Himes and Gallin, 2000]. The principal contribution of neutrophils to wound healing is microbial sterilization. However, the role of neutrophils is larger in this domain since they are also involved in the formation of "pus", that is, the active liquefaction of tissue [Weiss, 1989]. This mechanism serves to disassemble the extracellular matrix that impedes neutrophil-bacterial contact and puts pressure on local tissue. This pressure is thought to collapse surrounding vessels, thereby cutting off bacterial escape routes and trapping the microbes into a toxic mixture. Moreover, neutrophils have also been implicated in the regulation of the program that switches the state of the injured tissue from pro-inflammatory to anti-inflammatory. The ingestion of apoptotic neutrophils by tissue macrophages (M∅s), induces a decrease of the IL-23 production by these cells, which indirectly inhibits the proliferation of neutrophils in the bone marrow and consequently downregulates the inflammatory response [Stark *et al.*, 2005]. |**Fig.4**

3.1.3. Neutrophil: an instructor

In addition to their role in clearance of intruders, neutrophils can also instruct and shape immune responses. For example, Wittamer and collaborators have shown that neutrophils were able to attract dendritic cells (DCs) such as plasmacytoid dendritic cells (pDC) to the site of infection through the activation of the precursor of chemerin, a DC-attracting chemokine [Wittamer *et al.*, 2005]. |**Fig.4** It has also been found that neutrophils can function as a powerful suppressor of T cell activation. In patients with advanced cancer, neutrophils can indeed impair T-cell receptor (TCR) ζ-chain expression and cytokine production [Schmielau and Finn, 2001]. Moreover, it has been suggested that when activated by a cytokine combination made of granulocyte and M∅ colony stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), neutrophils might play the role of APCs [Yamashiro *et al.*, 2001].

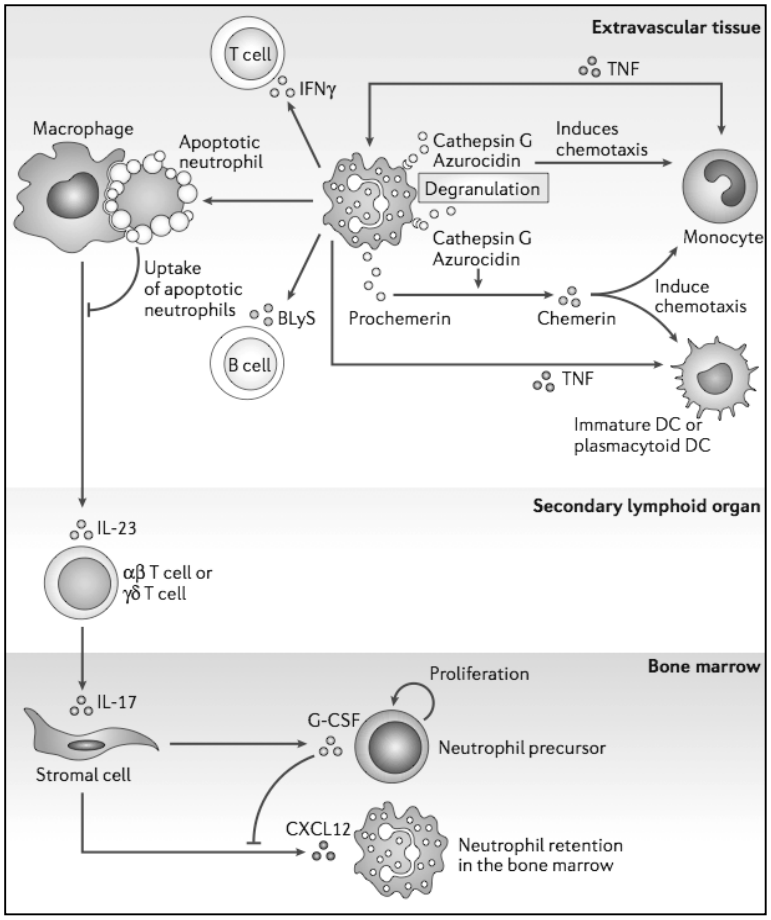


Figure 4| Neutrophils are major players of the immune response. During an infectious process, neutrophils recruit and activate immune cells and concurrently respond to their products. In addition, neutrophils control the inflammatory response by modulating their own development and proliferation. Granulocyte colony-stimulating factor (G-CSF) that triggers the release of neutrophils from the bone marrow, is regulated by Interleukine-17 (IL-17), itself governed by IL-23. Ingestion of apoptotic neutrophils provokes the suppression of the IL-23 production by extravascular macrophages. BLyS, tumor-necrosis factor-related ligand B-lymphocytes; CXCL12, CXC-chemokine ligand 12; IFN-γ, interferon-γ, TNF, tumor-necrosis factor. [Nathan, 2006]

3.2. Tissular macrophages (M∅)

M∅s are resident phagocytic cells in lymphoid and non-lymphoid tissues, which constitute a very heterogeneous population implicated in many physiological processes. They regulate a range of functions, including homeostasis, via the clearance of apoptotic cells, growth, through the production of growth factors, and survival by the destruction of invading microbes. Their ability to recognize a wide array of endogenous and exogenous patterns places these cells in a central position of the immune system [Taylor *et al.*, 2005].

3.2.1. Macrophages: a large family

Tissue M∅s originate from circulating bone marrow-derived precursor [Gordon and Taylor, 2005]. Studies of the expression of differentiation antigens have revealed that tissue M∅s form a phenotypically and functionally heterogeneous population |**Fig.5**. These cells are present in almost all tissues of the organism including lungs (alveolar M∅s), liver (Kupffer cells), nervous system (microglia), epidermis, and reproductive tract. They are also present within the *lamina propria* of gut and the interstitium of organs such as the heart, pancreas and kidney [Taylor *et al.*, 2005].

Various types of M∅s also populate lymphoid organs. The spleen, for example, is rich in subpopulations of M∅s that differ in receptor expression, microanatomical location, life history, and function |**Fig.5 (f)**. The so-called splenic red pulp mostly contains stromal type M∅s. These cells express high level of F4/80[3] antigen and are extensively involved in the disposal of senescent erythrocytes. The marginal zone[4] [Yang *et al.*] marks the boundary between the red pulp and the white pulp and represents the interface between splenic lymphoid tissue and the circulation. The MZ is constituted by a marginal sinus surrounded by two M∅s subtypes [Kraal, 1992]:

[3] Resident tissue M∅s, encompassing Kupffer cells of the liver and red pulp M∅s of the spleen, characteristically express the F4/80 molecule, a cell surface glycoprotein related to the seven transmembrane-spanning family of hormone receptors [Caminschi *et al.*, 2001]. To date, the function of F4/80 has not been determined, although speculation has suggested its involvement in M∅s adhesion events, a role in cell migration or as a G protein-coupled signaling component of M∅s [McKnight and Gordon, 1996; Stacey *et al.*, 2000].

[4] The marginal zone [Yang *et al.*] consists of open sinuses, sinus-lining reticular cells, MZ B cells, DCs, MM∅s, and MZ M∅s.

- Metallophilic MØs (MMØs) expressing high level of sialoadhesin are found in the inner layer of the MZ adjacent to the white pulp. Although their physiological role in the development of the immune response is still poorly understood, they might be decisive during viral infections and systemic infections [Eloranta and Alm, 1999; Taylor *et al.*, 2005]. MMØs are poorly characterized but can be specifically identified by the use of the monoclonal antibody MOMA-1[5].

- Phagocytic marginal zone MØs (MZMØs) are found on the other side of the marginal sinus. These MØs express a broad range of PRRs, which allow the MZMØs to play the role of biological filter of the circulation. This subpopulation is identified via the monoclonal antibody ER-TR9[6]

MØs are also represented within the white pulp by the so-called tingible body MØs, which eliminate apoptotic lymphocyte cells generated during processes that occur in the germinal centers [Taylor *et al.*, 2005].

3.2.2. Macrophages as "doctor Jekyll and mister Hyde"

The functions of MØs are markedly various and complex and of crucial importance for host immune response. Following phagocytosis, MØs destroy most of the microorganisms through the use of their bactericidal arsenal. By producing diverse messengers and presenting antigens to T cells, MØs orient the adaptive immune response leading to the expansion of specific adaptive mechanisms against the invaders [Gordon and Taylor, 2005]. In turns, inflammatory environment, as well as T_H1 or T_H2 effector T-cells and their products influence the state of activation of MØ populations |**Fig.6** [Mantovani *et al.*, 2002; Van Ginderachter *et al.*, 2006].

In this regard, MØs present an additional degree of complexity. Indeed, these cells can express different functional programs in response to specific microenvironment signals |**Fig.6** [Elgert *et al.*, 1998; Munder *et al.*, 1998]. For instance, a T_H1 cytokine context (IFN-γ, TNF-α) as well as recognition of microbial products or endogenous "danger" signals trigger the polarization of

[5] The antigen recognized by MOMA-1 has yet to be characterized, but its expression pattern is similar to that of sialoadhesin, a member of the sialic acid binding Ig-like lectin family [Oetke *et al.*, 2006].

[6] The monoclonal antibody ER-TR9 reacts with mouse SIGN-related 1 (SIGN-R1). Mouse SIGN-R1 is a C-type lectin, which mediates the uptake of encapsulated organisms and may be an important mediator for the uptake of microbes in spleen, particularly through the recognition of microbial polysaccharides [Kang *et al.*, 2003].

MØs to a "classical activation" profile. Classically activated MØs (M1MØ) typically produce high level of IL-12, a strong activator of T_H1 cells, inducible nitric oxide synthase (iNOS) and low level of IL-10 [Verreck *et al.*, 2004]. M1MØ are consequently strong promoters of T_H1 immune response (IFN-γ producing T_H cells). As such, M1MØs play an important role in protection against intracellular pathogens.

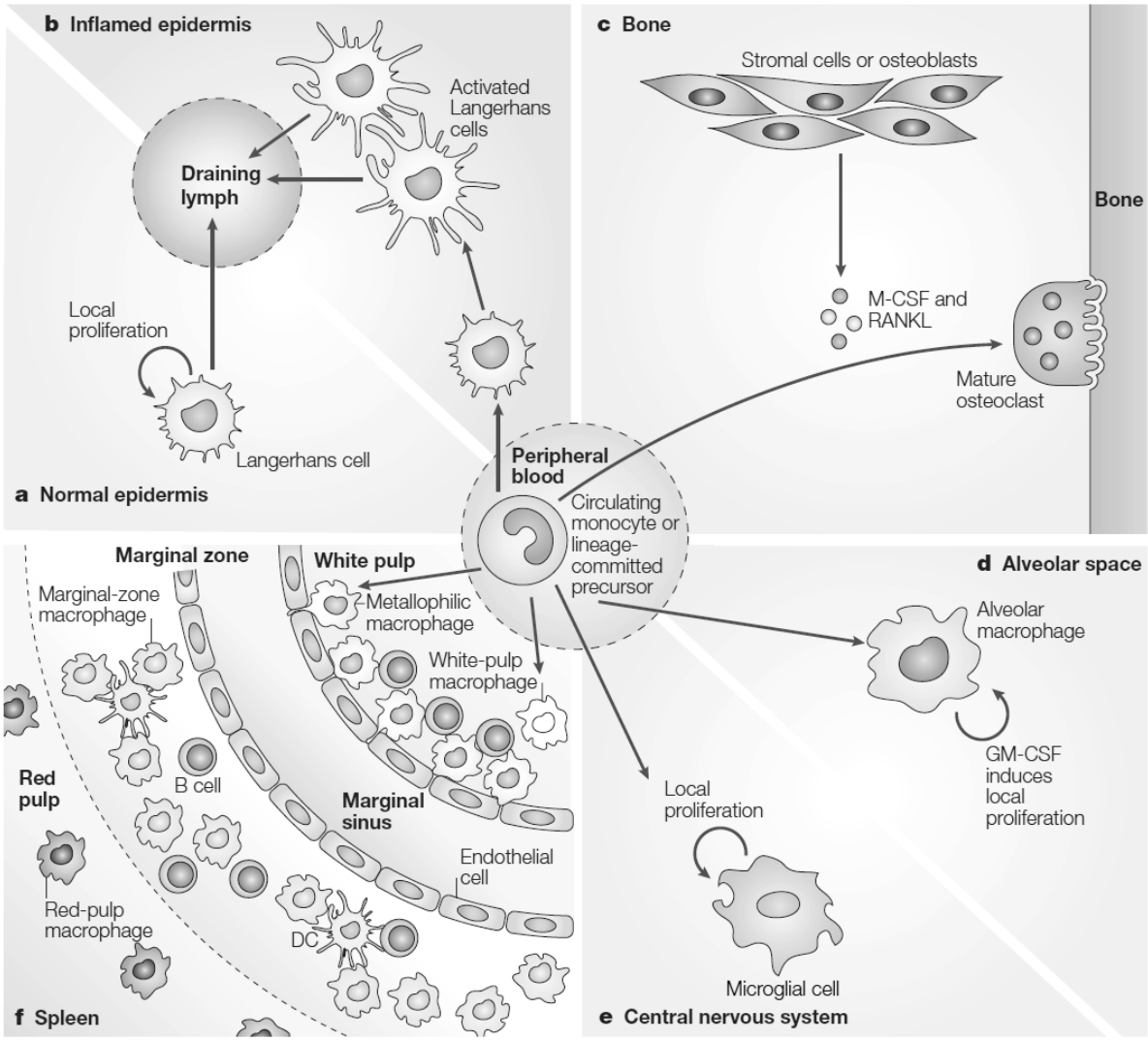


Figure |5 Origin and heterogeneity of differentiated cell in the adult mice. MØs, as well as most of DCs, originate from a common circulating precursor, the nature of which is unclear. (**a**) In the normal epidermis, Langerhans cells are replenished through a local auto-proliferation; after inflammation (**b**) the recruitment of circulating precursors to the inflamed epidermis allows the cell repopulation. In bone, (**c**) precursors recruited to the surface of the bone, undergo a maturation process to differentiate into mature osteoclasts, which are multinucleate. In the lungs (**d**), the bone marrow derived precursors recruitment is reinforced by a local proliferation to maintain the alveolar MØs population. In the central system (**e**), microglia cells are similarly maintained. Splenic MØs population (**f**) is thought to be sustained by a mixture of emigration of circulating monocyte pool and local proliferation. M-CSF, MØ colony-stimulating factor; RANKL, receptor activator of nuclear factor κB ligand; GM-CSF, granulocyte/macrophage colony stimulating factor. [Gordon and Taylor, 2005]

Distinct mediators have been reported to inhibit the M1 polarization and to attribute anti-inflammatory characteristics to MØs. These "alternative" MØs (M2MØs) produce high level of IL-10 in response to mediators such as T_H2 cytokines (IL-4, IL-13) and transforming growth factor (TGF) -β as well as apoptotic cells [Gough *et al.*, 2001; Verreck *et al.*, 2004]. As opposed to M1MØs, M2MØs promote T_H2 responses resulting in effector functions such as parasites killing and allergies phenomena. They are also implicated in the down-regulation of the immune response initiated by M1MØs. Moreover, they promote the fibrogenic activity of human fibroblast, and increase angiogenesis during acute inflammatory reactions. Finally, they exert selective immunosuppressive functions like the inhibition of T-cell proliferation [Brys *et al.*, 2005]. Importantly, fully polarized M1 and M2 MØs are to be considered as the extremes of a continuum of many other functional states [Van Ginderachter *et al.*, 2006].

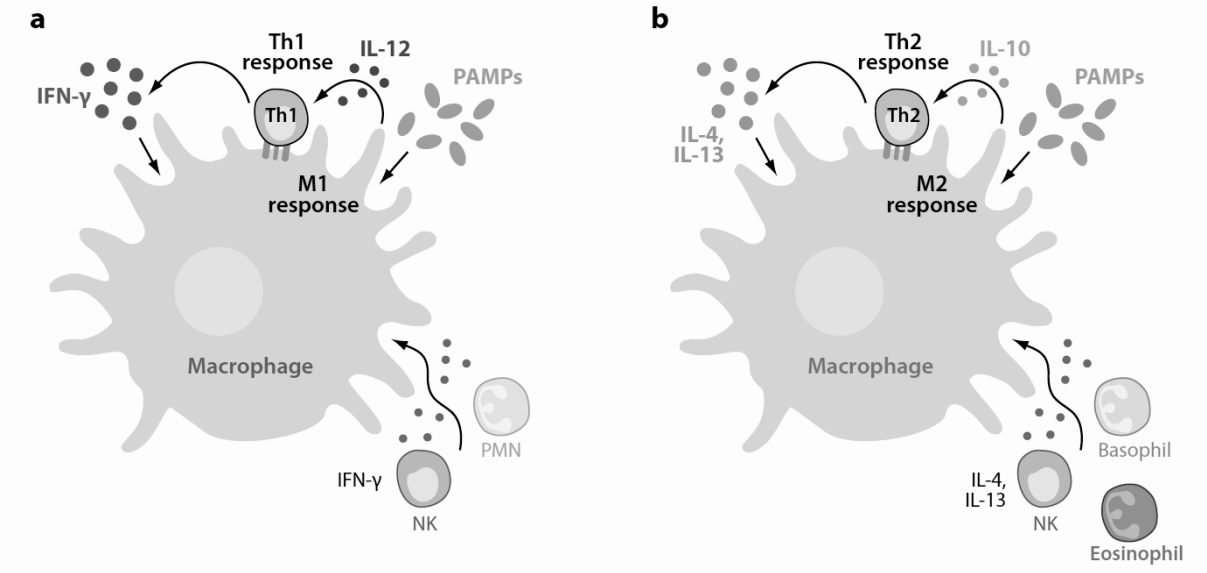


Figure |6 Programmed MØ activation model in the context of the immune response. Two essential signals are required for MØs full activation: recognition of microbial products by PRRs and stimulation with interferon (IFN)-γ. For Th1 response (**a**), engagement of PRRs provokes the secretion of chemokines that attract IFN-γ-producing innate immune cells such as Natural killer (NKs) to the site of infection. IFN-γ stimulates polarization of MØs to M1MØs, inducing Interleukin (IL)-12 secretion, a strong inducer for Th1 activation. Th1 activation induces the production of greater level of IFN-γ leading to a long-lasting M1 response. For Th2 response (**b**), stimulation induces the recruitment of IL-4 and IL-13-producing innate immune cells such as eosinophils and basophils. These cytokines stimulate the activation of M2MØs, which induced IL-10 secretion. [Martinez *et al.*, 2009]

3.3. Monocytes

Monocytes represent a pleiomorphic and pleiotropic population of circulating mononuclear cells that contribute, under baseline conditions, to homeostatic regulation of immune cell populations by supplying tissues with MØs, and DC precursors [Gordon and Taylor, 2005; Serbina *et al.*, 2008]. During an infectious process, however, the normal differentiation pathway of monocytes is temporarily refocused to differentiate into a broad spectrum of effector cells with distinct antimicrobial activities.

Within the bone marrow, myeloid restricted differentiation potential progenitor (MØ/DC progenitor, MDP) differentiates to $Ly6C^+$ monocytes and to the common DC progenitors. Among differentiated mature monocytes, at least two distinct phenotypic and functional subsets can be identified: $Ly6C^+/CCR2^+/CD11b^+/CX_3CR1^-$ monocytes, and $Ly6C^-/CCR2^-/CD11b^+/CX_3CR1^+$, referred as inflammatory monocytes and resident monocytes respectively [Auffray *et al.*, 2009] |**Fig.7 [Table 2]**.

Resident monocytes represent a functionally distinct monocyte subset [Auffray *et al.*, 2007]. These cells are characterized by small size, lack of expression of the chemokine receptor (CCR) 2, but exhibit a high expression of CX_3C chemokine receptor 1 (CX_3CR1, the receptor for fractalkine) [Tsou *et al.*, 2001]. This monocyte subset lasts in the circulation for longer periods and traffics into peripheral tissues under non-inflammatory conditions. In absence of infection, resident monocytes are thought to replenish the tissue resident MØs and DCs populations [Gordon and Taylor, 2005]. Their localization near vascular endothelium gives them an ideal emplacement to monitpr endothelial cells and surrounding tissue for damage or infection [Auffray *et al.*, 2007]. In addition, it has been proposed that these cells can promote healing by activating angiogenesis [Nahrendorf *et al.*, 2007] and induce the MØ polarization program M2 [Grunewald *et al.*, 2006] (section 3.2.2).

Inflammatory monocytes are so called because they give rise to MØs and DCs in a variety of infectious models [Serbina *et al.*, 2003; Narni-Mancinelli *et al.*, 2007]. They are characterized by a high expression level of the chemokine receptor CCR2, which is the main receptor for monocyte chemoattractant proteins (MCP)-1-2-3-5. In some cases, such as tumor, inflammatory monocytes play also the role of modulator of the immune response by differentiating to myeloid-derived suppressor cells (MDSCs) [Gabrilovich and Nagaraj, 2009]. MDSCs are a heterogeneous population of cells that expand during cancer and inflammation, and that have a remarkable ability to suppress T cell responses.

Table 2| Phenotypes of murine monocyte subsets			
Localization	Name	Markers	References
Bone Marrow monocyte Precursor	*MDP (monocyte, macrophage, dendritic cell precursor)*	Ter119$^-$, CD11b$^-$, CD11c$^-$, Nkp46$^-$, CD3$^-$, CD19$^-$, Gr1$^-$, MHC II$^-$, Sca1$^-$, IL-7Rα) CD117 (ckit) ^{int}, CD34$^+$, CD16$^+$, CX3CR1$^+$, CD115$^+$, CD135$^+$	[Fogg *et al.*, 2006; Auffray *et al.*, 2009; Auffray *et al.*, 2009; Liu *et al.*, 2009]
Circulating monocytes	*Ly6C$^+$/CCR2$^+$ monocytes*	CD117$^-$, NKp46$^-$, CD3$^-$, CD19$^-$, MHCII$^-$, Ly6G$^-$, CD115$^+$, CD11b$^+$, Ly6C$^+$, Ly6B(7/4)$^+$ F4/80^{low} CD62L$^+$ CD43$^+$, LFA-1$^+$, CX3CR1^{int}, CCR2$^+$, CD11c$^-$, CD135$^-$	[Palframan *et al.*, 2001; Geissmann *et al.*, 2003; Sunderkotter *et al.*, 2004; Saederup *et al.*, 2008; Serbina *et al.*, 2008; Auffray *et al.*, 2009; Auffray *et al.*, 2009; Liu *et al.*, 2009]
	Ly6C$^-$/CX$_3$CR1$^+$ monocytes	CD117$^-$, NKp46$^-$, CD3$^-$, CD19$^-$, MHCII$^-$, Ly6G$^-$, CD115$^+$, CD11b$^+$, Ly6C$^-$, Ly6B(7/4)$^-$, F4/80^{low}, CD62L$^-$ CD43^{hi}, LFA-1^{hi}, CX3CR1^{high}, CCR2$^-$, CD11c$^-$, CD135$^-$	[Palframan *et al.*, 2001; Geissmann *et al.*, 2003; Sunderkotter *et al.*, 2004; Auffray *et al.*, 2007; Varol *et al.*, 2007; Saederup *et al.*, 2008; Auffray *et al.*, 2009; Liu *et al.*, 2009; Swirski *et al.*, 2009]

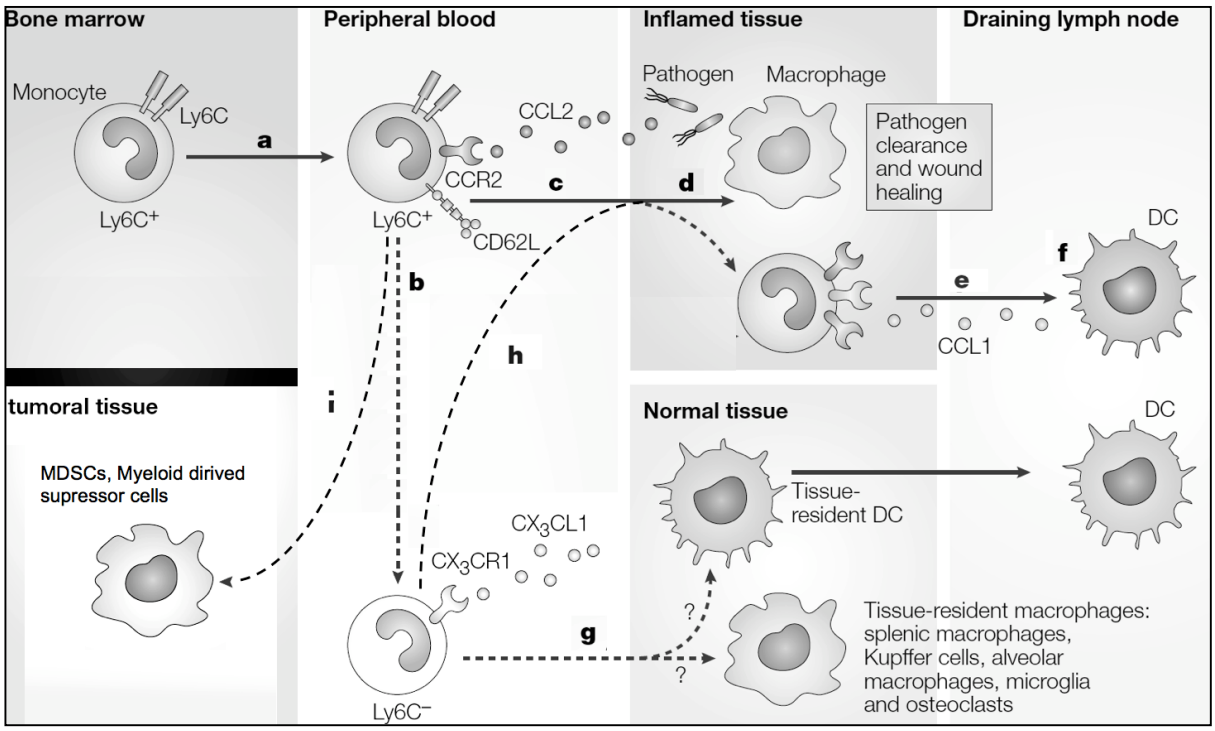


Figure \|7 Origin and functions of monocyte subsets in mice. In absence of infection, Ly6C$^+$ monocytes (**a**) are released in the circulation and (**b**) adopt a Ly6C$^-$ phenotype associated with the expression of CX$_3$C chemokine receptor 1 (CX$_3$CR1). During an infection process, both, Ly6C$^+$and Ly6C$^-$ respond to pro-inflammatory products (**c**) and can be recruited to the site of inflammation (**d,h**). In addition, (**e**) Ly6C$^+$ and Ly6C$^-$ monocytes emigrate directly from circulation to secondary lymphoid organs where (**f**) only Ly6C$^+$ acquire inflammatory DC-like features. In normal tissue Ly6C$^-$ monocytes enter the tissue and replenish the tissue-resident MØs and Dendritic cell populations (**g**). Ly6C$^-$ cells have also been associated to the wound healing of the inflamed tissue (**h**). In some cases, such as tumors (**i**), Ly6C$^+$ monocytes differentiate into myeloid derived suppressor cells (MDSCs). Solid arrows represent pathways that have been experimentally appreciated whereas dashed arrows correspond to pathways that are supported by speculations. CX$_3$CL1, CX$_3$C chemokine ligand 1. Adapted from [Gordon and Taylor, 2005].

3.4. Natural killer cells. (NK)

NK cells are large granular lymphocytes of the innate immune system that are found in organs such as liver, lungs and spleen as well as in the circulation. They are specialized to combat intracellular pathogens, mainly viruses, either directly through the induction of apoptosis of infected cells or indirectly via the production of cytokines, particularly IFN-γ [Gregoire *et al.*, 2007].

Their development is complex. NK cell precursor originates in the bone marrow from a lymphoid progenitor cell. However, it has been suggested that extramedullary NK cell development may occur in organs such as the lymph nodes or the thymus [Freud *et al.*, 2005; Vosshenrich *et al.*, 2006]. In mice, several stages of NK cell maturation [Hayakawa *et al.*, 2006], give rise to mature NK cells, which can be identified by the marker combination $NK1.1^{+}$ $CD3^{-}$ [Colucci *et al.*, 2003].

3.4.1. NK cells: "delivering the kiss…"

The balanced expression of activating and inhibitory ligands by host-cells, allows NK cells to take the decision whether to kill or not a targeted cell. The mechanism underlying the expression of these ligands by stressed cells is still poorly understood but seems to involve intracellular sensors of infection or cell-autonomous detection of excessive cellular stress [Lanier, 2005]. NK cells are able to sense changes in the expression of these ligands by integrating the information from two types of surface receptors, which together control their cytotoxic activity [Brown *et al.*, 2001; Robbins and Brossay, 2002]. One type is an activating receptor that triggers killing by the NK cell. This class includes members of the immunoglobulin-like and the C-type lectin families of proteins. The second set of receptors inhibits activation and prevents NK cells from killing normal host cells [Borrego *et al.*, 2002; Boyington and Sun, 2002]. These inhibitory receptors are specific for major histocompatibility complex (MHC) class I molecules[7].

[7] Because many intracellular pathogens have developed strategies that interfere with the presentation of their peptides on the surface of cells that they infect, altered expression of MHCI may be the signal for NK cells to kill the infected cells.

3.4.2. NK cells: "…of death"

During an inflammatory process, NK cells are recruited to the site of infection where they contribute to the milieu sterilization. These cells may kill infected neighboring cells by releasing cytotoxic proteins trapped into their cytoplasmic granules. Among these effector proteins, perforin acts in delivering granules content into the cytoplasm of the infected cell. Once introduced, cytotoxic proteins such as granzymes trigger apoptosis program by interacting with specific intracellular targets such as the protein BID or pro-caspase 3 [Trambas and Griffiths, 2003; Pipkin and Lieberman, 2007]. NK cells may induce the death of targeted cell by an additional signaling pathway implicating two proteins, the fas ligand (FasL) and its receptor (FasR). This mechanism has mainly been associated with immune tolerance, homeostasis and cancer [Andersen *et al.*, 2006] [French *et al.*, 1996; Greil *et al.*, 1998; Igney and Krammer, 2005] | **Box 1**.

Box 1 | The FasL- FasR signalling

NKs cells present an additional mechanism inducing programmed cell-death. It involves the interaction of a NKs cell expressed protein, Fas ligand (FasL), with its cognate receptor, Fas receptors (FasR), present on the surface of the targeted cells. FasL is a homotrimeric type II transmembrane protein that belongs to the tumor necrosis factor (TNF) family and signals through trimerization of FasR, which spans the membrane of the targeted cell. This trimerization induces the internalization of the complex and ultimately, leads to the activation of caspase 8, one of the first steps in the induction of apoptosis.
Notably, this mechanism has mainly been linked to cell homeostasis [Andersen *et al.*, 2006]. For example, the activation of T cells leads to the expression of FasL. T cells are initially resistant to Fas-mediated apoptosis during clonal expansion, but become progressively more sensitive the longer they are activated, ultimately resulting in apoptosis. This process is needed to prevent an excessive immune response and eliminate autoreactive T-cells. Humans and mice with deleterious mutations of Fas or Fas ligand develop an accumulation of aberrant T-cells, leading to lymphadenopathy, splenomegaly, and lupus erythematosus.

3.4.3. NK cells: beyond killing

Furthermore, NK cells are extensively implicated in the modulation of the immune response by their capacity to secrete immune mediators [Andoniou *et al.*, 2008]. In many infectious models, it has been shown that NK cells are the first source of IFN-γ production, which is an essential signal for T_H1 activation [Scharton and Scott, 1993; Kos and Engleman, 1995] [Kang *et al.*, 2008]. Moreover, these cells were shown to be required for the generation of anti-viral cytotoxic T cell responses and enhanced activation of B cells *in vitro* [Burlington *et al.*, 1984; Snapper *et al.*, 1994]. Finally, under some circumstances, NK cells have been reported to inhibit adaptive immunity [Su *et al.*, 2001; Barber *et al.*, 2007].

3.5. Dendritic Cells

Dendritic cells (DCs) are widely distributed hematopoietic cells that are specialized in the capture, processing and presentation of antigens (Ag) to T cells. Since their initial description by Steinman and Cohn, it has become evident that DCs are the main APCs responsible for the shaping of the immune response [Steinman and Cohn, 1973].

3.5.1. DCs: the hinge of the immune response

These cells are found widely throughout the body and act mainly as sentinels, able to warn the immune system for potential dangerous signals. In these tissues, they are mainly in an immature state and have a high capacity to recognize microbial determinant through specific PRRs [Schuler and Steinman, 1985; Romani *et al.*, 1989]. Following bacterial invasion, DCs capture Ag and subsequently migrate to the T cell area of secondary lymphoid organs, where, after maturation, they interact with rare Ag-specific T cells, and thereby initiate their activation and expansion. Depending on the nature of the information given by the DCs, the activated T-cells mature to a particular T_H cell subset, which permit the setting up of an adapted immune response (see section 2.4) [Coquerelle and Moser, 2010]. After interaction with lymphocytes, DCs die by apoptosis [Matsue and Takashima, 1999; Guermonprez *et al.*, 2002; Yen and Ganea, 2009].

3.5.2. DCs: omnibus viis DCs pervenitur *(all roads lead to DCs)*

DCs, together with all blood cells, are originated from hematopoietic stem cells (HSCs) differentiation. However, when compared with other hematopoietic cell lineages, DCs generation presents a remarkable developmental flexibility. A series of studies have indeed shown that these cells could be generated from myeloid-biased precursors as well as lymphoid-biased precursors (for review see [Shortman and Naik, 2007]).

Actually, regardless the nature of the precursor, DCs generation strongly depends on the expression of the receptors FLT3[8] (FMS-related tyrosine kinase3 also known as FLk2) on the surface of the progenitor cell [Waskow *et al.*, 2008]. The choice of the progenitor type that will generate DCs *in vivo* depends on the availability of precursors, the local environment and the tissue involved [Karsunky *et al.*, 2003].

3.5.3. DCs or not DCs

DCs constitute a complex system of cells, which, under different microenvironmental conditions, can induce many different responses. Although all DCs share phenotypic and functional properties, the expression of certain cell-surface markers has pointed out a substantial DC heterogeneity **[Table 3]**. These DCs subsets differ in location, migratory pathways, immunological functions and dependence on infectious stimuli for their generation. According to the Shortman and Naik's classification, the DCs family can be divided into three subtypes: Precursors-DCs (pre-DCs), Classical DCs (cDCs), and Inflammatory DCs (iDCs) [Shortman and Naik, 2007].

3.5.4. Precursor DCs: "ready to transform"

Pre-DCs are cells without immediate DCs forms and functions, which correspond to the last stage before DCs generation. They are relatively long-lived cells present in bone marrow and all peripheral organs. Different pre-DCs produce different types of DCs following their activation by exogenous stimuli such as microbial products. Examples of pre-DCs are plasmacytoid DCs (pDCs) and the immediate precursors of steady-state splenic cDCs. pDCs, also known as the natural interferon-producing cells [Liu, 2005], are specialized DCs that respond to viral infections with massive production of type I interferons (IFNs) [Corcoran *et al.*, 2003]. pDCs also play the role of APCs and can control T-cell responses [Colonna *et al.*, 2004].

8 Fms-like tyrosine kinase 3 (FLT3) is a cytokine tyrosine kinase receptor that plays important roles in the proliferation, survival, and differentiation of hematopoietic stem/progenitor cells (HSPCs), particularly myeloid and lymphoid progenitors [McKenna *et al.*, 2000].

3.5.5. Classical DCs: "ready to harvest"

cDCs are specialized APC furnished with high phagocytic activity as immature cells and high cytokine-production capacity as mature cells [Banchereau and Steinman, 1998; Mellman and Steinman, 2001]. These cDCs can be further separated into migratory DCs and lymphoid-tissue resident cDCs.

Migratory cDCs are the classical DCs. They are highly motile cells that can convey Ag from tissues to T cells area of secondary lymphoid organs via afferent lymphatics and high endothelial venules [Bell *et al.*, 1999]. Particularly, Langerhans cells (LCs) of epidermis are the model of migratory DCs [Romani *et al.*, 2003]. By contrast, lymphoid resident cDCs do not migrate and rather collect Ags by sampling it in a soluble form from the circulation or from other APCs via the phenomenon of cross-presentation[9] [Albert *et al.*, 1998]. These cells include most of the DC of the thymus and the spleen [Ardavin, 1997; Vremec *et al.*, 2000] |**Fig.8**. In mice, splenic resident cDCs can be further separated into $CD8^+$ cDCs that express high level of the T-cell marker, CD8α, on cell surface and $CD8^-$ cDCs lacking this marker [Vremec *et al.*, 2000; Wilson *et al.*, 2003]. The $CD8^-$ cDCs in turn are subdivided into $CD4^+$ $CD8^-$ ($CD4^+$) and $CD4^-$ $CD8^-$ (double negative) cDCs subsets [Vremec *et al.*, 2000]. $CD8^+$ cDCs are located in the T-cell area of the spleen, whereas $CD8^-$ cDCs reside in the MZ of this organ. These two subsets of cDCs differ in immune functions [Coquerelle and Moser]. It has been found that $CD8^-$ cDCs induced activation of cells secreting high levels of IL-4, Il-5 and Il-10, whereas $CD8^+$ cDCs triggered cells producing Il-2, IL-12 and IFN-γ. Regarding these observations, several studies concluded that the splenic subclasses of lymphoid resident cDCs have the potential of differentially modulate cytokine production toward T_H1 or T_H2 bias (for review see [Coquerelle and Moser]).

[9] Cross presentation correspond to the initiation of a $CD8^+$ T-cell response to an Ag that is not initially present within the cytoplasm of APCs. This exogenous Ag must be taken up by APCs and then re-routed to the MHC class I pathway of Ag presentation. [Kurts *et al.*]

Table 3| Phenotypes of murine Dendritic cells (DCs) subsets			
Type	Name	Markers	References
Bone Marrow DCs Precursor	*CDP (common DC precursor)*	Ter119$^-$, CD11b$^-$, CD11c$^-$, Nkp46$^-$, CD3$^-$, CD4$^-$, CD8α^-, CD19$^-$, Gr-1$^-$, MHC II$^-$, Sca1$^-$,IL-7Rα^- CD117$^{int/low}$, CX3CR1$^+$, CD115$^+$, CD135$^+$	[Liu *et al.*, 2009] [Onai *et al.*, 2007] [Naik *et al.*, 2007]
Precursor DCs	*pDCs (plasmacytoid DCs)*	CD45RA (B220)$^+$, Siglec-H$^+$, BST2$^+$, Ly6C$^+$, CD62L$^+$, CX3CR1^{low} CCR2$^+$ MHCII^{low} CD11c^{int},CD115$^-$, CD11b$^-$, NKp46$^-$, CD3$^-$, CD19$^-$, Ly6G$^-$, F4/80$^-$ CD43$^+$	[Colonna *et al.*, 2004]
	Blood/spleen precursor cDCs:	CD115^{low}, CD11b$^+$, CD11c$^+$, F4/80$^-$, CX3CR1$^+$, CD117$^-$, CD135$^+$, MHCII$^+$	[Liu *et al.*, 2009]
	Derived intestinal lamina propria DCs	**CD103$^+$ CD11b$^-$:** CD103$^+$, CD11b$^-$, CD11c^{hi}, CD14$^-$, CX3CR1$^-$, **CD103+ CD11b+:** CD103$^+$, CD11b$^+$, CD11c^{hi}, CD14$^-$, CX3CR1$^-$	[Bogunovic *et al.*, 2009; Varol *et al.*, 2009]
Classical DCs	*Langerhans DCs (migratory)*	CD4$^-$, CD8^{low}, CD205$^+$ CD11b$^+$, CD11c^{hi}, CD207$^+$	[Shortman and Liu, 2002]
	classical DCs CD8$^+$	CD115$^-$, CD11c^{high}, CD8α^+, CD4$^-$, DEC205$^+$, 33D1$^-$, MHCII$^+$, CD11b$^-$, CX3CR1^{neg}, CD80^{high}, CD86^{high}	[Shortman and Liu, 2002; Dudziak *et al.*, 2007; Liu *et al.*, 2009]
	classical DCs CD4$^+$	CD115$^-$, CD11c^{high}, CD4$^+$, CD8α^-, DEC205$^-$, 33D1$^+$, MHCII$^+$, CD11b$^+$, CX3CR1^{int}, CD80^{high}, CD86^{high}	
	classical DCs CD8$^-$ CD4$^-$(double neg)	CD115$^-$, CD11c^{high}, CD8α^-, CD4$^-$, DEC205$^-$, 33D1$^+$, MHCII$^+$, CD11b$^+$, CX3CR1^{int}, CD80^{high}, CD86^{high}	
Inflammatory DCs	*TIP-DCs (TNF-iNOS DCs)*	CD115$^+$, CD11b$^+$, CD11c^{int}, NKp46$^-$, CD3$^-$, CD19$^-$, Ly6G$^-$, Ly6C$^+$, CX3CR1$^+$, CCR2$^+$,MHCII$^+$,CD80^{high}, CD86^{high}, iNOS$^+$, TNFα^+	[Serbina *et al.*, 2003; Serbina *et al.*, 2003; Serbina and Pamer, 2006; Serbina *et al.*, 2008; Auffray *et al.*, 2009]

3.5.6. Inflammatory DCs: "ready to act"

Inflammatory DCs (iDCs) constitute an effector DCs population, which is not present in the steady state of the organism but appears crucial in several infection models |**Fig.8**. These cells have been firstly identified in spleens of *Listeria monocytogenes* infected mice by Pamer's team [Serbina *et al.*, 2003]. They found that these iDCs were not essential for T-cell priming but mediated immune defense by secreting massive concentration of iNOS and TNF-α. Consequently, these cells were termed TNF-α and iNOS producing (Tip) DCs. In this infection model, the majority of TipDCs were not infected but were recruited to the site of infection in a CCR2-dependant fashion. On the contrary, TipDCs were found to be the major infected population in the lesions and the draining lymph nodes of *Leishmania major* infected mice and appear to be a major class of T_H1 regulated effector cell population [De Trez *et al.*, 2009]. Moreover, Lowes reported that TipDCs could be the direct inflammatory effector cells implicated in psoriatic lesions [Lowes *et al.*, 2005]. Other analyses have also suggested that these TipDCs directly induce IgA class switching of naïve B cells [Tezuka *et al.*, 2007].

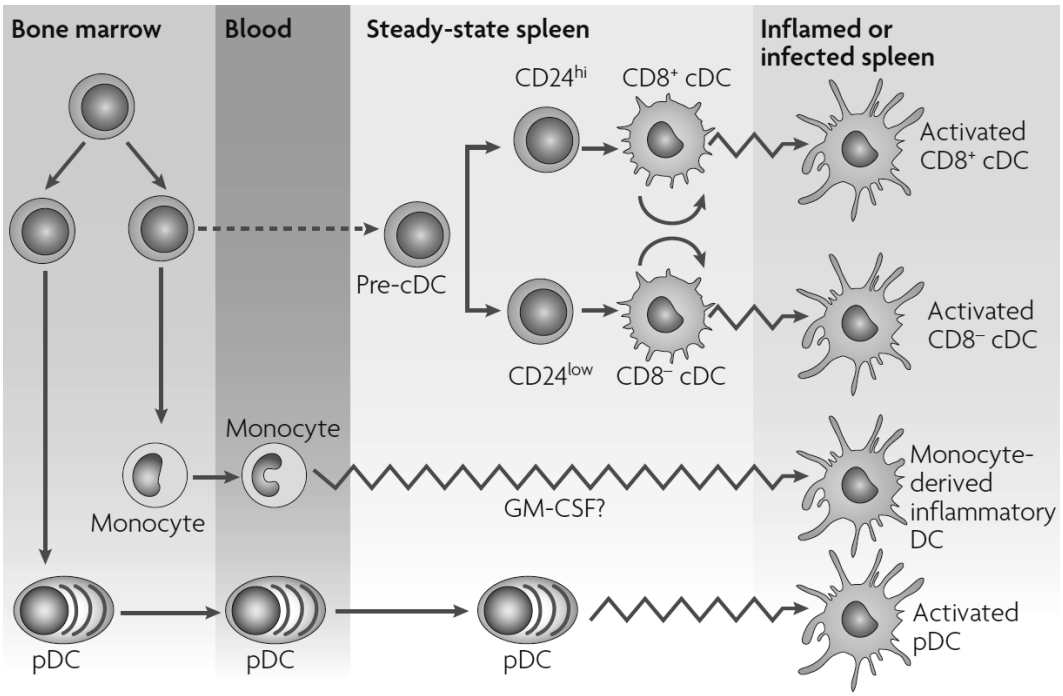


Figure |8 Origin and Heterogeneity of splenic DCs. Steady state conventional splenic DCs (cDCs) originate from an intrasplenic precursor, a pre-cDCs, derived from the differentiation of an earlier progenitor generated in the bone marrow. Later in the splenic cDCs development, Pre-cDCs give rise to two populations that are pre-committed to form either $CD8^+$ or $CD8^-$ cDCs subsets. In contrast, the plasmacytoid DCs found in the spleen arrive there from the bloodstream. During an inflammatory process, a DC subset that is not present in the steady state of the mouse is recruited in the spleen. This subset derives from a monocyte precursor that expresses high level of Ly-6C cell surface marker. GM-CSF, granulocyte MØ colony-stimulating factor. [Shortman and Naik, 2007]

Importantly, analysis realized by Wick and collaborators revealed that TipDCs were actually a subdivision of an iDCs family characterized by the potential to differentiate into distinct subsets defined by specialization in the production of antimicrobial molecules such as TNF-α, IL-12 and iNOS [Tam and Wick, 2006]. Using five-color flow cytometry and double intracellular cytokine staining, they indeed detected phenotypically similar $CD11c^+$, $CD11b^+$ cells with distinct capacities to produce TNF-α/IL-12 or TNFα/iNOS in *Listeria* infected tissues. Furthermore, production of TNF-α ,iNOS and IL-12 was differentially dependent on cellular localization of the bacteria. They concluded that iDCs were a heterogeneous group of innate immune cells.

3.6. Eosinophils, basophils and mast cells

Eosinophils, basophils, and mast cells are members of the granulocytes family and form a host-defense module mainly involved in the defense of parasitic helminthes, tissue injury and allergic diseases [Hogan *et al.*, 2008] [Galli *et al.*, 2005].

Mast cells are present in mucosal and connective tissue, whereas eosinophils traffic mainly into the *lamina propria* of all segments (except esophagus) of the gastrointestinal tract and basophils are recruited to the site of infection from the circulation [Mishra *et al.*, 1999] [Arock *et al.*, 2002] [Kalesnikoff and Galli, 2008].

The mechanisms underlying the activation of eosinophils, basophils and mast cells are still poorly understood. The induction of these cells has been shown to involve most notably the T_H2 cells products IL-4, IL-5, IL-9 and IL-13 [Stetson *et al.*, 2004]. Moreover, it has been found that some pathogenic structural components such as one of the major parasite associated cell wall component, chitin, were able to induce the recruitment of these cells [Reese *et al.*, 2007].

Eosinophils, basophils and mast cells contain different populations of sercretory granules, which are constituted of a broad range of toxic components such as neurotoxins, peroxidases, and basic proteins [Hogan *et al.*, 2008]. The main defensive strategy used by these cells against parasites does not seem to target the pathogens directly. Instead, host tissues, particularly vasculature, smooth muscles, and the mucosal epithelia, are the main target of these immune components. The mediators released by these cells affect the targeted tissue in such a way that limits the spread of the parasite and promote its expulsion from the organism [Hogan *et al.*, 2008; Kalesnikoff and Galli, 2008].

4. Sensing Pathogen and Shaping Immune Response

The capacity of the innate immune cells to engage qualitatively distinct immune responses against different microbial infections firstly arises from their abilities to sense and discriminate invading organisms. Unlike adaptive immune detection, innate immune recognition occurs via receptors that are expressed on all cells of a given class. Ultimately, the goal of these receptors is to discriminate between infectious nonself and noninfectious self. Failure to make this distinction can lead to destruction of host tissue and autoimmunity on the one hand or overwhelming infection on the other.

Through the course of evolution, several mechanisms have been developed by metazoans to deal with this crucial task. Conceptually, three strategies exist:

- Pattern recognition
- Missing-self recognition
- Altered or Induced -self recognition

Pattern recognition is based on the detection of conserved molecular patterns that are essential products of microbial physiology [Janeway, 1989]. These invariant structures are referred as “pathogen-associated molecular patterns (PAMPs), although they are not unique to pathogens and are also present in all microorganisms, pathogenic or not. On the other hand, PAMPs are not produced by the host and are invariant among microorganisms of a given class [Janeway, 1989; Medzhitov and Janeway, 1997]. The best studied examples of PAMPs include bacterial components such as lipopolysaccharide (LPS), the major glycolipidic component of gram-negative bacteria and lipoteichoic acid of gram-positive bacteria. These and other PAMPs are recognized directly by innate immune cells via specific transmembrane and cytosolic receptors termed PRRs |**Fig.9**.or indirectly through soluble PRRs.

Missing-self recognition is based on the presence of ubiquitous self-molecules on the membrane of host cells [Ravetch and Lanier, 2000; Raulet *et al.*, 2001]. These markers engage inhibitory receptors on innate immune cells such as NK cells and thereby, prevent their activation. Absence or downregulation of these markers lead to the disengagement of the inhibitory signal and to the subsequent activation of patrolling cells that respond by inducing the death of the target lacking self-marker.

The third strategy, recognition of altered or induced -self, is based on the detection of self-markers that are either induced upon various stresses such as infection or released in the extracellular milieu following non-physiological cell death | **Box 2**.

**Box 2	Induced or Altered –self receptors**	
Induced Self receptors	Upon infection, activating receptors expressed on innate immune cells recognize induced self-markers expressed on infected cells. This recognition initiates the death by apoptosis of the altered self-marker expressing cells. Notably, several families of these receptors serve as ligands for activating NK cells and some T-cells.
Altered Self receptors	The innate immune cells have also receptors that detect some intracellular molecules that are normally "hidden" whithin of cells and that are only revealed after necrosis. In this case, necrotic cells death is considered to be a signal of potential threat to the organism that consequently stimulate the immune system by releasing endogenous molecules termed, damage-associated molecular patterns (DAMPs). Note that necrosis can also generate extracellular DAMPs by releasing other molecules (such as hydrolases) that act on extracellular components (for example, fragments of extracellular matrix such as hyaluronic acid).

In this chapter, we will focus on how pattern recognition is used by innate immune system to distinguish self from non-self and how this discrimination is translated into induction of effective response. In particular, we will describe the Toll-like receptor (TLRs) family.

4.1. PRRs: checkpoints controlling the adaptive immunity

From the activation of responses to the effector versus memory generation, the stepwise decisions taken by the adaptive immunity necessitate a complex control of information provided by various signals harvested during infection through a panel of PRRs |**Fig.9**. These receptors determine the nature of the Ag, the type of infection (pathogen class), the magnitude and duration of infection, and finally, the requirement for memory response (for review see [Palm and Medzhitov, 2009]).

All PRRs can detect the presence and type of microbial infection and activate the appropriate innate immune response. However, not all PRRs are equal in terms of their ability to induce adaptive immune response [Iwasaki and Medzhitov, 2004].

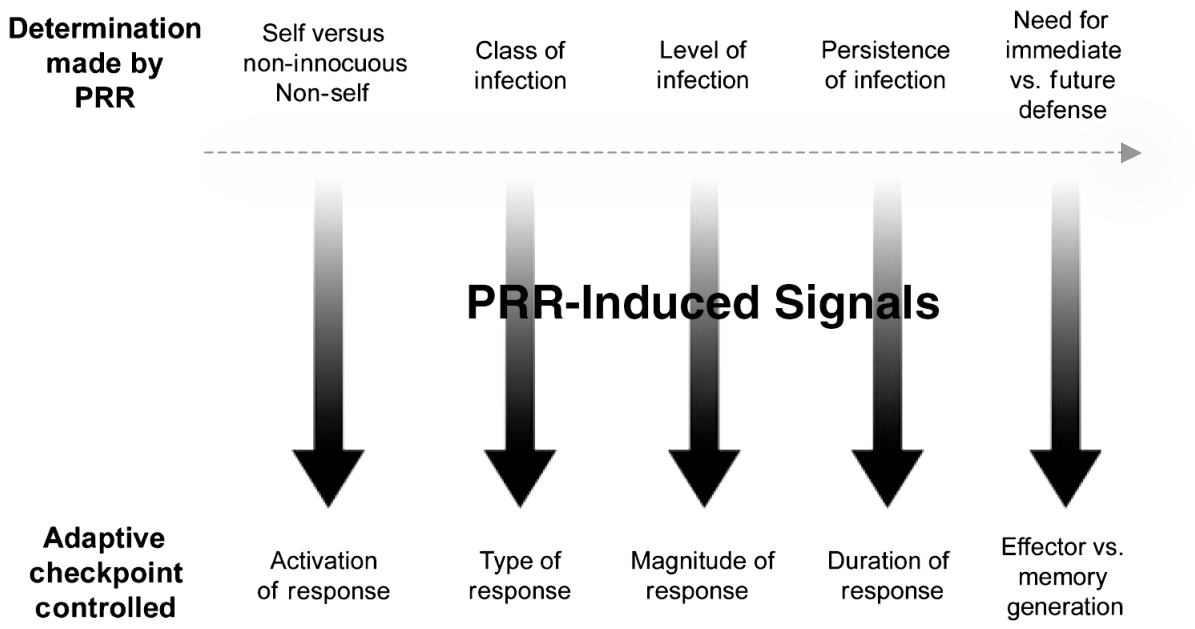


Figure |9 Checkpoints of adaptive immunity controlled by PRRs. Adaptive immunity is a multistep process. Each step is controlled before the onset of every subsequent stage to ensure a productive immunity. PRRs detect the presence and feature of an infection and PRRs-mediated signals control adaptive immunity accordingly at various checkpoints. A failure to properly regulate immunity at each checkpoint can lead to various immune disorders including autoimmunity [Palm and Medzhitov, 2009].

Over the past few years, the list of known PRRs has never stopped to growth, underscoring the importance of this strategy in recognition of infectious nonself. Following this huge expansion, categories have emerged considering either structural criteria such as the presence of homology domains[10] or geographical distribution. Accordingly, PRRs can be divided into three categories: Soluble PRRs, Cytoplasmic PRRs and Transmembrane PRRs **| Box 3.** For the purpose of this work, one family of PRRs that belongs to the transmembrane PRRs, the toll like receptors (TLRs), are further discussed below.

**Box 3	Classes of PRRs**	
Soluble PRRs	Soluble PRRs are secreted proteins found in the body fluids. This class is essentially produced in the liver and notably includes the collectins (e.g. mannose-binding lectin family [Ip *et al.*, 2009]), ficolins and pentraxins of the acute phase proteins family [Holmskov *et al.*, 2003; Bottazzi *et al.*, 2006; Endo *et al.*, 2006], as well as other proteins such as the LPS-binding protein (LBP) [Shimazu *et al.*, 1999].
Cytoplasmic PRRs	Cytosolic PRRs are intracellular receptors recognizing bacterial and viral –associated molecular patterns. These receptors can directly detect cytosolic PAMPs and subsequently activate various signaling cascades [Meylan *et al.*, 2006; Takeuchi and Akira, 2007]. This group includes: - Nod (nucleotide-binding oligomerization domain) 1 and 2 -like receptors (NLRs), which recognize structurally distinct bacterial peptidoglycan fragments. - RIG-I (retinoic acid-inducible gene I)-like helicases (RLHs), the MDA5 (melanoma differentiation-associated gene 5) protein, which both sense RNA viruses - DAI (DNA-dependant activator of interferon-regulatory factors) regulator, which detects viral-DNA. Cytosolic PRRs also includes members that are not activated by their direct binding to the PAMPs but need additional signals that result from intracellular perturbations such as destabilization of phagosomal compartment, potassium efflux, or generation of reactive oxygen species (ROS) [Bryant and Fitzgerald, 2009]. Accordingly, such PRRs are able to associate the presence of microbes to the lost of integrity of the infected cell [Brodsky and Monack, 2009]. These PRRs are members of the NLR family that are implicated in the formation and activation of large multimeric protein complexes referred as inflammasomes[11] [Martinon and Tschopp, 2004].
Transmembrane PRRs	Transmembrane PRRs recognize PAMPs in the extracellular space and/or intracellular compartments such as the phagosme of phagocytic cells. This class includes various receptor families such as TLRs, members of the C-type lectin (CLRs) family (e.g., Dectin-1 [Brown and Gordon, 2001]) and scavenger receptors [Stuart and Ezekowitz, 2008].

[10] Several types of protein domains have been found to be involved in pattern recognition, the most prominent being the C-type lectin domain, scavenger receptor cysteine-rich domain and leucine-rich repeat (LRR) domain.

[11] Inflammasome is a process that controls the recruitment and cleavage of pro-caspase-1, and the secretion of caspase-1-dependent cytokines such as IL-1β and IL-18, through a process of cellular self-destruction known as pyroptosis [Vazquez-Boland *et al.*].

4.2. Toll Like Receptors (TLRs)

TLRs are membrane-bound PRRs whose ligand-binding domains, composed of leucine rich repeats (LRRs), point toward the extracellular milieu or to the lumen of membrane-enclosed intracellular compartments [Akira and Takeda, 2004]. The cytoplasmic domains of these receptors, known as Toll/IL-1 receptor [Barquero-Calvo *et al.*] domain, transduce their signals through phenotypic interaction with cytoplasmic adaptor proteins, which also contain a TIR domain (see section 4.2.2) [Akira and Takeda, 2004]. Ligand-induced receptors and adaptor proteins activation result in recruitment of additional signaling proteins and in the triggering of host defense responses that involve the production of protein mediators such as cytokines, and costimulatory molecules.

4.2.1. The TLRs family and their ligands

TLRs constitute a family of 10 (TLRs 1-10) and 13 (TLRs 1-9 and 11-13) members in human and mice, respectively, that are widely distributed among APCs and other cells [Ishii *et al.*, 2008] [Alexopoulou and Kontoyiannis, 2005]. TLR 3, 7, 8 and 9 are located in the membrane of endosomes and the remaining TLRs are found in the cell-surface membrane. According to the molecular characteristics of their ligands, TLRs can be divided into three categories: lipid-, nucleic acid- and protein- recognizing TLRs | **Table 4**.

Table 4 | Toll-like receptors and some of their ligands

Categories	TLRs	Ligands	References
Lipid-recognizing TLRs	*TLR4*	Enterobacterial Lipopolysaccharide	[Akira *et al.*, 2006]
	TLR2/TLR2	Peptidoglycan, zymosan, lipoarabinomannan	[Akira *et al.*, 2006]
	TLR2/TLR1	Di-acyl lipopeptide	[Takeuchi *et al.*, 2001; Akira *et al.*, 2006]
	TLR2/TLR6	Tri-acyl lipopeptide	[Takeuchi *et al.*, 2002; Akira *et al.*, 2006]
Nucleic acid-recognizing TLRs	*TLR3*	Double-stranded RNA	[Alexopoulou *et al.*, 2001]
	TLR7	Imidazoquinolines, single stranded RNA	[Hemmi *et al.*, 2002; Heil *et al.*, 2004]
	TLR8	Single-stranded RNA	[Heil *et al.*, 2004]
	TLR9	Unmethylated CpG* DNA motif	[Hemmi *et al.*, 2000]
Protein recognizing protein	*TLR5*	Flagellin	[Hayashi *et al.*, 2001]
	TLR11	Uropathogenic bacteria-associated PAMPs. Profilin-like molecule derived from *Toxoplasma gondii*	[Zhang *et al.*, 2004; Yarovinsky *et al.*, 2005]

* Cytosine-Phosphate-Guanosine dinucleotides

Among these receptors, TLR2, TLR4, TLR5 and TLR9 are the most important for defense against bacterial infection as they recognize lipoproteins, LPS, flagellin and CpG (Cytosine-Phosphate-Guanosine) dinucleotides respectively [Akira *et al.*, 2006].

4.2.2. Tir-domain containing adaptors in TLR signaling

The biological functions of TLRs rely on transduction pathways triggered downstream of these ligand-binding receptors. Each TLRs signaling has a distinct function, which largely depends on intracellular adaptor proteins involved. At present, five intracellular molecules carrying the TIR domain have been reported. They are briefly described in **Box 3** (for review see [O'Neill and Bowie, 2007]).

Among these adaptor proteins, the TIR domain-containing adaptor myeloid differentiation factor 88 (Myd88) is common to all TLR-mediated signaling (except TLR3), and can interact with IL-1 receptor. In terms of signaling, the situation regarding Myd88 is complex and explains why this receptor is crucial to innate immune responses. **Fig.10** describes the functions of Myd88. Upon stimulation by TLRs ligands or inflammatory cytokines including IL-1, IL-18 and INF-γ, Myd88 recruits a family of IL-1 receptor-associated kinases (IRAKs). IRAKs then activate tumor receptor-associated factor 6 (TRAF6), thereby inducing activation of mitogen-activated protein kinase (MAPK). This leads to the activation of transcription factors such as NF-κB, p38 and JNK (JUN N-terminal kinase) [Akira, 2004; O'Neill and Bowie, 2007]. Critical roles of this pathway were clarified through analysis of proinflammatory gene expression in Myd88-deficient mice. In this context, induction of *NF-κB* was completely abolished upon stimulation with IL-1 family members, IL-1 and IL-18, as well as with TLR-2, -7, and -9 agonists. Myd88 also couples to interferon-regulatory factor 5 (IRF5), IRF1 and IRF7. IRFs was identified as being crucial for the induction of proinflammatory cytokines and type I IFNs by all TLRs tested [Takaoka *et al.*, 2005]. Taken together, these observations justify the central position held by Myd88 in innate immune recognition.

Box 4| Toll-like receptors and some of their ligands		
Adaptor Protiens	Functions	References
Myd88 (Myeloid differentiation factor 88)	It is the universal adaptor protein for TLRs (except TLR3) and is also a member of the IL-1 receptor family. It has been found to be implicated in regulation of the immune response to numerous bacterial infectious models by inducing activation of mitogen-activated protein kinase (MAPK) and NF-κB.	[Alexopoulou *et al.*, 2001] [Kawai *et al.*, 1999] [Hacker *et al.*, 2000; Hemmi *et al.*, 2002] [Medzhitov *et al.*, 1998]
MAL/TIRAP (Myd88-adaptor-like/TIR-associated protein)	It acts as an essential bridging adaptor for Myd88 in the context of TLR 2 and TLR 4 signaling.	[Yamamoto *et al.*, 2002]
TRIF/TICAM-1 (TIR domain-containing adaptor inducing *IFN-β* also called TIR-domain-containing adaptor molecule 1)	It is the sole adaptor protein implicated in the TLR 3 signaling pathway in response to double-stranded RNA In addition, TRIF is also associated with the LPS-recognition. It has been observed that LPS could trigger the expression of the proinflammatory gene, *NF-κB*, through TLR-4 signalling pathway in a TRIF-dependant manner.	[Yamamoto *et al.*, 2003] [Covert *et al.*, 2005]
TRAM/TICAM-2 (Toll-receptor-associated molecule also called TIR-domain-containing adaptor molecule 2)	It is a linking adaptor allowing the activation of the TRIF-dependent pathway in response to LPS.	[Oshiumi *et al.*, 2003]
SARM (Sterile alpha motif and armadillo motif domain-containing protein)	The last of the five adaptors identified. Initially discovered in *Caenorhabditis elegans,* SARM has been described to downregulate the TRIF-dependant pathway by acting directly on TRIFF adaptor. However, its function remains unclear.	[Mink *et al.*, 2001] [Couillault *et al.*, 2004]

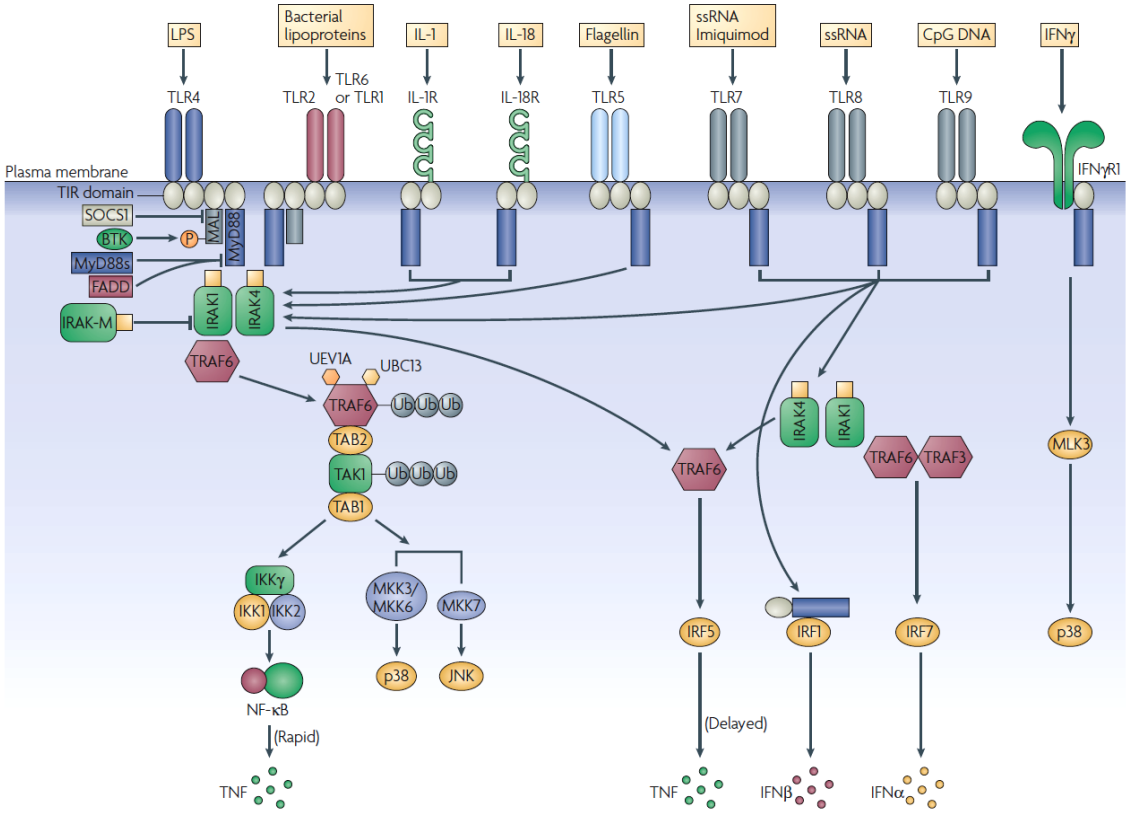


Figure 10 Myd88 signaling. MyD88 is the key signaling adaptor for all TLRs (with the exception of TLR3 and certain TLR4 signals), IL-1R and IL-18R. Its main role is the activation of NF-κB. My88 acts to recruit IRAK4 (IL-1R-associated kinase 4). This leads to a pathway involving IRAK1, TRAF6 (tumour-necrosis-factor-receptor-associated factor 6), TAK1 (transforming-growth-factor-β-activated kinase) and the ubiquitylating factors UEV1A (ubiquitin-conjugating enzyme E2 variant 1) and UBC13 (ubiquitin-conjugating enzyme 13), which modify and activate TRAF6 and TAK1. This leads to the activation of the inhibitor of NF-κB kinase [Lander *et al.*] complex and NF-κB, and the upstream kinases for p38 and JNK (JUN N-terminal kinase). MyD88 is targeted by negative regulators, such as a shorter form known as MyD88s, which interferes with IRAK4 recruitment, and FADD (FAS-associated via death domain). IRAK-M can also inhibit signalling by preventing the release of IRAK1 and IRAK4 from MyD88. MyD88 also couples to interferon-regulatory factor 5 (IRF5) and IRF1. In the latter case, MyD88 traffics to the nucleus with IRF1. In the case of TLR2 and TLR4 signalling, a bridging adaptor, MAL (MyD88-adaptor-like protein), is required for MyD88 recruitment. This is subject to regulation by BTK (Bruton's tyrosine kinase) and SOCS1 (suppressor of cytokine signalling 1), which promotes MAL degradation. In the case of signalling by TLR7, TLR8 and TLR9, the MyD88–IRAK4 pathway also leads through TRAF6 and TRAF3 to the activation of IRF7. Finally, IFNγR1 (interferon-γ receptor 1) can also engage with MyD88, and through mixed-lineage kinase 3 (MLK3) leads to p38activation. Target genes for each of these pathways are shown. MKK, mitogen-activated protein kinase kinase; TAB, TAK1-binding protein; TNF, tumour-necrosis factor; Ub, Ubiquitin. [O'Neill and Bowie, 2007]

4.2.3. Pleiotropic function of TLRs: initiation of innate immune response

TLRs are expressed on a wide variety of cell types including innate immune cells (M∅s, DCs, PMNs) and adaptive immune cells (B and T cells), but also non hematopoietic cellular lineages such as fibroblasts, endothelial and epithelial cells. TLRs are directly linked with a variety of defense mechanisms involved in bacterial killing or preventing viral replication [Takeda *et al.*, 2003]. For example, the engagement of TLRs signaling in M∅s and PMNs, induces production of reactive oxygen and nitrogen intermediates, antimicrobial proteins and peptides, as well as other effector molecules that help to destroy infectious agent [Huang *et al.*, 2001] [Medzhitov, 2007] (See chapter 5).

In addition, TLR signals are also involved in the regulation of defense processes such as autophagy[12] [Sanjuan *et al.*, 2007]. In human monocytes infected with the intracellular parasite *Mycobacterium tuberculosis*, TLR2/1 stimulation has been shown to robustly activate antibacterial autophagy through vitamin D receptor signaling activation and cathelicidin induction [Shin *et al.*]. In terms of inflammatory response, TLRs also induce M∅s to produce pro-inflammatory cytokines, including TNF, IL-1β and IL-6, which coordinate local and systemic inflammatory mechanisms [Medzhitov, 2007]. In addition to driving inflammatory responses, TLRs also regulate cell proliferation and survival which serves to expand useful immune cells and integrate inflammatory responses and tissue repair processes [Li *et al.*]. For supplementary information concerning the subject of this section see [Kawai and Akira].

[12] In cell biology, autophagy, or autophagocytosis, is a catabolic process involving the degradation of a cell's own components through the lysosomal machinery [Kundu and Thompson, 2008]. Autophagy is also emerging as a key antimicrobial strategy involved in intracellular pathogen resistance [Schmid and Munz, 2007].

4.2.4. Pleiotropic function of TLRs: Initiation of adaptive immune response.

Some of the TLRs-induced signals also have important roles in the initiation of the adaptive immune responses and are involved in many regulatory pathways [Banchereau and Steinman, 1998] [Hemmi and Akira, 2005].

The most important aspect of TLR functions in terms of adaptive immunity is the control of APC activation. Accumulating evidence indicates that TLR are capable of inducing distinct responses in DCs and other APCs, and can direct T cell differentiation in opposing directions. The generation of such varied responses is achieved through selective utilization of adaptor molecules linking TLRs to distinct signal transduction pathways. Reader are referred to the following reviews for more details [Zanoni and Granucci; Kaisho and Akira, 2003; Re and Strominger, 2004]

Notably, it has been shown that TLRs expressed on DCs link microbial recognition with T cell activation and consequently play a central role in the innate control of the adaptive immunity. When localized in the tissues, before they have encountered pathogens, DCs are in a so-called immature state characterized by high endocytic capability and low T-cell stimulation ability. During an infectious process, immature DCs scan the inflamed tissue and recognize pathogens through their PRRs. TLR-mediated recognition initiate intracellular signaling pathways that lead to induction of a differentiation process called DC maturation. First, TLRs engagement initiates the upregulation of genes involved in antigen processing and presentation. Second, TLRs ligation leads to the expression of costimulatory molecules on DCs, greatly improving their ability to prime naïve T-cells. Third, TLRs activation induces the migration of DCs from tissues to lymphoid organs. Finally, DCs that have recognized pathogens via TLRs express high level of cytokines such as IL-12 [Dalod *et al.*, 2002]. The ultimate goal of mature DCs is to present microbial-derived peptides to naïve T-cells in the right context to initiate the effective adaptive response. In this sense, the recognition of infectious nonself by TLRs, link APCs to induction of adaptive immunity.

5. Killing Pathogen and Shaping Immune Response

The goal of the innate immune cells is to mount the most rapid and effective immune response against invading pathogens without causing excessive tissue damage and in the meantime to facilitate and stimulate the development of a specific and lasting adaptive immunity [Medzhitov, 2008].

In order to control microbial replication and spread, the innate immune cells use a collection of distinct strategies that carry out different functions in host defense. These "modules" include phagocytosis, reactive oxygen and nitrogen species production and granulomic aggregation. These mechanisms are at the edges of a large range of means employed by the innate immunity to subvert the strategies of successful pathogens.

5.1. Phagocytosis

The internalization and subsequent destruction of pathogens by innate immune cells are fundamental steps of the immune response that promote Ag presentation and development of adaptive immunity. Central to these events is the generation of a phagosome, the organelle that forms around internalized material by a process termed, phagocytosis.

Phagocytosis is the mechanism of taking in particulate material (≥ 0,5 μm), in an actin dependent manner, by specialized cells. It involves a chain of carefully orchestrated molecular processes including, adhesion, ingestion, and digestion [Flannagan *et al.*, 2009], which begin with the engagement of phagocyte receptors and culminates by the activation of numerous signaling pathways. These cascades coordinate an orderly progression of changes including rearrangement of the cytosqueleton, membrane remodeling, and phagosome maturation [Blander and Medzhitov, 2006; Hall *et al.*, 2006].

Importantly, phagocytosis is an essential cellular mechanism performed by unicellular organisms and many cell types found in metazoans such as the slime mould, *Dictyostelium discoideum* [Chen *et al.*, 2007]. In higher organisms, the phagocytosis process is a predominant feature of several motile cells of hematopietic origin known as "professional" phagocytes, including MØs, PMNs and DCs. These cells present a remarkable ability to internalize foreign material and can engulf particles larger than their own surface area [Djaldetti *et al.*, 2002].

Two main classes of targets are removed by phagocytosis: microorganisms and "altered self" particles, like apoptotic cells[13].

5.1.1. Phagocytosis: a matter of housekeeping

Engulfment of apoptotic cells is an essential mechanism implicated in tissue remodeling, embryogenesis and tissue homeostasis that occurs when excess cells undergo programmed cell death [Tepass *et al.*, 1994; Zhou *et al.*, 1995; Blander and Medzhitov, 2006]. This scavenging function is realized through the detection of a number of molecules that are normally absent on live and healthy cells [Gardai *et al.*, 2006]. These include surface expression of calreticulin and exposure of phosphatidylserine on the outer leaflet of the plasma membrane, as well as the expression of amino sugars and lysophospholipids, which are recognized by various types of receptors. In contrast normal cells express specialized signals such as CD47 that engage inhibitory receptors such as SIRPα that blocks phagocytosis [Oldenborg *et al.*, 2000; Gardai *et al.*, 2006].

Phagocytosis of apoptotic cells is accompanied by triggering of anti-inflammatory responses, such as production of TGF-β and can specifically inhibit the expression of several cytokines, including IL-12 [Kim *et al.*, 2004; Henson and Hume, 2006]. Of note, the specific receptors involved in the suppression of the inflammatory response are poorly characterized but they seem to involve the Tyro-3-family receptor tyrosine kinases [Aderem and Underhill, 1999].

[13] A cell that has died because of a genetically regulated program of of cell death. Cell contraction, chromatin condensation, cell membrane budding and DNA fragmentation characterize this process. Eventually, the cell blow up into many-membrane bound apoptotic bodies, which are phagocytosed by neighboring cells.

5.1.2. Phagocytosis: a matter of host-defense

During pathogen invasion, phagocytosis is also the spearhead of the early innate immune response and the main host-defense mechanism of many specices [Hoffmann *et al.*, 1999].

Phagocytosis of microorganisms is a process designed to eliminate pathogens, but it also contributes to generate an inflammatory context, including TNF-α, IL-1 and IL-6, which is essential to warn the immune system of the presence of an infection [Aderem and Underhill, 1999]. The difference in outcome for apoptotic cells versus microbial cell phagocytosis is determined initially by the engagement of TLRs during inflammatory phagocytosis of pathogens and not during noninflammatory phagocytosis of by-products of the host physiology[11] [Blander and Medzhitov, 2006].

Upon phagocytosis of microbial pathogens, surface TLRs, including TLR2 and TLR4, are recruited and react with bacterial cell wall components [Underhill *et al.*, 1999]. Then, TLRs signaling activates an inducible mode of phagosome maturation characterized by distinct kinetics and functional consequences such as increased clearance and phagolysosomal fusion rate as well as controlled of DCs maturation[14] [Blander and Medzhitov, 2006] [Heath and Carbone, 2001]. The uptake of microbes is not *per-se* mediated by TLRs but rather by a number of specific phagocytic receptors (see next section) that cooperate with TLRs in the induction of inflammatory responses [Gantner *et al.*, 2003; Brown, 2006].

The central role of phagocytosis in host-defense is emphasized by observations that many pathogen factors directly manipulate the outcome of the phagosome (see chapter 6) |**Fig.12**.

[14] Notice that TLRs engagement has been linked to the internalization of apoptotic cells in the context of DCs maturation. DCs can phagocytose apoptotic cells, which do not activate them in the absence of TLRs but rather result in a tolerogenic state that often leads to deletion of autoreactive T cells [Heath and Carbone, 2001].

5.1.3. Cell-surface recognition: the ability to make the difference

Phagocytosis is initiated following the ligation of cell-surface receptors that either directly bind to the particle or to opsonins[15] that are deposited on the particle surface [Litvack and Palaniyar; Taylor *et al.*, 2005]. The impressive variety of existing phagocytosis-inducing receptors illustrates the importance of the phagocytic process in cellular physiology (for review [Taylor *et al.*, 2005]) | **Table 5**. The precise nature of these receptors as well as their interactions and functions are still largely elusive. Principally, these are classified regarding the nature of recognized-ligands. For instance, scavenger receptors are structurally unrelated multi-ligand receptors which share the ability of binding polyanionic ligands [Taylor *et al.*, 2005; Stuart and Ezekowitz, 2008]. They have been described to attach a wide range of pathogens and modified self-ligands, and are implicated in both, the housekeeping and the protection responses.

Interestingly, the ability of these receptors to discriminate among different classes of organisms, as well as modified host cells, remains poorly understood and may not fit with the concept of PRRs [Taylor *et al.*, 2005].

5.1.4. The phagosome: a great complexity behind a simplistic appearance.

After engulfment, the microbe is trapped in a vacuole derived from the plasma membrane, termed phagosome, which undergoes a maturation process to acquire the bactericidal and degradative features required to destroy the pathogen |**Fig.11**. This conversion, known as phagosomal maturation, is realized through a choreographed sequence of vesicles fusion and fission with the maturing endosomes that ultimately deliver its content to lysosomes for degradation. Rab proteins and ADP-ribosylation factor GTPases are important protein actors implicated in directing and tethering the endomembrane to form the phagosome, whereas soluble NSF-attachment protein receptor (SNARE) proteins mediate docking and fusion of cellular membrane (for review see [Stow *et al.*, 2006]).

[15] Opsonins are soluble host factors, such as soluble PRRs, immunoglobulin G (IgG), and components of the complement cascade that when attached to the pathogen surface, acquire a conformation recognized by phagocyte receptors, such as Fcγ receptors (FcγRs) and the complement receptor 3 (CR3) and enhances uptake of the particle by phagocytosis [Palaniyar] [Daeron, 1997].

Table 5| Overview of MØs receptors implicated in phagocytosis [Taylor *et al.*, 2005]		
Receptor family	Example	Function (example)
Scavenger receptor (SR) (collagenous)	SR-A	Phagocytosis of bacteria and apoptotic cells, Endocytosis of modified low density lipoprotein, Adhesion
Scavenger (noncollagenous)	CD36	Phagocytosis of apoptotic cells, Diacyl lipid recognition of bacteria
GPI-anchored	CD14	LPS-binding protein/interactions MD2/MyD88, TLR signaling, Apoptotic cell recognition
Integrin	Complement Receptor (CR) 3 (CD18/11b)	Complement receptor (C3bi) mediated phagocytosis, Adhesion to endothelium
Ig Superfamily	FcR (ITAM/ITIM)*	Antibody-dependent binding, uptake, and killing
Seven transmembrane	TREM-1 (ITAM)*	Regulation of inflammation
	CCR2	Receptor for MCP-1
	C5aR	Chemotaxis, degranulation
	EMR2 (EGF-TM7)*	Myeloid cell adhesion, Chondroitin sulphate binding
NK-like C-type lectin-like	Dectin-1 (ITAM-like)	β-glucan receptor, fungal particle ingestion TNFα release/interaction TLR2

*ITAM, immunoreceptor tyrosine-based activation motif; TREM, Triggering Receptors Expressed by Myeloid Cells; ITIM, immunoreceptor tyrosine-based inhibition motif; EMR, epidermal growth factor (EGF)-module-containing mucin like hormone receptor;

The phagosome maturation starts immediately after scission from the surface membrane. Then, the nascent phagosome undergoes successive fusions with early endosomes, late endosomes, and lysosomes [Desjardins *et al.*, 1994] **| Box 5**. The sequential fusion events provoke a remodeling of the membrane of the phagosome that is accompanied by changes in the composition of its lumen, which becomes progressively an acidic, oxidative and degradative environment. This maturation process culminates with the formation of the so-called phagolysosome, the ultimate bactericidal organelle. Phagolysosomes are equipped with a sophisticated arsenal to eliminate microorganisms. They are highly acidic (lumen pH lower than 4,5), enriched in antimicrobial proteins and peptides, and characterized by a high production of reactive oxygen and nitrogen species.

Box 5 | Stages of phagosome maturation	
The early phagosome	A mildly acidic compartment (pH 6,1 – 6,5) that has a propensity to fuse with sorting and recycling endosomes and that are refractory to fusion with lysosomes [Desjardins *et al.*, 1997]. This stage is mainly governed by the small GTPase Rab5, which integrates the tethering and fusion of early endosome and is involved in the dynamic of the organelle [Bucci *et al.*, 1992; Kitano *et al.*, 2008].
The late phagosome	The late stage is characterized by a more acidic luminal pH (5,5 – 6,0) resulting from the acquisition of additional proton-pumping V-ATPase and an enrichment of proteases and lysosome–associated membrane proteins (LAMPs), which are either imported from the golgi complex or acquired by fusion with late endosomes [Desjardins *et al.*, 1994]. The small GTPase Rab7A is a characteristic marker of this organelle, and is known to mediate the traffic up to lysosomes [Harrison *et al.*, 2003].
The phagolysosome	The phagolysosome is the ultimate microbicidal organelle, which is endowed with a complete and sophisticated arsenal to eliminate and degrade microorganisms. They originate from the fusion with lysosomes trhough a Rab7A-dependent process and are highly acidic (pH 4,5). This organelle can be differentiated from late phagosomes by their elevated mature cathepsin content and by their lack of mannose-6-phosphate receptors [Griffiths *et al.*, 1988; Flannagan *et al.*, 2009].

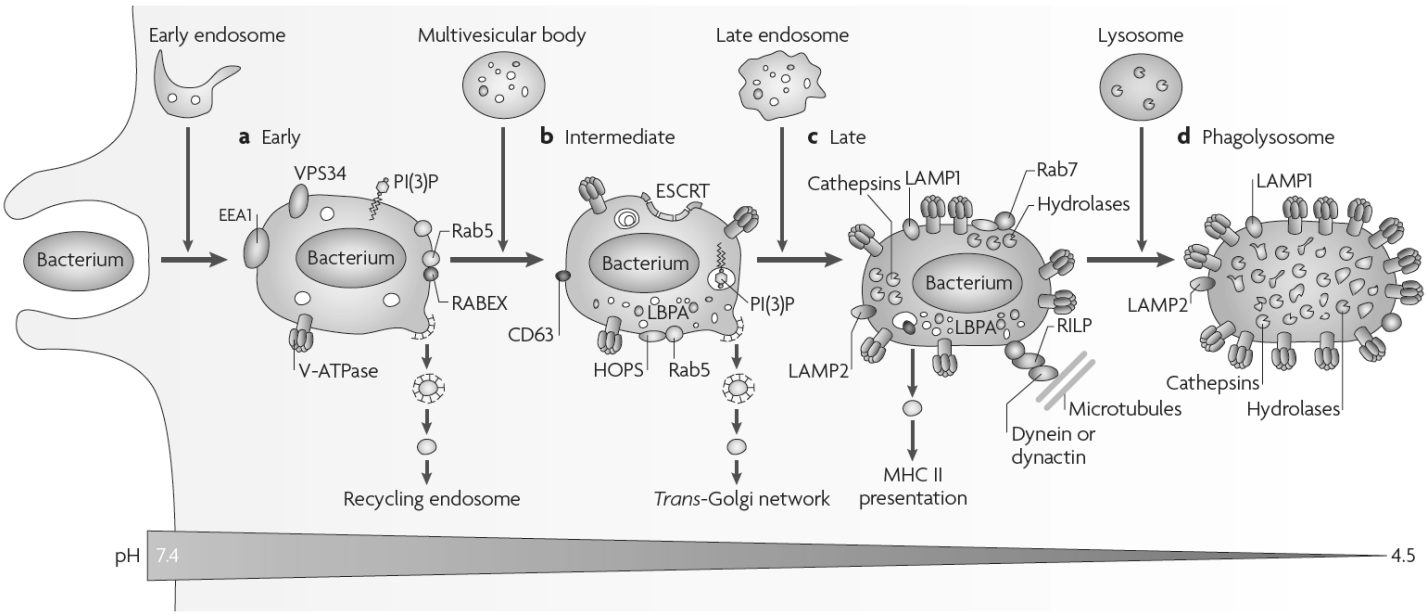


Figure |11 The phagosome maturation. Following the engulfment of the microbe, the phagosome interacts with sevral components of the endocytic pathway leading to a sequential transformation process that culminates with the formation of phagolysosomes. The maturation of the phagosome can be divided into four distinct stages: early phagosome (a), intermediate phagosome (b), late phagosome (c), phagolysosome (d). During transformation, the phagosome acquire a series of proteins such as hydrolase and undergoes a progressive acidification resulting from the action of V-ATPase. EEA1, early endosome antigen; ESCRT, endosomal-sorting complex required for transport; HOPS, homotypic protein sorting; LAMP, lysosomal-associated membrane protein; LBPA, lysobisphosphatidic acid; PI[Cole *et al.*]P, phosphatidylinositol-3-phosphate; MHCII, major histocompatibility complex II; RILP, Rab-interacting lysosomal protein. [Flannagan *et al.*, 2009]

5.1.5. The phagosome: better not to be inside

Acidification of the phagosome, production of antimicrobial proteins and peptides, and generation of reactive radicals are elements of the sophisticated microbicidal arsenal developed by the phagolysosomes |**Fig.12**.

- *Acidification of the phagosome*: Phagosomal acidification arises from the activity of a cytoplasmic V_1 complex that hydrolyses adenosine tri-phosphate (ATP) and transfers the energy to a membrane-embedded V_0 complex that translocates H^+ across the lipid bilayer of the organelle [Beyenbach and Wieczorek, 2006]. This progressive accumulation creates a hostile environment that impedes microbial growth [Huynh and Grinstein, 2007]. Besides the direct impact on the bacterial metabolism, the transmembrane H^+ gradient generated by the V-ATPase also favors the activity of many hydrolytic enzymes that have acidic pH optima and extrudes essential microbial nutrients from the phagosomal lumen.

- *Antimicrobial proteins and peptides:* More-direct mechanisms deployed by phagosomes to antagonize bacterial growth involve a set of proteins that either limit the availability of essential nutrients inside the compartment or directly compromise the integrity of the microorganisms. Lactoferrin, a glycoprotein belonging to the first group of protein, is released into the phagosome lumen, where it sequesters iron that is essential to bacteria [Masson *et al.*, 1969]. Natural resistance associated M∅s protein 1 (NRAMP1) exerts a bacteriostatic effect by extruding Fe^{2+}, Zn^{2+}, Mn^{2+} from the phagosome lumen [Cellier *et al.*, 2007]. These divalent cations are cofactors of microbial housekeeping and key protective enzymes. Phagocytes also contain small peptides such as defensin and cathelicidins that act by permeabilizing the inner membrane of Gram-positive bacteria and the outer and inner membranes of Gram-negative bacteria. In addition, phagosomes are equipped with an assortment of endopeptidases, exopeptidases and hydrolases that degrade various microbial components [Claus *et al.*, 1998; Pillay *et al.*, 2002] [Lehrer *et al.*, 1993; Zanetti, 2005].

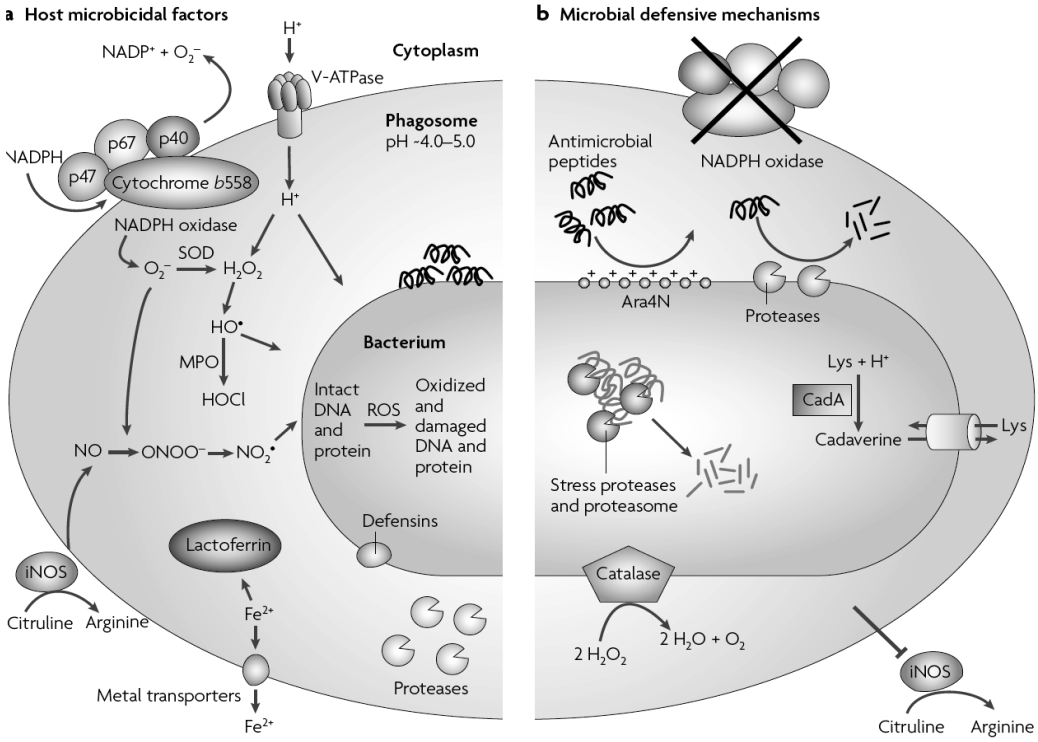


Figure 12 David versus Goliath: the bactericidal arsenal of the phagolysosome facing the defense mechanisms of microorganisms. (**a**) The host microbicidal armamentarium includes radical species, iron scavenger and exporters such as lactoferrin and natural resistance-associated MØs protein 1 (NRAMP1), as well as antimicrobial peptides and proteins that permeabilize and degrade the bacteria. (**b**) The bacterial defensive factors include surface modified structures that resist or break down antimicrobial peptides and expression of a garrison of enzymes, such as catalase and stress proteases, that neutralize reactive species and degrade antimicrobial peptides, respectively. [Flannagan *et al.*, 2009]

5.2. Reactive oxygen and nitrogen species (ROSs, RNSs)

Phagocyte-derived reactive oxygen and nitrogen species are of crucial relevance for host resistance against microbial invaders. Both are expressed in phagocytic cells, although the amount of ROSs produced by neutrophils is significantly greater than that generated by MØs and conversely, MØs produce considerably more RNSs than neutrophils [Nathan and Shiloh, 2000].

Superoxide ($O_2^{\cdot -}$) radicals and other oxygen-derived intermediates that can modify organic molecules are referred as ROS, whereas nitric oxide ($NO^{\cdot}$) and its derivatives are collectively known as RNSs. ROSs and RNSs are part of the most powerful micobicidal mechanisms of phagocytic cells. They are generated by the NADPH phagocyte oxydase (also know as phox) and inducible (or independent) nitric oxide synthase (iNOS) pathways, respectively. Beyond their antimicrobial role, both ROSs and RNSs have essential functions in a broad range of physiological processes. It includes signal transduction, regulation of vascular tone, tissue injury and also control of inflammation. Readers are referred to [Fang, 2004] for a relevant source of information.

Experiments using phox- and iNOS- deficient mice have sufficiently proved the relevance of these enzymes in the growth control of many pathogens such as *Mycobacterium tuberculosis*, *L.monocytogenes*, and *Leishmania* spp (reviewed in [Nathan and Shiloh, 2000]). These and other studies have emphasized the sequential roles of ROSs and RNSs in the destruction of pathogens. ROSs seem to have an meaningful role in the early host response to infection, after which RNSs have a sustained function on limiting residual bacterial replication [Mastroeni *et al.*, 2000; Nathan and Shiloh, 2000]. According to this hypothesis, it can be suggested that RNSs might have a particularly purposeful role in the maintenance of latent infections [Stenger *et al.*, 1996; Scanga *et al.*, 1999; Voskuil *et al.*, 2003].

ROSs and RNSs can interact with numerous targets in a microbial cell, including thiols, metal centers, protein tyrosines, nucleotide bases and lipids [Nathan and Shiloh, 2000]. Moreover, although the products catalyzed by the phox and iNOS are $O_2^{\cdot -}$ and $NO^{\cdot}$, respectively, subsequent reactions involving both molecules can generate additional intermediates with different reactivities and biological activities [Fang, 2004] |**Fig.13**. This variety of ROSs and RNSs endows the phagocytic cells with an impressive and effective armory.

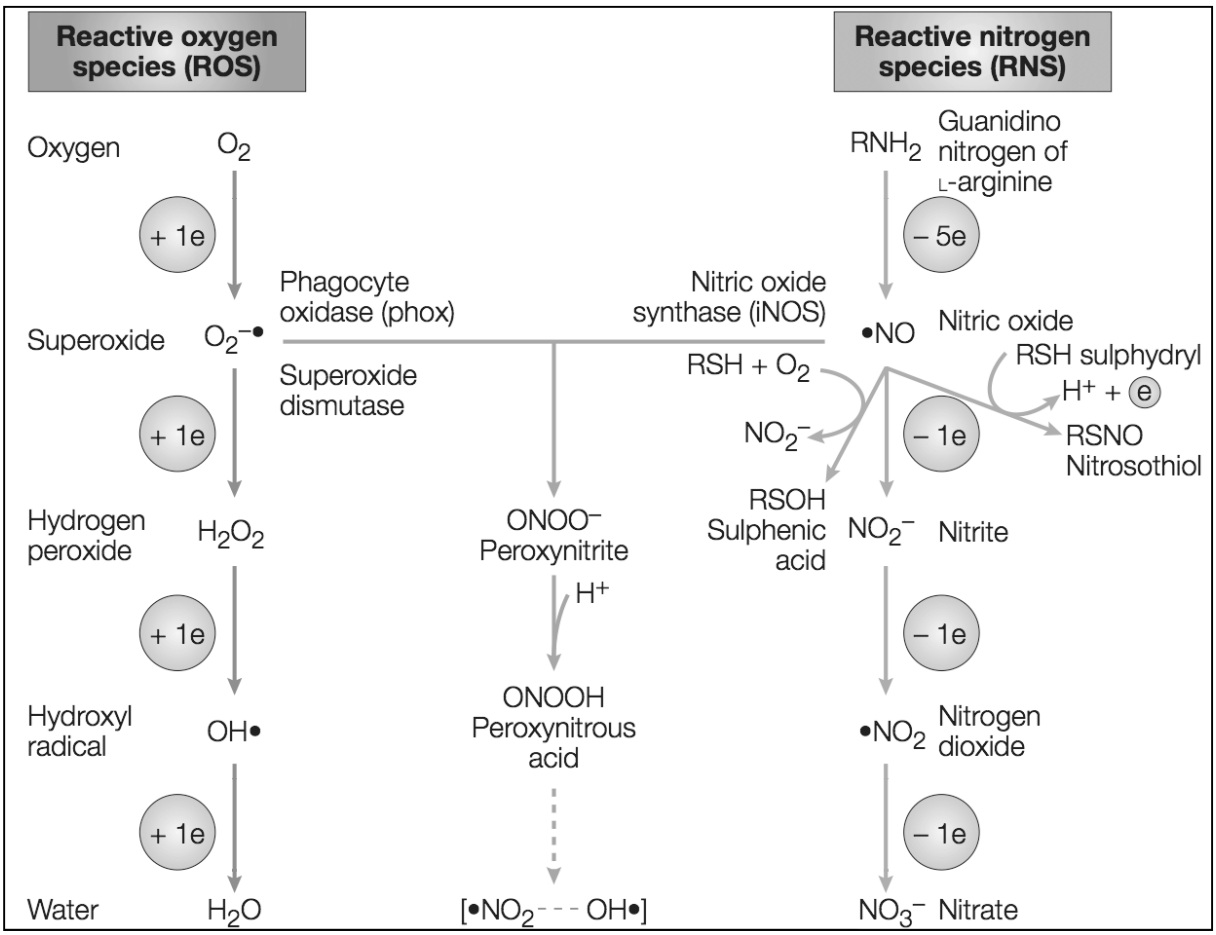


Figure |13 Reactive oxygen and nitrogen intermediates production in mammalian cells. ROS and RNS are generated by different enzymatic pathways in phagocytes. ROS cascade begin with the reduction of di-oxygen (O_2) to superoxide ($O_2^{\cdot-}$) by the phagocyte oxide (phox) The production of NO and its intermediates derive from the oxidation of one molecule of L-arginine at a guanidine nitrogen by the inducible nitric oxide synthase (iNOS). The reaction of ROS and RNS leads to the generation of peroxynitrite ($ONOO^-$) and peroxynitous acid (ONOOH), which spontaneously decompose to a series of species that resemble the reactive radical hydroxyls ($OH^{\cdot}$) and nitrogen dioxide ($NO_2^{\cdot}$). [Fang, 2004]

5.3. Granuloma Formation: an arm wrestling between Host and Pathogen

When the innate immune cells are not able to eliminate intracellular microorganisms by the sole utilization of their bactericidal armament, the immune system may engage a sophisticated confinement program, which consists in the recruitment and gathering of innate immune cells at individual infected foci to form cellular complex called granuloma. The pathological definition of a granuloma is an organized collection of differentiated cells (mostly MØs and lymphocytes) with a characteristic morphology [Adams, 1976]. Although the function and cellular composition can be different, granulomas are formed in response to several persistent pathogens, such as *Mycobacterium, Listeria* or *Brucella* spp. as well as to foreign bodies [Cosma *et al.*, 2003] [Kang *et al.*, 2008] [Davis *et al.*, 2002] [Stavitsky, 2004].

5.3.1. The Granulomas formation: Mycobacterium *shows the way*

Being inhaled as droplets, *Mycobacterium tuberculosis*, the causative agent of tuberculosis, is immediately trapped by alveolar MØs in the lungs where it causes a local proinflammatory response that leads to the recruitment of mononuclear cells from neighbouring blood vessels [Adams, 1976; Davis *et al.*, 2002]. Recruited monocytes undergo a further maturation process reflected by an increase in their size and bactericidal capabilities and followed by a subsequent differentiation into epithelioid cells [Adams, 1976; Bouley *et al.*, 2001] that may fuse to form giant syncytial cells. Then, effector T lymphocytes and other immune cells are recruited to the site of infection and fortify the scaffold of the granulomas structure. This step defines the "containment" phase of the infection during which there is no clinical manifestation of the disease. Changes in the immune status of the host lead to modifications in the granulomas structure reflected by the emergence of areas of caseous necrosis, that is, regions of acellular debris that have distinct pathological appearance [Adams, 1976; Cosma *et al.*, 2003].

5.3.2. Behind the structure

The molecular mechanisms involved in granulomas formation are not yet understood and are likely to be complex. Notably, TNF-α, a key protective cytokine in tuberculosis, was thought to mediate protection by promoting granulomas formation, at least in part by modulating the expression of key chemokines and their cognate receptors [Flynn and Chan, 2001; Algood *et al.*, 2003; Peters and Ernst, 2003]. The precise role of this cytokine is a controversial topic. However, TNF-α signaling is not required for the primordial step of the structure formation [Clay *et al.*, 2008]. Rather, its role in early protection appears to be associated to the maintain of the overall architecture integrity as observed in a murine model, where TNF-α neutralization therapy resulted in fatal reactivation of persistent tuberculosis that was characterized by severe histopathological deterioration in the mouse lungs [Mohan *et al.*, 2001; Miller and Ernst, 2008]. More recent data demonstrate that TNF-α may contribute to the granulomatous response by exerting an anti-inflammatory effect through modulation of the expression of pro-inflammatory mediators [Chakravarty *et al.*, 2008].

As *M. tuberculosis* establishes an infection, the local organization of the lung is modified to facilitate innate cells infiltration and initiation of granulomas formation. While the underlying mechanism is still poorly understood, it has been found that matrix metalloproteinases (MMPs) play a role [Volkman *et al.*; Elkington and Friedland, 2006]. The MMPs are members of a family of zinc-dependent proteases, which have the ability to degrade the stable collagen backbone of the lung extracellular matrix (ECM) [Page-McCaw *et al.*, 2007]. Infection with *M. tuberculosis* has been shown to induce the secretion of MMP-1, -2, -7, -9 from cultured peripheral blood mononuclear cells and human airway epithelial cells [Elkington *et al.*, 2005; Rand *et al.*, 2009]. In advanced human tuberculosis, induced expression is reported in some monocytes and multinucleated giant cells driving necrotic centers of lymph node granulomas and in epithelial cells proximal to lung granulomas [Volkman *et al.*; Price *et al.*, 2003; Zhu *et al.*, 2007]. Importantly, analysis of *M. marinum* granulomas in zebrafish larvae has shown that MMP expression was strictly localized to epidermal cells immediately adjacent to infected M∅s, whereas overlying peridermal cells did not produce these proteins [Volkman *et al.*]. Strikingly, every granulomas, including the smallest identifiable M∅s aggregate had proximate *MMP*-expressing epithelial cells. Thus, MMPs induction seems to be critical for granulomas formation from the very earliest stages and probably in later stages.

5.3.3. *The* Listeria monocytogenes *Granulomas*

In a mouse model, Kang and his collaborators have also described the hierarchical organization of a granulomatous response after an infection with *L. monocytogenes* [Kang *et al.*, 2008]. Their experiments have shown that the granulomas formation is firstly orchestrated by infected DCs in the spleen. By a process involving the production of IL-12 and IL-18, monocytes and neutrophils rapidly cluster at the foci of bacterial infection localized near the terminal arterioles in the T lymphoid sheath of the splenic white-pulp. Chemoattractants, including CCR5 ligands, then recruit and localize NK cells around the nascent cellular aggregate. This novel cellular arrival results in IFN-γ expression, which is essential for the maturation of monocytes to TipDCs. Their finding, consistent with previous reports suggests that granulomas facilitate the entry and early expansion of *Listeria* within these collection of clustered white blood cells in an attempt to localize and restrain the infection to sites allowing later controls by the adaptive immune system.

5.3.4. *Granulomas: illustration of the "*cui bono*" concept*

If granulomas have long been considered as a host defense mechanism for containing persistent pathogens, Davis and Ramakrishnan have recently suggested, using imaging techniques in transparent zebrafish embryos, that granuloma formation are not necessarily beneficial to the host, at least in the early stages of infection [Davis and Ramakrishnan, 2009] |**Fig.14**. Instead, they have shown that mycobacterial expansion in early granulomas is driven by a continual cycle of apoptosis of infected cells and their subsequent phagocytosis by newly recruited M∅s. The initiation of the adaptive immunity, which correlates with the formation of secondary granulomas, then seems to halt bacterial expansion not by forming new granulomas structures as suggested by previous model [Andersen, 1997; Saunders and Cooper, 2000] but rather by modifying the early granulomas into a state of equilibrium between host and pathogen [Davis and Ramakrishnan, 2009].

Moreover, although TNF-α is generally considered to be required for host containment of tuberculosis as discussed above, a recent study indicates that *M. tuberculosis* increases TNF-α levels secreted from infected M∅s by direct interference with intracellular second messenger signaling such as intracellular cyclic adenosine monophosphate (cAMP) [Agarwal *et al.*, 2009]. Following infection, a *M. tuberculosis* adenylate cyclase, was found to elicit an increase

cAMP response-element-binding protein (CREB) activation, and a significant TNF-α upregulation. *M. tuberculosis* mutant lacking this protein survives poorly in mouse infection and gives reduced lung pathology. This study raises the possibility that at certain phases of the infection cycle, the microbe provokes an excessive TNF-α response as part of a virulent strategy.

Another example that emphasized how *M. tuberculosis* can stimulate and facilitate granulomas formation, is given by its capacity to modulate the expression of MMPs [Taylor *et al.*, 2006]. The mycobacterial protein 6-kd early-secreted antigenic target (ESTA-6) induced MMP-9 production by epithelial cells close to infected MØs [Volkman *et al.*]. As already discussed, MM9 enhanced the recruitment of MØs, which contributed to nascent granulomas maturation. Studies of *M. tuberculosis* infection in MMP-9 deficient mice indeed revealed that MMP9 is required for full bacterial proliferation, as these animals showed reduced bacterial burden and decreased lung MØs recruitment compared with wild type. Concomitantly, the MMP9 knockout mice displayed reduced ability to produce well-formed granulomas.

Together, these observations suggest that mycobacterial granulomas, thought to curtail infection for over a century, is now viewed as an essential innate immune mechanism for the microbe expansion.

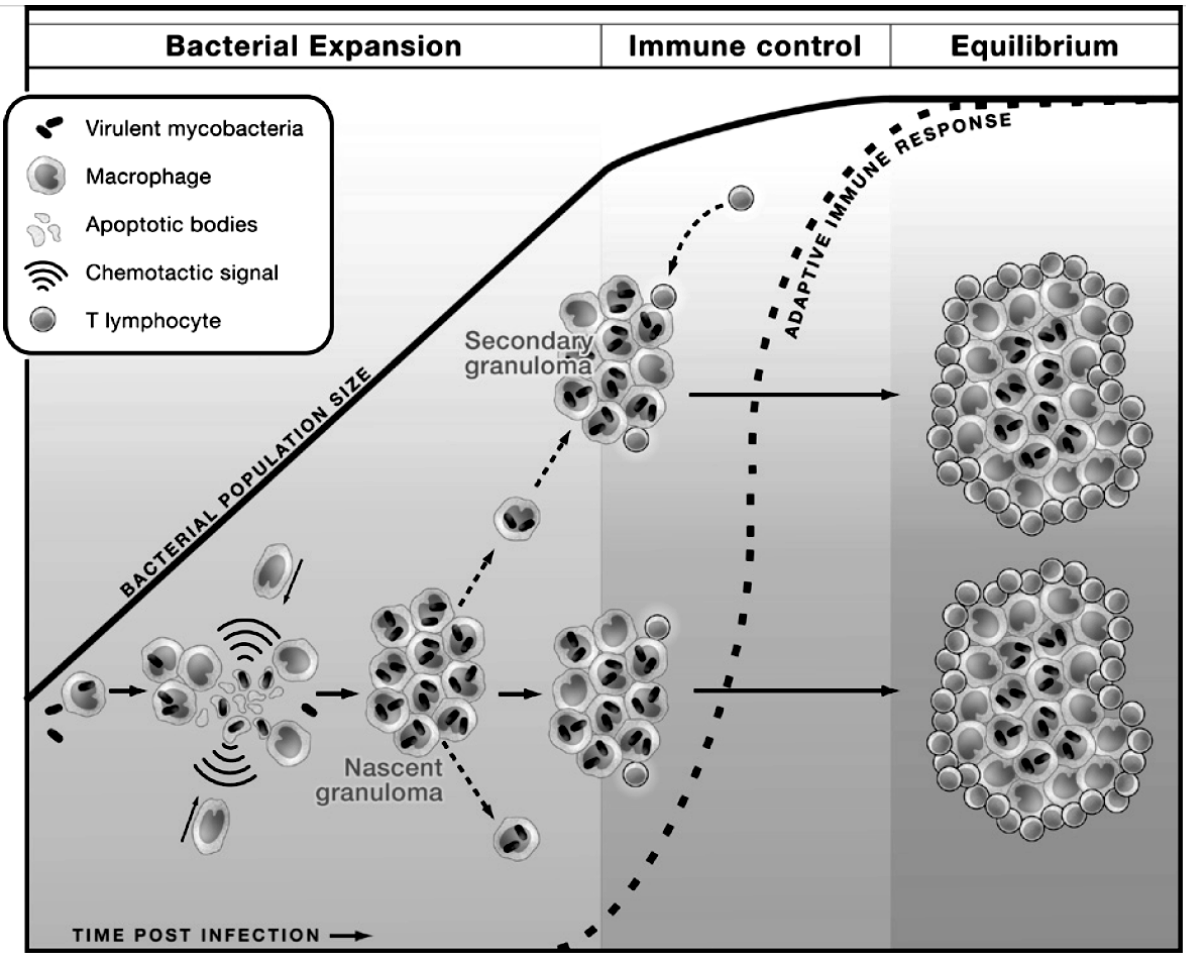


Figure 14 Stages of granulomas maturation during *Mycobacterium* Infection. The granulomas formation starts with the clustering of innate immune cells to the site of infection, which facilitates the early expansion of the bacterium. Microbe multiplication occurs through the phagocytosis of apoptotic-infected particles by recruited uninfected macrophages. Some newly infected cells egress from the nascent granulomas and contribute to the formation of secondary granulomas in the tissue. The initiation of adaptive immunity is correlated to the control of bacterial spreading. The mature granulomas represents an equilibrium between the bacterium and the host immune response. [Bold and Ernst, 2009]

6. Immune response to successful intracellular pathogens

The concept of "detrimental-granulomas" illustrates the host-pathogen relationship complexity: multicellular organisms have evolved sophisticated immune mechanisms to counter the continued microbial insult of the environment. However, millions of years of co-evolution have endowed successful pathogens with a range of strategies for hijacking antimicrobial defenses and surviving within an infected host.

Although the rudiments of these strategies are similar for most pathogens i.e., – activation of virulence programs that enable them to access, develop and persist in host compartments that are generally off limits to microbes –, the underlying cellular and molecular mechanisms vary substantially. Depending on the niche that they colonize, pathogens have elaborated virulence factors that allow a range of activities such as penetration of surface epithelia, invasion of intracellular compartments and evasion of host defense mechanisms. Accordingly, host immune responses to different invaders are diversified, being influenced by the route of infection, the cell type that is invaded and the microbial molecules that are revealed to innate immune receptors of the host cells. Therefore, it is not surprising that immune responses to different pathogens differ markedly.

Because the present work is focused on the intracellular pathogen, *Brucella melitensis*, the following section will present the immune system-pathogen relationships for two well-studied intracellular microbes, *Listeria monocytogenes* and *Mycobacterium tuberculosis*, which preferentially enter the host via the gut and the lungs, respectively. These two bacteria not only differ enormously with regard to their impact on human health[16], they also diverge according to virulence and course of infection. *L. monocytogenes* causes acute disease and is rapidly eliminated once the adaptive immunity response has been established. *M. tuberculosis*, in contrast, is a slow growing microbe that causes long lasting infection and persists in the host even in the face of a fully-activated adaptive immune response. Hence, these two pathogens serve as good examples for discussing distinct and common mechanisms of antibacterial immune responses.

[16] *M. tuberculosis* is one of the major bacterial infection world wide. Every years, more than 1,5 million people die of tuberculosis, and 8 million new cases are reported [W.H.O., 2009]. Nowadays, one-third of the world's population is believed being infected. *L. monocytogenes* plays minor role in human health morbidity and mortality statistics by only affecting young, old, pregnant and immunocompromised individuals. Listeriosis represent less than 1% of all food-borne illness in the USA each year with a fatality rate of 20% [Vazquez-Boland *et al.*, 2001].

6.1. *Listeria monocytogenes*: the intracellular lifestyle of a multifaceted model

The gram-positive bacterium *L. monocytogenes* is a ubiquitous facultative intracellular pathogen found in various environments such as soil, water, various food products, animals and humans. Listeriosis caused by this microorganism is acquired by the ingestion of contaminated food products, and mainly affect immunocompromised individuals.

In the gastriointestinal tract, *L. monocytogenes* invades intestinal epithelial cells in a process that requires the interaction of the bacterial surface protein internalin A [Quinlan *et al.*] with epithelial cadherin (E-cadherin), a surface protein expressed by host epithelial cells [Gaillard *et al.*, 1991]. Once crossed the intestinal barriers, this pathogen reaches the bloodstream and colonizes organs such as the liver and the spleen where they are internalized by hepatic and splenic M∅s [Pamer, 2004].

6.1.1. Inside the cell

After entering a host cell, *L. monocytogenes* escapes the phagosome by secreting the pore-forming protein listeriolysin O (LLO), a virulence factor that destroys the phagosomal membrane[17] [Bielecki *et al.*, 1990] [Gedde *et al.*, 2000]. LLO has two functions: first, to avoid the maturation of the pathogen-containing vacuole by dissipating the pH gradient, and second, to form a channel that allows bacterial proteins to reach the cytoplasm that then achieve dissolution of the intracellular compartment [Portnoy *et al.*, 2002].

Invasion of the cytosol is a critical step to trigger protective immunity. Since immunization of mice with strains that cannot reach the cytoplasm does not induce long-term, T-cell mediated protective immunity [Berche *et al.*, 1987] [Muraille *et al.*, 2005].

Once released from the phagosome, motility of the bacterium through the cytoplasm and into neighboring cells is ensured by bacterial-mediated polymerization of host cell actin filaments. Polar expression of the actin assembly inducing protein A (ActA), a bacterial protein that nucleates actin to the pole of the microbe, results in actin polymers formation that propel the bacterium inside

[17] Invasion of cytosol of some cell types such as HeLa cells, can be mediated by phospholipases, independently of LLO [O'Riordan *et al.*, 2002]

the cell, thereby allowing pathogen dissemination [Domann *et al.*, 1992; Kocks *et al.*, 1992; Cameron *et al.*, 2000]. Of note, although LLO- and ActA deficient bacteria are both highly attenuated in mice, invasion of the cytosol by ActA deficient bacteria triggers immune responses that are essential for development of protective immunity [Goossens and Milon, 1992] [Auerbuch *et al.*, 2001] **| Fig. 15**.

6.1.2. Innate immunity: first fruits for a T_H1 condemnation

Following inoculation with *L. monocytogenes*, innate immune responses are rapidly triggered and are sufficient for resistance to the infection at early time points as illustrated by studies using severe combined immunodeficient (SCID) mice[18] and nude mice[19] [Nickol and Bonventre, 1977; Bancroft *et al.*, 1991; Unanue, 1997].

Initial control of the infection is mediated by the recruitment of innate immune cells such as PMNs, monocytes, and NK cells to sites of bacterial infection [Conlan and North, 1994; Rogers *et al.*, 1996; Unanue, 1996]. Whereas PMNs and M∅s exert their antibacterial activities by directly killing the pathogen, NKs cells contributes to the clearance of *L. monocytogenes* indirectly by producing IFN-γ [Buchmeier and Schreiber, 1985; Havell, 1989; Conlan and North, 1994; Cousens and Wing, 2000]. Early NK cells-derived IFN-γ results in differentiation of circulating monocytes expressing CCR2 to inflammatory TipDCs, which are the main TNF-α and iNOS producing cells during a *L. monocytogenes* infection [Serbina *et al.*, 2003; Kang *et al.*, 2008]. At the cellular level, PMNs, M∅s and TipDCs are thought to eliminate the bacterium by generating reactive species, principally NO· [Shiloh *et al.*, 1999; Serbina *et al.*, 2003]. Consistent with these observations, mice lacking, IFN-γ, TNF-α, iNOS or CCR2 have markedly decreased resistance to *L. monocytogenes* infection [Buchmeier and Schreiber, 1985; Havell, 1989; Pfeffer *et al.*, 1993; Shiloh *et al.*, 1999; Serbina and Pamer, 2006]. Accordingly, *L. monocytogenes* is a strong inducer of T_H1 responses.

[18] Severe combined immunodeficient (SCID) mice don't have any B and T -cells.

[19] Nude mice have a mutation that causes both hairlessness and defective formation of the thymus. These mice do not have mature T cells.

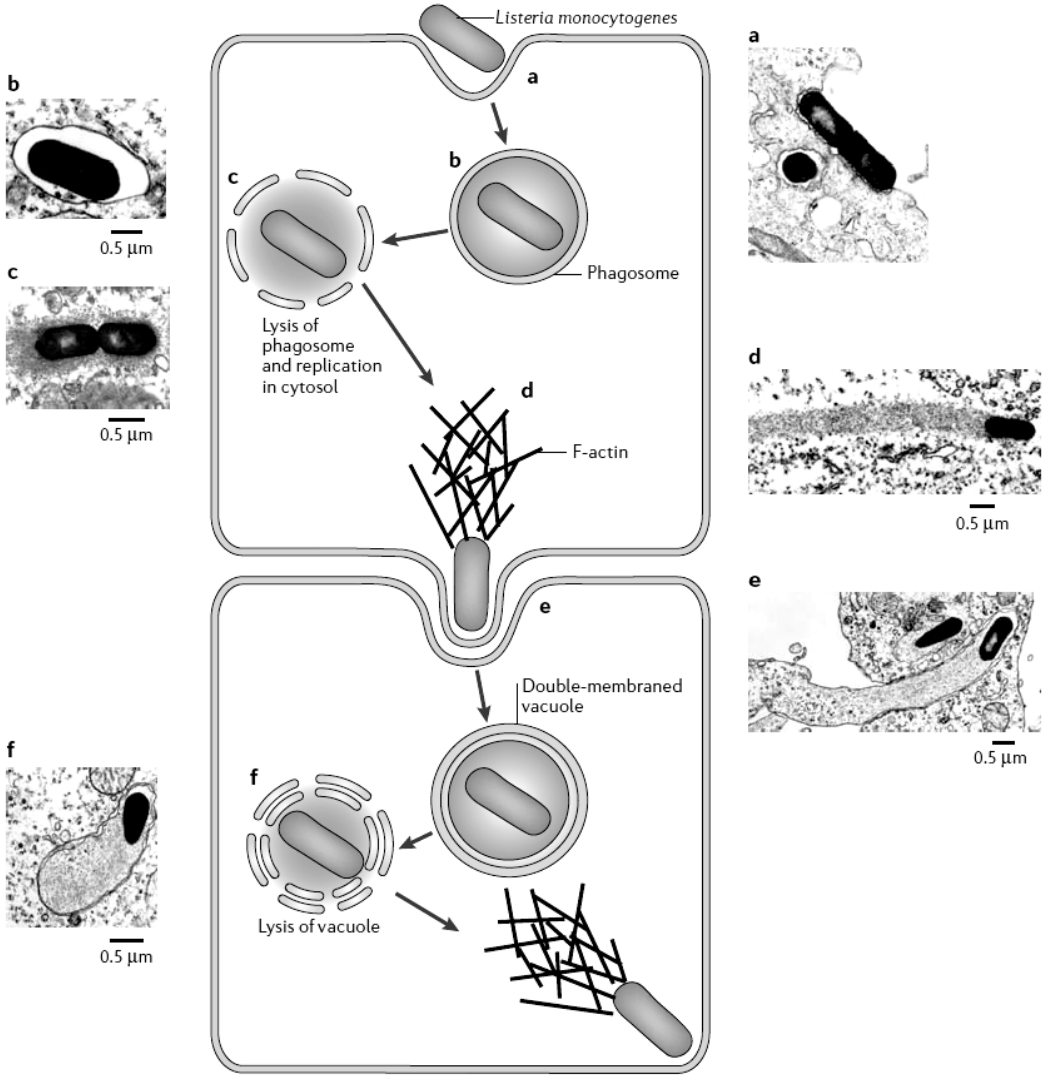


Figure 15 *Listeria monocytogenes* intracellular life cycle. *L. monocytogenes* expresses cell-surface and secreted proteins that enable **(a)** attachment to host cells, **(b)** internalization and **(c)** escape from the phagocytic vacuole. Then, actin polymerization **(d)** allows bacteria to be propelled through the cytosol and to pass into a neighboring cell by forming protrusions in the plasma membrane. On entry into the adjacent cell, **(e)** bacteria are enclosed in a double-membrane vacuole from which **(f)** they escape to perpetuate the cycle (from **[Hamon, 2006]**).

6.1.3. Entering the blood stream: the filter function of the spleen

The highly organized microarchitecture of the spleen forms an inevitable step for blood-borne pathogens. In the MZ, the blood leaves the terminal arteriol and moves into open sinuses, where blood-borne particles are mainly entrapped by MM∅s and MZM∅s [Aichele *et al.*, 2003]. Selective depletion of both M∅s leads to a rapid and uncontrolled dissemination of *L. monocytogenes* that ultimately causes the death of the infected mice [Aichele *et al.*, 2003]. Notably, pathogen trapping in the MZ is highly enhanced by opsonins such as natural antibodies. In mice genetically deficient of B cells, the capture of *L. monocytogenes* by MZ phagocytes is dramatically compromised [Ochsenbein and Zinkernagel, 2000].

Once in the spleen, $CD11b^+$ $CD11c^-$ monocytes as well as $CD11c^+$ DCs rapidly carry *L. monocytogenes* to the splenic T-cell zone of the white pulp [Conlan, 1996] [Muraille *et al.*, 2005] [Aoshi *et al.*, 2008; Kang *et al.*, 2008]. Concurrent with but independent of these early events, CCR2-mediated signals[20] promote emigration of monocytes from bone marrow into the spleen, which aggregated around the infected cells [Serbina and Pamer, 2006; Jia *et al.*, 2008]. Finally, NKs cells colocalize at site of infection and form a necklace surrounding aggregate monocytes that differentiate to TipDCs, which produce high amounts of TNF-α and NO· limiting thus bacterial replication [Kang *et al.*, 2008] | **Fig.16**.

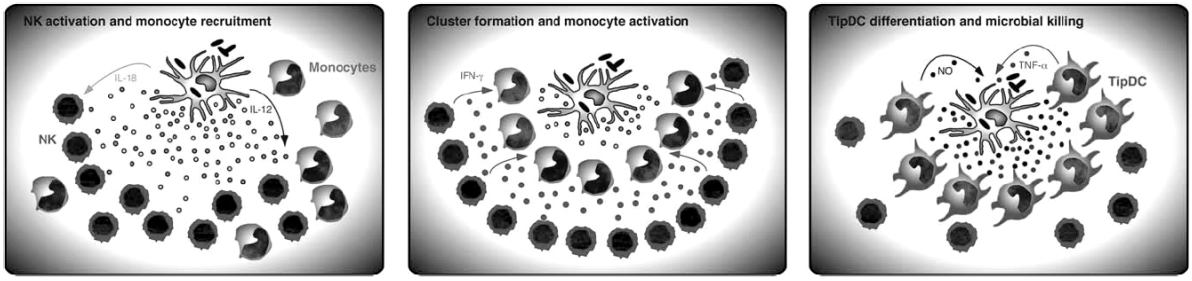


Figure |16 Spatial organization of the innate immune response during *L. monocytogenes* infection. After systemic administration of *L. monocytogenes*, infected cell ferry bacteria to the periarteriolar region of the white pulp, where they initiate secretion of mediators required for the recruitment and activation of NK cells and monocytes **(left panel)**. Recruited cells are spatially organized: infected cells are surrounded by monocytes border and NK cells form a coat at the periphery **(middle panel)**. NKs cells-derived IFN-γ orchestrates monocytes differentiation that upregulate MHC class II and iNOS expression and subsequent differentiation into TipDCs. TipDCs secrete $NO^{\cdot}$ and TNF-α, ultimately leading to restriction of bacterial replication **(right panel)** [Serbina and Pamer, 2008].

[20] The chemokine receptor CCR2 binds CC-chemokine ligand 2 (CCL2), a chemokine produced by M∅s that have internalized *L. monocytogenes*. [Serbina and Pamer, 2006]

6.1.4. Role of TLRs

Peptidoglycan is the main constituent of gram-positive bacteria, including *L. monocytogenes*, that interacts with the transmembrane PRRs, TLR2. Although some studies have described an impact on the viability of mice deficient in TLR2 infected with *L. monocytogenes* [Torres *et al.*, 2004], other works did not show impaired resistance as compared with wild-type mice [Edelson and Unanue, 2002; Seki *et al.*, 2002; Serbina *et al.*, 2003].

Flagellin purified from *L. monocytogenes* activates TLR5. However the role of TLR5 *in vivo* is unclear as *L. monocytogenes* deficient in bacterial flagellin displays no significant effect on virulence [Hayashi *et al.*, 2001] [Way *et al.*, 2004].

The absence of the intracellular adaptor protein, Myd88, drastically contributes to the expansion of the microbe [Seki *et al.*, 2002]. Mice lacking Myd88 have been shown to be more susceptible to *L. monocytogenes* infection than mice lacking either IFN-γ or both IL-12 and IL-18 [Edelson and Unanue, 2002; Seki *et al.*, 2002]. This observation places Myd88 at the crossroads of the *L. monocytogenes* eradication strategies.

Remarkably, despite its essential role in the control of bacterial dissemination, Myd88 was found to be dispensable to mount normal T-cell responses, indicating that Myd88-signalling does not contribute to the priming or differentiation of pathogen-specific T cells [Way *et al.*, 2003]. Furthermore, the induction of CCL2 chemokine by infected cells and the subsequent recruitment of $CCR2^+$ monocyte to the site of infection occur independently of Myd88 [Serbina *et al.*, 2003]. Indeed, characterization of splenocytes from Myd88-deficient mice infected with *L. monocytogenes* showed that CCL2-dependent differentiation of TipDCs was intact. However, unlike TipDCs from wild-type mice, the production of inflammatory mediators like iNOS and TNF-α was abrogated in TipDCs from Myd88-deficient mice. So, Myd88-mediated signals are essential for the activation of inflammatory cells, but they are not required for the recruitment of these cells [Serbina *et al.*, 2003].

Innate immune activation by virulent *Listeria monocytogenes* is thus a multistep process where temporally, anatomically, and chemically distinct signals coordinate innate immune responses during *in vivo* infection **| Fig.17**.

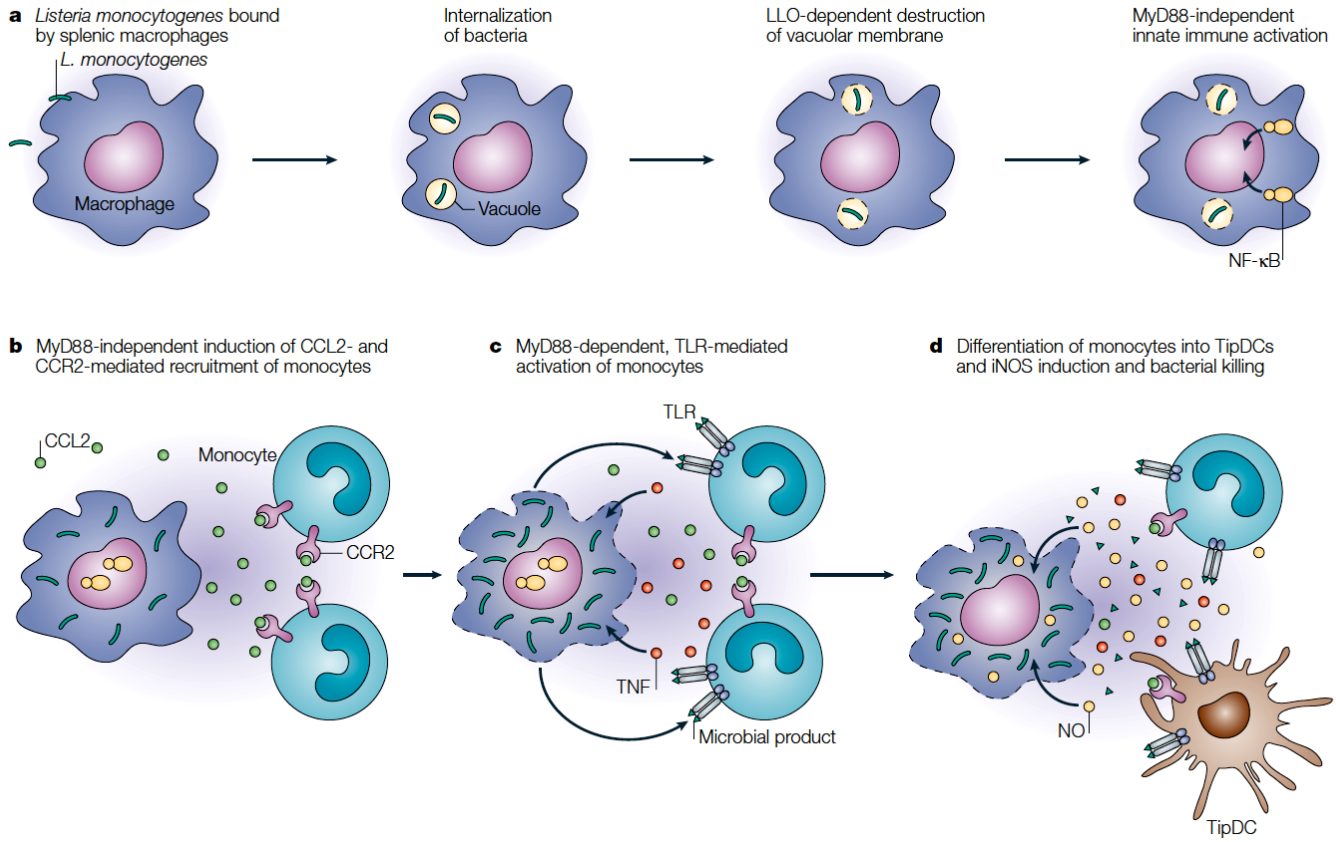


Figure 17 Sequential activation of innate immune responses during *Listeria monocytogenes* infection. (a) Circulating *L. monocytogenes* interact with splenic phagocytes and are internalized. In the endosome, bacteria secrete LLO which lyses the vacuolar membrane and activates expression of genes that lead to production of chemokines such as CCL2. **(b)** This production induces the recruitment of bone marrow monocytes expressing CCR2 to the T cell area of the spleen. **(c)** Engagement of TLR borne by these monocytes provokes their differentiation in a MyD88–dependent manner. **(d)** As a result, monocytes differentiate into TipDCs, which promote bacterial elimination. [Pamer, 2004]

6.2. Mycobacterium tuberculosis: "a pathogen with one length ahead…"

M. tuberculosis, the causative agent of tuberculosis in human, is an obligate aerobe and slow-growing[21] facultative intracellular pathogen that is shielded by a unique peptidoglycan wall supplemented with an extensive array of complex molecules, including long-chains fatty acids, glycolipids and other components. As a result, approximately 250 genes within the *M. tuberculosis* genome are devoted to fatty-acid metabolism [Cole *et al.*, 1998]. This robust shell contributes to the capacity of the bacterium to survive in host phagocytes and reflects its ability to resist desiccation in the environment [Russell, 2001] [Kaufmann, 2001].

Off all bacteria, *M. tuberculosis* is one of the most effective pathogens, with one-third of the world's population (about 2 billions people) being infected. In 2007, the last year for which data are available, more cases of tuberculosis were registered than at any prior point in world history [W.H.O., 2009]. Among the factors that contribute to the expansion of the illness, are the efficiency of human-to-human transmission by the aerosol route, the ability of the causal agent to persist despite development of host immune responses, and the synergistic impact that may have additional diseases on the host-resistance to tuberculosis, as for instance acquired immune deficiency syndrome (AIDS) [Comas *et al.*].

6.2.1. Tuberculosis: from acute to latency and reactivation

Although *M. tuberculosis* can manifest itself at any tissue site, the lung represents both the main entry and an important place of disease development. After entering in the respiratory tract, the bacteria are phagocytosed by alveolar MØs and DCs, and induce a localized inflammatory response that leads to recruitment of additional mononuclear cells from neighboring vessels [Kaufmann, 2001; Wolf *et al.*, 2007]. These cells constitute the building block of granulomas (see section 5.3). In the mediastinal lymph node, specific T cells are stimulated and traffic rapidly to the lungs where they contribute to bacterial containment. Then, a dynamic balance between bacterial persistence and host defense develops **| Fig.18**. Importantly, this equilibrium might be lifelong, so that the individual is infected but remains asymptomatic. For not

[21] *M. tuberculosis* has a replication time of approximately 20 hours.

well-defined reasons, 10% of infected individuals will develop clinical disease during their lifetimes among which 50% of untreated patients will die. Only in immunologically incompetent individuals, such as newborns, elderly people and the human immunodeficiency virus (HIV)-infected patients, does primary infection habitually transform into disease [Kaufmann *et al.*, 2005].

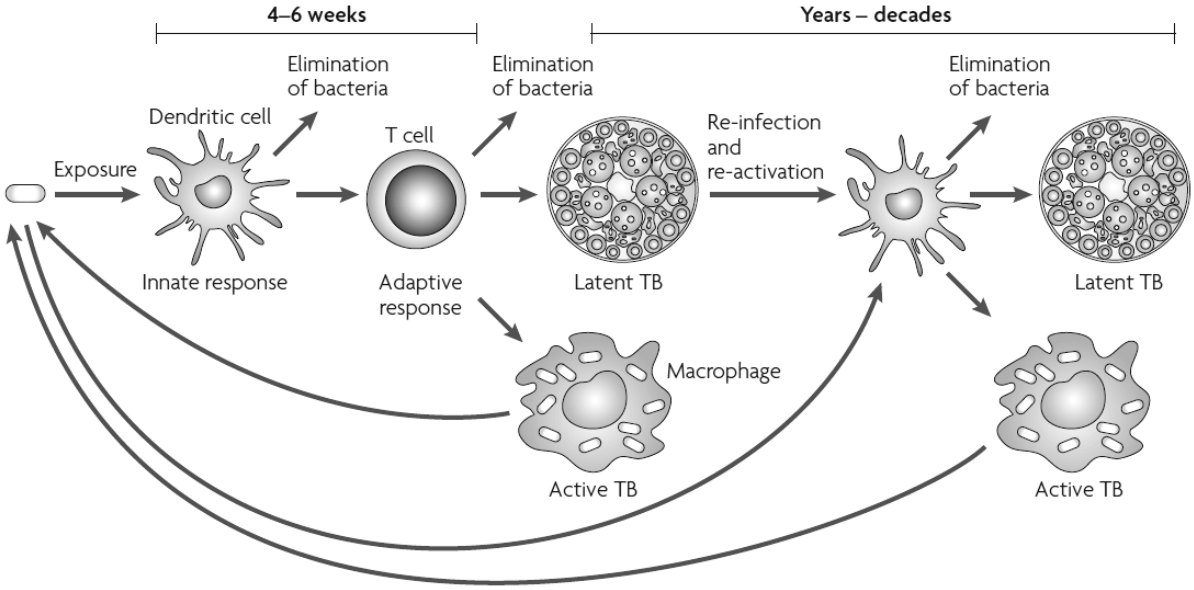


Figure 18 Chronological events after inhalation of *M. tuberculosis*. Following *M. tuberculosis*-containing droplets inhalation, several scenarios may occur. Bacteria may be destroyed by innate immune cells, in which case no real infection will take place. Alternatively, *M. tuberculosis* may persist, and so an immune response consisting in formation of granulomas will develop. Most often, the infection is stabilized at this point. In a minority of cases primary infection become an active disease. Years afterwards, usually in conditions of failing immune surveillance, latent infection may reactivate. [Young *et al.*, 2008]

6.2.2. M. tuberculosis*: a pathogen that doesn't go unnoticed*

In vivo and *ex-vivo* studies of mice following aerosol infection with *M. tuberculosis* show that several types of APCs in the lung are infected, including alveolar MØs and DCs [Wolf *et al.*, 2007], [Gonzalez-Juarrero *et al.*, 2003]. One of the paradoxes of the host-pathogen interaction in *M. tuberculosis* infection is that this organism is rich in cell wall components and other PAMPs that are recognized by a large array of APCs receptors. For example, *M. tuberculosis* stimulates MØs receptors such as MØs mannose receptor (MMR), DC-specific ICAM3-grabbing non-integrin (DC-SIGN), dectin 1, and TLR-2, -4, and -9 [Tailleux *et al.*, 2003; Neyrolles *et al.*, 2006]. In addition, class A SR and CD14, as well as CR1 and CR3 are involved in the attachment and uptake of the bacterium by professional phagocytes [Tailleux *et al.*, 2003; Neyrolles *et al.*, 2006; Yadav and Schorey, 2006; Korbel *et al.*, 2008] | **Table 6**.

At the single cell level, interaction with such receptors directly influences the intracellular fate of the pathogens. Whereas binding of most of the above quoted receptors lead to activation of pro-inflammatory cascades, some of them, including DC-SIGN and MMR have also been associated to the secretion of IL-10, which dampens pro-inflammatory responses mediated by T_H1 cells. Concomitantly, it has been shown that the presence of IL-10 favours *M. tuberculosis* replication [Korbel *et al.*, 2008; Cooper, 2009]. **Table 6** lists some well-studied surface effector molecules involved in innate immune response against *M. tuberculosis*.

Table 6| Overview of molecules implicated in innate immune response against *M. tuberculosis*		
Molecule	Effector function (example)	References
SR-A	Opsonin-independent uptake	[Gough and Gordon, 2000; van Crevel *et al.*, 2002]
CD44	Binding and phagocytosis	[Leemans *et al.*, 2003]
CR 1	Opsonin-dependent uptake	[van Crevel *et al.*, 2002]
CR 3	Opsonin-dependent as well as Opsonin-independent uptake	[Ernst, 1998]
FcγR	Opsonin-dependent uptake	[van Crevel *et al.*, 2002]
MMR	Opsonin-independent uptake, Immune downmodulation	[van Crevel *et al.*, 2002]
DC SIGN	Opsonin-independent uptake, MAN-LAM specific; Immune downmodulation	[Geijtenbeek *et al.*, 2003; Kaufmann and Schaible, 2003; Tailleux *et al.*, 2003]
TREM-1	Synergistic recognition of TLR-2 and TLR-4 signalling pattern	[Bleharski *et al.*, 2003]
TLR-2/6/1	Recognition of LAM, 19-kDa protein; Immune activation	[Abel *et al.*, 2002; Reiling *et al.*, 2002; van Crevel *et al.*, 2002]
TLR-4/CD14	Recognition of undefined, heat-labile culture supernatant factor, PIM4-6; Immune activation	[Ernst, 1998; Reiling *et al.*, 2002; van Crevel *et al.*, 2002] [Abel *et al.*, 2002]
TLR-9	Recognition of bacterial DNA	[van Crevel *et al.*, 2002]
Cholesterol	Internalization; Phagosome targeting	[Pieters, 2001]

6.2.3. M. tuberculosis *and MØs. "Who leads the dance?"*

The pathogenicity of *M. tuberculosis* is largely attributed to its ability to survive within MØs by blocking phagosomal maturation [Pethe *et al.*, 2004] | **Fig.19**. Once phagocytosed, the bacterium-containing vacuole fails to mature and to become a phagolysosome and is instead arrested in an early stage, which is characterized by the presence of the early endosomal GTPase, Rab5 and the absence of the late endosomal GTPase, Rab7 [Fratti *et al.*, 2001]. Indeed, *M. tuberculosis* expresses a large molecular arsenal, which is involved in maintaining a block on vacuole maturation. For example, the mycobacterium lipoarabinomanan (LAM), a component of the cell wall which is secreted by live bacteria throughout the endocytic network, prevent the cytosolic increase in calcium concentration that normally accompanies the phagosome maturation [Beatty *et al.*, 2000; Vergne *et al.*, 2003]. In addition, *M. tuberculosis* can also transiently disrupt phosphatidylinositol-3-phosphate (PI(3)P) accumulation in the vacuolar membrane by producing the phosphatase, SapM, which specifically hydrolyses PI [Cole *et al.*]P [Vergne *et al.*, 2005]. Depletion of PI [Cole *et al.*]P from early endosome prevents the transition to the later stages of maturation.

Importantly, *M. tuberculosis* not only arrests phagolysosome formation but can also escape the phagosome [van der Wel *et al.*, 2007]. In a recent study, van der Wel *et al.*, used electron microscopy to demonstrate that over the course of human MØs infection, *M. tuberculosis* progressively accumulates in the host cell cytosol [van der Wel *et al.*, 2007]. The authors interpret their observations as evidence that *Mycobacterium* is the unique pathogen adapted to replicate both in the vacuole and in the cytosol.

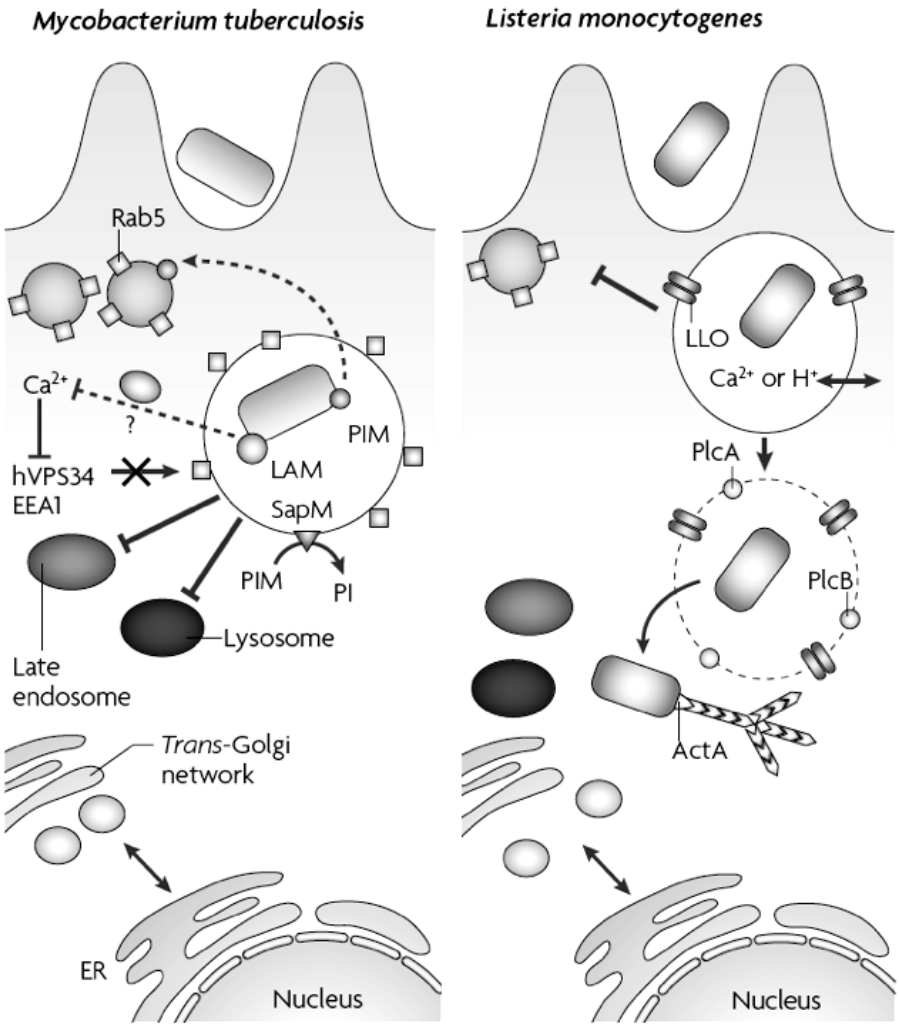


Figure 19 Comparison of strategies used by *M. tuberculosis* and *L. monocytogenes* to modulate phagosome maturation. *M. tuberculosis.* Following internalization, the bacterium uses a set of effector molecules, including the lipids phosphatidylinositole mannoside (PIM) and lipoarabinomannan (LAM), and the phosphatidylinositol-3-phosphate (PI(3)P) phosphatase SapM to arrest phagosome maturation at an early stage. *L. monocytogenes*. This pathogen escapes the phagosome by the bias of listeriolysin O (LLO) and two phosphatases, PlcA and PlcB. Once in the cytoplasm, the bacterium becomes motile by using actin "comet tails" generated by the effector ActA. Adapted from [Flannagan *et al.*, 2009].

6.2.4. M. tuberculosis. *"A pinch of adaptive but not too much".*

Soon after the infection, myeloid DCs are rapidly infected by the pathogen and represent the predominant cells that contain *M. tuberculosis* in the mediastinal lymph node [Wolf *et al.*, 2007], where the adaptive immune response is engaged. During such infection, DCs are the primary APCs and are essential to prime T cells to become effector and memory cells, whereas MØs, although excellent T cells activators, present a weak contribution for priming adaptive immune cells *in vivo* (reviewed in [Harding and Boom]). The differences between these cells in activation of adaptive immunity in response to *M. tuberculosis* were shown to result from an opposite effect of the internalized bacterium on MHC class II Ag presentation and regulation. MØs and DCs differ in how MHC expression is regulated in response to TLR signaling. Prolonged TLR 2 signaling (that is, signaling for more than 24 hours) reduces MHC class II molecule levels in MØs [Noss *et al.*, 2000; Gehring *et al.*, 2004]. By contrast, TLR 2 agonists drive DCs to undergo maturation, which includes their migration and enhanced levels of MHC class II molecules and co-stimulators, resulting in increased Ag presentation to naïve T cells in lymph node [De Smedt *et al.*, 1996; Cella *et al.*, 1997].

Additionally, the adaptive immune response to *M. tuberculosis* is delayed compared with that of other infections or immunization. This phenomenon contributes to the early bacterial population expansion in the lungs and is thought to be the consequence of a delay in the transport of *M. tuberculosis* Ag to lymphoid organs [Wolf *et al.*, 2008]. However, whether the bacteria are present in cells that intrinsically lack the ability to migrate, or whether they actively inhibit migration of DCs from the lungs to the lymph node is unknown.

Taken together, these observations could explain, in part, one of the main paradoxes of the host-pathogen interaction in *M. tuberculosis* infection: that an organism that elicits strong innate immune responses, through multiple adjuvant-like molecules in its cell wall, as well as strong T cell responses is able to survive and persist in APCs, which are the "starting block" of the host's adaptive immune system.

6.2.5. Innate immune cells recruited during M. tuberculosis *infection*

Aerosol infection of mice with *M. tuberculosis* leads to the trafficking of several innate immune cells into the bronchoalveolar space, including monocytes, NKs cells and PMNs [Gonzalez-Juarrero *et al.*, 2003].

MONOCYTE

Recruited monocytes have been shown to express CD11b, F4/80, and CCR2, which correspond to the typical signature of inflammatory monocytes (see section 3.3) [Peters *et al.*, 2004]. CCR2 deficiency is characterized by significant reduction of $F4/80^+$ cells migration, and results in early mortality following high-dosis intravenous and aerosol challenges [Peters *et al.*, 2000; Peters *et al.*, 2001]. In contrast, CCR2-deficient mice survive low-dose aerosol infection despite reduced recruitment of inflammatory monocytes, diminished iNOS levels, and delayed T cell influx [Scott and Flynn, 2002]. Thus, these cells have been associated to protection against *M. tuberculosis* when bacterial burdens are high, such as in systemic infection. Accordingly, these observations lead to the hypothesis that bactericidal mechanisms of resident M∅s may be sufficient to control bacterial growth following low-dose aerosol infection with *M. tuberculosis.*

NATURAL KILLERS

Although in recent years, it has become clear that NKs cells are capable of mounting a vigorous response to *M. tuberculosis*, their exact function *in vivo* remains enigmatic. Among their potential roles, it has been demonstrated that NKs cells can actively restrict *M. tuberculosis* growth in an apoptosis-dependent but FasR/FasL-independent manner, *in vitro* [Brill *et al.*, 2001]. Experimental infections with *M. tuberculosis* have shown that NKs cells accumulate in the lungs of mice and secrete IFN-γ during the first 2 weeks of a low dose aerosol infection [Junqueira-Kipnis *et al.*, 2003]. However, depletion of NKs cells prior to and during infection did not affect control of pathogen expansion. This observation indicates that even though NKs cells are able to adequately respond to *M. tuberculosis* infection, they may have functions that overlap with other immune cells (e.g. IFN-γ-producting T cells).

NEUTROPHILS

Most publications suggest that PMNs cells become activated, secrete numerous chemokines, and cytokines in response to *M. tuberculosis*, and actively restrict mycobacterial growth, at least *in vitro* (reviewed in [Korbel *et al.*, 2008]). For example, antimicrobial contents of PMNs granules are deleterious for *Mycobacterium* persistence *in vitro*. Moreover, in addtion to T_H1 cells, it has been suggested that protective immunity to *M. tuberculosis* requires IL-17 producing T_H17 cells that attract PMNs to the site of infection [Khader and Cooper, 2008]. IL-17 production is promoted synergistically by IL-12 and IL-23 (both sharing the IL-12p40 subunit) [Weaver *et al.*, 2007]. In contrast to vaccine strains which induces high amount of IL-23 and thereby induce the recruitment of PMNs, wild type

M. tuberculosis produce low quantities of this cytokine and therefore is characterized by a weak PMNs influx [Khader *et al.*, 2007]. Finally, PMNs could also modulate the induction of adaptive immunity by DCs during a *M. tuberculosis* infection [Aleman *et al.*, 2005; Aleman *et al.*, 2007].

6.2.6. Innate immune responses against mycobacteria

Recognition of *M. tuberculosis* by phagocytic cells leads to cell activation and production of molecules that play a crucial role in the immune response and the outcome of mycobacterial infection. Among the important proteins, TNF-α, IFN-γ, Myd88 and iNOS are essential performers.

- ***TNF-α.*** Stimulation of monocytes, M∅s [Valone *et al.*, 1988] and DCs [Henderson *et al.*, 1997] with mycobacterial products stimulates the production of TNF-α. This cytokine exerts its action on multiple cell types and plays a critical but incompletely characterized role in immunity to *M. tuberculosis*. TNF-α induces M∅s activation, presents immunoregulatory properties [Orme and Cooper, 1999; Tsenova *et al.*, 1999] and clearly contributes to maintenance of granulomas [Clay *et al.*, 2008; Miller and Ernst, 2008]. Regarding this last point, blockade of TNF-α signaling may have multiple effects on maintenance of granulomas: (1) it can lead to augmentation of bacterial replication and alter bacterial gene expression; (2) decrease of chemokines, chemokine receptors, and/or adhesion protein expression, and [Cole *et al.*] increase of T cell apoptosis that may cause altered host and bacterial gene expression and promote pathogen expansion [Clay *et al.*, 2008].

- ***IFN-γ.*** The protective role of IFN-γ is well established, primarily in the context of MØs activation. Phagosomal maturation is activated by IFN-γ which stimulates anti-mycobacterial mechanisms in phagocytes, notably ROSs and RNSs production [Chakravortty and Hensel, 2003] [Schaible *et al.*, 1999]. Unlike the phagosome in resting MØs, IFN-γ activated MØs overcome the block that the bacterium exerts over phagosome maturation and acidify the vacuole to pH 5,2 [Via *et al.*, 1998]. Moreover, within stimulated MØs, it has been observed that *M. tuberculosis*-containing phagosomes are sequestred by autophagic compartment that ultimately fuse with lysosomes leading to the destruction of the pathogen [Jo; Gutierrez *et al.*, 2004]. Additionally, it has been reported that protective immunity to tuberculosis also requires IFN-γ reponsive nonhematopoietic cells, which activate by regulating IL-17 expression and neutrophilc inflammation in the lungs [Desvignes and Ernst, 2009]. Early production of IFN-γ is mainly assumed by NK cells, either in response to IL-12 and IL-18 [Garcia *et al.*, 1999] or directly by exposure to mycobacterial oligodeoxynucleotides [Iho *et al.*, 1999]. In addition, this early production has also been attributed to alveolar MØs [Wang *et al.*, 1999] and T cells bearing limited T cell receptor diversity, including T cell expressing γδ T cells receptors (γδ T cells) and CD1-restricted T cells[22] [Janis *et al.*, 1989; Moody *et al.*, 2000; Moody *et al.*, 2000].

[22] CD1-restricted T cells do not react with mycobacterial protein Ag in the context of MHC class I and MHC class II molecules. Instead, these cells react with mycobacterial lipid and glycolipid Ag bound to CD1 molecules on APCs [Stenger *et al.*, 1997]. CD1 molecules have close resemblance to MHC class I but are relatively nonpolymorphic. CD1-restricted T cells display cytotoxic activity and are able to produce IFN-γ [Stenger *et al.*, 1997].

- ***iNOS.*** The role of RNSs in controlling infections with *M. tuberculosis* has been extensively studied (reviewed in [Chan *et al.*, 2001]), and the prime role of iNOS in the control of the pathogen expansion has been clearly demonstrated. The infection studies on knock-out mice deficient in *iNOS* or *phox* indicated that RNSs are primarily required to control the infection, whereas ROSs have a minor contribution. Other reports indicated a dual role of NO· as a direct antimicrobial effector as well as an anti-inflammatory agent controlling the cellular influx to *M. tuberculosis* infected tissue sites [Cooper *et al.*, 2002]. Following a low-dose aerosol challenge, it has been shown that the granulomas structures in the *iNOS* knock-out mice background are much larger, contain PMNs and tend to become necrotic. These observations support a role for iNOS in reducing inflammation. Of note, among many roles, NO· is know to impede adherence and transmigration of monocytes and granulocytes [Grisham *et al.*, 1998]. Therefore, it has been postulated that it could be the absence of such restriction signal that contributes to the increased size of the lesions [Cooper *et al.*, 2002].

- ***Myd88.*** The adaptor molecule Myd88 was demonstrated to be crucial in the control of *M. tuberculosis* expansion during the early steps of the infection [Scanga *et al.*, 2004]. IFN-γ-dependent NO· production and concomitantly restriction of mycobacterial growth is severely impaired in Myd88 knock-out mice. Interestingly, this observation is not redundant with the phenotype of TLR -2/ -4/ -9-triple-deficient MØs, which keeps the infection under control for at least 3 months in a similar manner to wild type mice [Holscher *et al.*, 2008]. This suggests that TLR -2, -4 and -9 are of minor importance in protective immunity against *M. tuberculosis* whereas Myd88 is essential for protection throughout a more extensive contribution to the immune response.

7. *Brucella*: A hidden immigrant in a blind system

Brucella spp. are facultative intracellular gram-negative coccobacilli that represent some of the world's major zoonotic pathogens responsible for important economic losses and considerable human morbidity [Godfroid *et al.*, 2005; Pappas *et al.*, 2006]. Belonging to the α-2 subclass of Proteobacteria [Moreno *et al.*, 1990], *Brucella* spp. are phylogenetically close to microorganisms capable of establishing associations with eukaryotic cells in a wide range of lifestyles. For example, members of the genus *Agrobacterium* are pericellular pathogens of plant cells, whereas *Bartonella*, and *Sinorhizobium* are facultative intracellular bacteria that behave as pathogens or endosymbionts, and *Rickettsia*, *Ehrlichia* and *Wolbachia* are obligate intracellular pathogens of mammals and arthropods [Batut *et al.*, 2004].

Comparative analysis revealed the existence of remarkable parallels between mechanisms employed by these bacteria to established successful interactions with their plants and animals hosts [LeVier *et al.*, 2000; Batut *et al.*, 2004]. Notably, pathogens belonging to this group present the passive ability to avoid immediate recognition by innate immunity [Barquero-Calvo *et al.*, 2007]. Such abilities, rely mainly on the absence, modification and inaccessibility of PAMP-bearing molecules, and contribute to the elaboration of a "stealthy strategy" by which these pathogens establish chronic infection [Batut *et al.*, 2004]. Accordingly, it has been proposed that *Brucell*a spp. have evolved from a free-living α-Proteobacteria ancestor that was initially endowed with structures prone to low recognition by the immune system. Representing a first step towards innate immune evasion, this evolution was accompanied by:

- → The removal of stimulating-molecular determinants (capsules, fimbriae)
- → The modification of surface molecules (flagella, LPS, lipoproteins)
- → The acquisition of virulent systems (effector molecules secreted by a type IV secretion system)

7.1. *Brucella* species

Although characterized by extremely high levels of nucleotide similarity[23] [Whatmore, 2009], *Brucella* species vary widely in host tropisms, microbial pathogenicity and disease seriousness. Accordingly, these bacteria are mainly differentiated by regard with their preferential hosts |**Table 7**.

B. melitensis, *B. abortus* and *B. suis* infect goats and sheep, cattle and swine, respectively and cause abortion and infertility in their natural hosts. These bacteria are also responsible for a human severe illness known as brucellosis or "undulant fever" as the result of contact with infected animals or their products |**Box 6.** [Pappas *et al.*, 2006]. Among these species, *B. melitensis* is the most frequent cause of human brucellosis [Corbel, 1997].

B. ovis and *B. canis* are natural pathogens of sheep and dog, respectively where they preliminary cause epididymitis and infertility in males and occasionally abortion in females [Wanke, 2004]. Human infection with *B. canis* is rare and human infection with *B. ovis* has not been reported.

Marine mammals strains of *Brucella* have been isolated from human patients indicating that these strains are potential zoonotic pathogens [Jahans *et al.*, 1997]. This group includes *B. pinnipedialis* and *B. ceti* isolated from seal and dolphin respectively [Cloeckaert *et al.*, 2001].

Although poorly studied, additional *Brucella* species have been reported, including *B. neotomae, B.microti* and *B. inopinata.* As far as it is known *B. neotomae* infect desert wood rat [Stoenner and Lackman, 1957], whereas *B. microti* was isolated from clinical specimens of the common vole *Microtus arvalis* [Scholz *et al.*, 2008] and *B. inopinata* has been recently cultivated from a breast implant infection of a 71 year old female patient with clinical signs of brucellosis [Scholz *et al.*].

[23] The majority (>90%) of *B. suis* and *B. melitensis* genes share 98-100% identity at the nucleotide level [Paulsen *et al.*, 2002].

Table 7| Currently described *Brucella* species and host associated	
Species	Major host(s)
Classical species	
B. abortus	Cattle
B. melitensis	Sheep, Goats
B. suis	Pigs, Hares, Reindeer, Rodents*
B. canis	Dogs
B. ovis	Sheep
B. neotomae	Desert Wood Rat
Newly described species	
B. pinnipedialis	Seals
B. ceti	Dolphins, Porpoises
B. microti	Voles
B. inopinata	Unknown

*Host preference and Biovar dependent.

Box 6 | Brucellosis: an insidious disease

For more than 2000 years, brucellosis is an illness that has accompanied human civilization expansion [Capasso, 2002]. As a zoonotic disease, naturally occurring human brucellosis is strictly the consequence of direct or indirect exposure to animals[24]. Transmission mainly happens via cuts or abrasions, or through ingestion of unpasteurised dairy products [Young, 2005]. People may also be infected by inhalation of contaminated particles or aerosols[25].

Human Infection with *B. abortus, B. melitensis* or *B. suis* may present varying forms, often becoming chronic but rarely being lethal. The "insidious" aspect of the disease comes from the fact that the first symptoms are like those associated with many other febrile diseases. However, although the majority of patients exhibit undulant fever, malaise, sweats and lymphadenopathy, complications may occur, including arthritis, and epididymoorchitis. If untreated, the disease can leads to focalizations and can become chronic. The focalizations of brucellosis occur usually in bones and joints and spondylodiscitis of lumbar spine accompanied by sacroiliitis is very characteristic of this disease. More serious and hard to treat complications include, neurobrucellosis and endocarditis, the latter being potentially fatal [Young, 2005].

In natural hosts *Brucella* causes generalized infections with a bacteraemic phase followed by localization in the reproductive organs and reticuloendothelial system. The colonization of the trophoblastic tissue is often concomitant with abortions in pregnant animals. Such an abortion expels contaminated tissue and fluid containing high loads of *Brucella* spp. (e.g. 10^{13} CFU/gr of tissue in fetal cotyledons) [Alexander *et al.*, 1981] into the surrounding environment, thus representing a major route of transmission. The infection also reduces fertility, causes weight loss, and lowers the milk yield of animals, accounting for the economic significance of this pathogen.

[24] Transmission of brucellosis has rarely been reported between humans. Only 2 cases of probable sexual transmission of *Brucella* from husband to wife have been described [Meltzer *et al.*].

[25] It is important to note that the efficiency of aerosolized *Brucella* to cause a human infection classifies brucellosis at the first place of the most common laboratory acquired pathogen worldwide [Fugier *et al.*, 2007], and explains its inclusion in the potential biological weapon list [Pappas *et al.*, 2006].

7.2. The targeted host cells: professional or not?

Brucella spp. are easily grown on artificial media and were for a long time referred as being facultative intracellular parasites. Nevertheless, it has been proposed that they are more appropriately termed "facultative extracellular pathogens" since they have no environmental reservoir and they predominately maintain an intracellular lifestyle within their mammalian hosts [Moreno and Moriyon, 2002]. The chronicity of brucellosis reflects the fact that *Brucella* is able to survive and replicate within host cells by adapting to intracellular conditions and modulating the immune system [Kohler *et al.*, 2003; Baldwin and Goenka, 2006; Gorvel, 2008; Roop *et al.*, 2009]. This intracellular niche provides a safe haven for the bacterium, protecting it from extracellular aggressions such as humoral components of the immune system.

It is well documented that *B. melitensis, B. suis* and *B. abortus* species are capable to survive and replicate in a broad spectrum of host cell lines |**Fig 20**. These cells include professional phagocytes such as osteoblastic cells [Delpino *et al.*, 2009], MØs [Forestier *et al.*, 1999], PMNs [Riley and Robertson, 1984] and DCs [Billard *et al.*, 2005] and non professional phagocytes, including placental trophoblasts [Anderson *et al.*, 1986], epithelial cells [Pizarro-Cerda *et al.*, 1998], fibroblastic cells [Detilleux *et al.*, 1990] and lymphocytes [Cheville *et al.*, 1996]. It is important to note that most of experiments that have studied interactions between *Brucella* and its host cells have been performed *in vitro* and mainly served to elucidate intracellular trafficking and phenotypes of potentially virulent mutants.

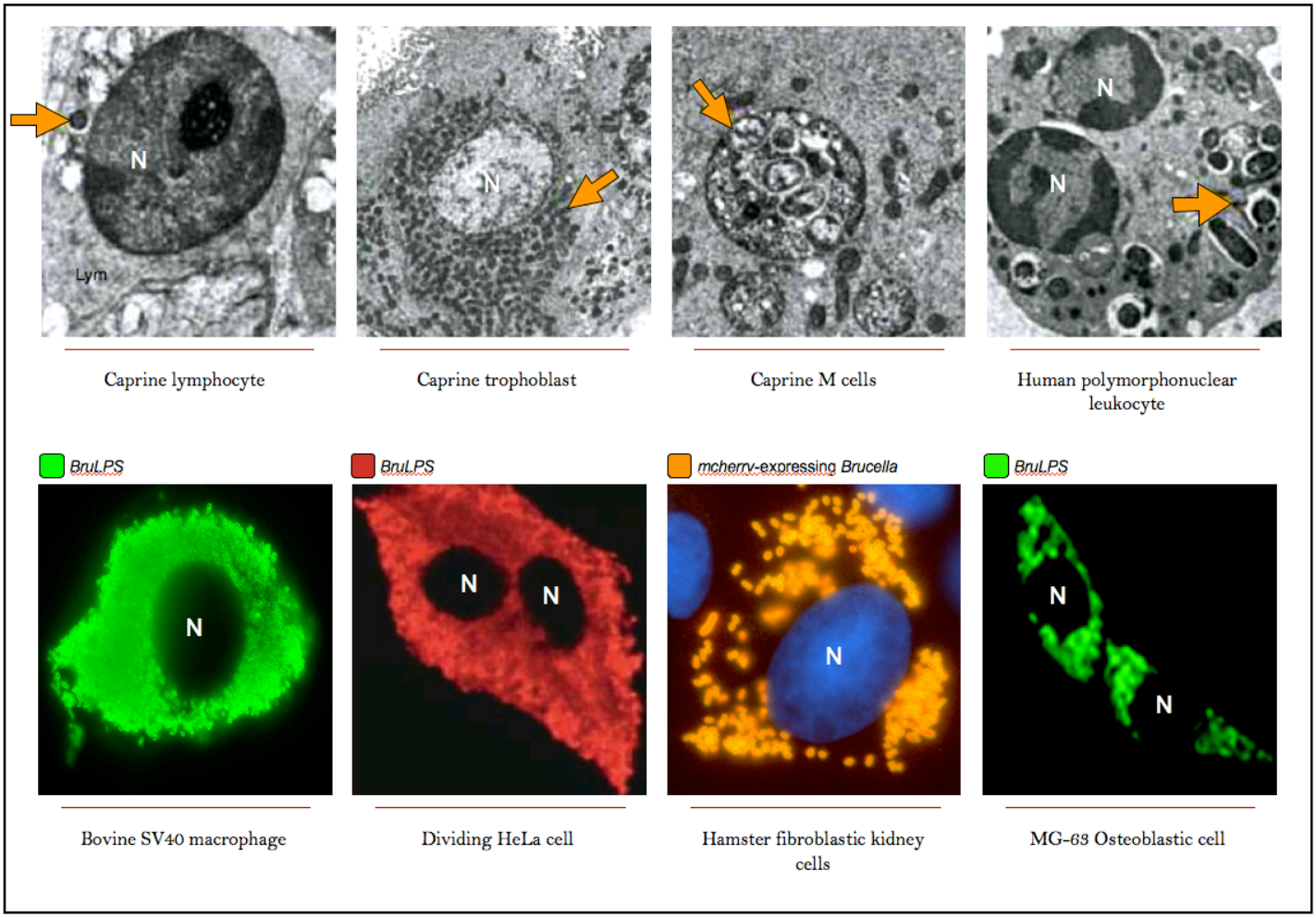


Figure 20 Electron microscopy and fluorescent imaging of *Brucella* in different host cells. *B. melitensis, B. abortus and B. suis* are able to infect a wide range of mammals host cells. It is important to note that only pictures of infected caprine trophoblast and caprine M cells result from *in vivo* experimental procedures. Top row references: [Cheville *et al.*, 1996], [Anderson *et al.*, 1986], [Ackermann *et al.*, 1988], [Riley and Robertson, 1984]. Down row references: this work, [Moriyon, 2006], this work, [Delpino *et al.*, 2009].

7.3. *Brucella*: "a journey to the center of the cell"

Upon entry in the host organism, *Brucella* gains access to circulation by crossing the mucosal barriers. Although the precise process has yet to be verified experimentally, it has been proposed that interactions with host epithelial cells at these locations represent an important point of initial contact between the pathogen and its host [Enright *et al.*, 1990]. However, the efficiency with which *Brucella* invades these cells has been shown to be low compared with bacterial pathogens that are considered as truly "invasive" [Moreno, 2004]. Consequently, the extent of the contribution of epithelial cells invasion makes to the initiation of *Brucella* infection is unclear, and some investigators have proposed that M cells located at the mucosal surface could serve as major site of entry for *Brucella* strains into their host [Moreno, 2004; Paixao *et al.*, 2009].

Internalization mechanisms employed by virulent *Brucella* to invade professional and non-professional cells are still poorly understood. However, it has been reported that lipid raft-containing regions might play an important role [Porte *et al.*, 2003]. Although the underlying process remains unknown, interaction between the *O*-chain of the smooth *Brucella*-LPS (*Bru*LPS) and lipid rafts on the surface of the host-cell seem to mediate entry in a manner that leads to the expansion of the bacterial population[26] [Porte *et al.*, 2003]. Moreover, additional studies have shown that *Brucella* adheres to epithelial cells and M∅s via cellular receptors containing sialic acid and sulphated residues [Castaneda-Roldan *et al.*, 2004], and are internalized by an active mechanism involving Rho GTPases and minor cytoskeletal rearrangements [Guzman-Verri *et al.*, 2001].

Some proteins have been proposed as being important for mediating the entrance of *Brucella* inside the cell, even though their respective contributions are still controversial:

- The class A SR (SR-A) [Kim *et al.*, 2004]. It has been reported that *B. abortus* internalizes into macrophages by using SR-A as specific receptor and that the bacterial VirB type IV secretion system (T4SS) regulates signal transduction dependent on this interaction to form a replicative phagosome. However, this model is criticized since several studies have shown that the T4SS was not involved in the early stages of infection. [Celli *et al.*, 2003; Lapaque *et al.*, 2005].

[26] Importantly, it has been proposed that only a fraction of *Brucella* population that enters into the cells is able to survive and replicate [He *et al.*, 2006].

- The 41 kDa surface protein (SP41) [Castaneda-Roldan *et al.*, 2006]. This *Brucella* protein has been involved in bacterial adherence and invasion of non-professional phagocytes. Biochemical analysis revealed that SP41 is the predicted product of the *ugpB* locus, which showed significant homology to the glycerol-3-phosphate-binding ATP-binding cassette (ABC) transporter protein found in several bacterial species.

7.3.1. The Brucella*-containing vacuole: special ferry to the endoplasmic reticulum*

Upon entry into M∅s, unopsonized[27] *Brucella* strains reside within a membrane bound compartment, the *Brucella*-containing vacuole (BCV). The maturation of this vacuole is controlled by the pathogen and generates a replicative organelle known as replicative phagosome [Kim *et al.*, 2002], replicative vacuole [Moreno, 2004] or brucellosome [Kohler *et al.*, 2003] |**Fig.20**. As illustrated by the acquisition of makers such as LAMP1, trafficking of the BCV undergoes transient interactions with late endosomes, and is accompanied by an increase of acidification [Starr *et al.*, 2008]. This vacuole then begins to interact extensively with the endoplasmic reticulum (ER) where it acquires numerous ER markers including the translocator sec61 and the lectin chaperones calnexin and calreticulin. Notably, the final replication niche coincides with ER exit sites (ERES) and is dependent on the T4SS system-encoding operon *virB* [O'Callaghan *et al.*, 1999; Foulongne *et al.*, 2000; Delrue *et al.*, 2001] |**Fig.21**.

It is important to note that the majority of virulent brucellae fail to survive intracellularly following phagocytosis, and, thus, the parasitism of the cells results from a small percentage of surviving organisms. It has been shown that there is an initial decline in organisms following internalization, during which up to 90-99% of *Brucella* may be killed, while the surviving bacteria subsequently replicate [Jiang *et al.*, 1993; Caron *et al.*, 1994; Rittig *et al.*, 2001].

[27] Internalization of pathogenic strains opsonized with hyperimmune IgG results in the formation of BCVs that undergo transient association with the lysosomal compartment and become acidified, but do not interact extensively with the ER [Bellaire *et al.*, 2005].

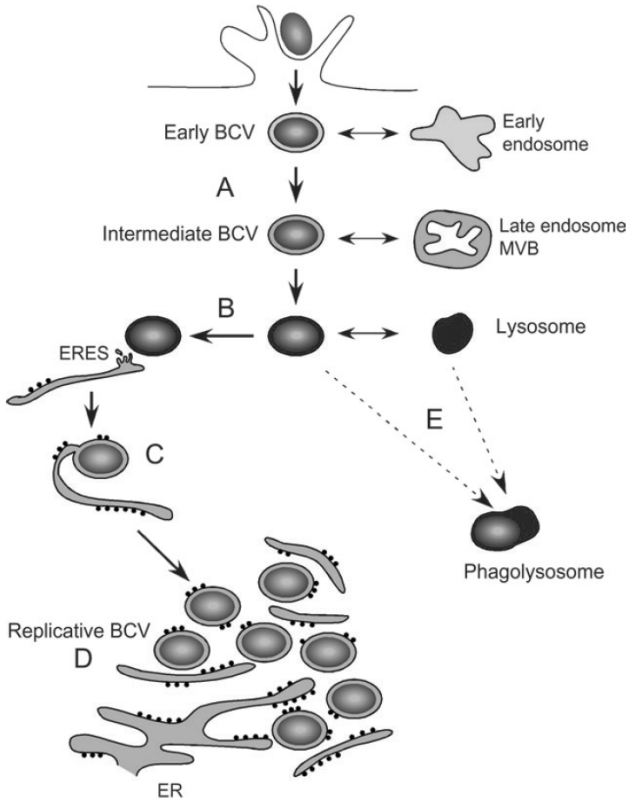


Figure 21 *Brucella* intracellular trafficking within professional phagocytic cells.
Upon entry (**A**), newly formed BCVs traffic along the endocytic pathway, sequentially interacting with early compartments of the endocytic pathway and late endosomes and multivesicular bodies (MVB) before fusing with lysosomes. Such interactions are required for further trafficking (**B**), whereby intermediate BCVs interact with ER exit sites (ERES) and subsequently fuse with the ER (**C**) to generate ER-derived, replicative BCVs (**D**). BCVs containing defective mutants that cannot sustain interactions with the ER and/or limit fusion with the lysosomal compartment and mature into a bactericidal vacuole (**E**) adpted from [Starr *et al.*, 2008].

Such ER-associated replication niche represents an obvious beneficial compartment for the bacterium, rich in various metabolites and protected from lysosomal fusions. However, unlike intracellular pathogens that block phagocytic maturation or escape from the early endosome (see section 6.1 and 6.2), *Brucella* still encounters formidable environmental stresses during their deviated trafficking within the M∅s. These stresses include ROSs and RNSs, exposure to acidic pH, and at least transient nutritional deprivation and exposure to the antimicrobial peptides. Correspondingly, *Brucella* is well equipped to withstand these environmental stresses [Kohler *et al.*, 2003; Roop *et al.*, 2004]. This feature undoubtedly plays an important role in the success with which *Brucella* strains maintain prolonged residence in their host phagocytes. Such bacterium "resistance" factors are summarized in **Box 7** and will not be further described in this work.

7.4. Brucella as Mr "Hide"

As previously mentioned, the ability of *Brucella* to establish long lasting infection result from its capacity to behave as a stealthy parasite that avoids its recognition by the immune system. Unlike most of the studied pathogens, a systemic inoculation of *Brucella* in mice did not induce classical inflammatory responses as illustrated by the absence of leukocyte recruitment, the lack of humoral or cellular blood changes, and the weak induction of pro-inflammatory cytokines such as TNF-α [Barquero-Calvo *et al.*, 2007].

Even though it does not preclude development of determinants that actively hamper the triggering of immunity at later stages, such furtive capacity seems to be mainly connected to physiological and structural features of the bacterium [Barquero-Calvo *et al.*, 2007]. In fact, it seems likely that *Brucella* has "opted" for a furtive strategy by eradicating, modifying and hiding active PAMPs-bearing molecules, to the point that they are practically not recognized by the corresponding receptors. For example, *Brucella* is devoided of classical surface structures and presents an outer membrane associated with distinct structural characteristics including a low number of lipoproteins, and the presence of phosphatidylcholine and non-stimulating flagella [Lapaque *et al.*, 2005]. *Brucella* also disposes of a non-classical LPS, the *Bru*LPS, which by itself possesses all characteristics needed for facilitating the life of an intracellular furtive pathogen **|Box 8 |Fig.22.**

Box 7 | List of *Brucella* virulent genes implicated in the intracellular trafficking			
Virulent factors	Function (example)	Effect of deletion	References
Base excision repair (*xthA*)	Protecting *B. abortus* from oxidative damage *in vitro*	Increased susceptibility to ROSs and ROS/RNSs	[Hornback and Roop, 2006]
***Brucella* virulence factor A (*bvfA*)**	May play a role in the establishment of the intracellular niche	Attenuated in BALB/c mice and J774A.1 cell line	[Lavigne *et al.*, 2005]
Catalase	Protecting cells from oxidative stress	Increased sensitivity to hydrogen peroxide	[Kim *et al.*, 2000; Gee *et al.*, 2004]
Cyclic β-1, 2-glucans (CβG)	Interferes with cellular trafficking by acting on lipid rafts found on host cell membranes and preventing the phagosome-lysosome fusion cycle	Attenuated in BALB/c mice and HeLa cells	[Briones *et al.*, 2001; Arellano-Reynoso *et al.*, 2005]
Cytochrome oxidase (*cydDCAB*)	Adaptation to low oxygen tension, preventing the buildup of oxidative free radicals and detoxifying the intracellular compartment	High sensitivity to the respiratory inhibitors sodium azide and zinc, highly reactive to ROSs, low pH, and attenuated virulence in BALB/c mice	[Endley *et al.*, 2001]
Lipopolysaccharides (LPS)	Protecting against bactericidal cationic peptides, plays a role in entry and early survival inside M∅s, prevent the synthesis of immune mediators, inhibit host cell apoptosis	Sensitive to normal serum and complement attack, attenuated in BALB/c mice	[Moreno *et al.*, 1981; Allen *et al.*, 1998; Forestier *et al.*, 1999; Forestier *et al.*, 2000; Lapaque *et al.*, 2005]
Nitric oxide reductase (*norD*)	Survival under very low oxygen tension, NO detoxification	Attenuated in BALB/c mice and in an activated J774A.1 cell line	[Loisel-Meyer *et al.*, 2006]
Superoxide dismutase (*Sod*)	Protects from the respiratory burst	Attenuated in C57BL6J mice and corresponding peritoneal M∅s but not in BALB/c mice and J77 cell lines	[Latimer *et al.*, 1992; Tatum *et al.*, 1992; Gee *et al.*, 2005]
Type IV secretion system (*virB*)	Essential for reaching ER-associated vacuole	Unable to acquire ER membranes, lost their ability to multiply in HeLa cells and cleared from infected BALB/c mice	[O'Callaghan *et al.*, 1999; Celli and Gorvel, 2004; Delrue *et al.*, 2004]
(p)ppGpp synthetase (*rsh*)	Essential for adaptation to low-nutrient environments in the infected cell	Severely attenuated in cell lines and BALB/c mice	[Dozot *et al.*, 2006]

Box 8 | The *Brucella* LPS or the invisible cloak of *Brucella*

The smooth phenotype of *Brucella* is due to the presence of a complete LPS, which is composed of a lipid A, a core oligosaccharide, and a perosamine *O* side-chain polysaccharide |Fig 22. The *Bru*LPS exhibits properties distinct from other LPS [Caroff *et al.*, 1984; Iriarte M, 2004] |Fig 22. In contrast to classical enterobacterial LPS, those of *Brucella* species are several hundred times less active and less toxic [Lapaque *et al.*, 2005]. As a result, this non-classical structure has direct implication on the pathogenecity of the bacterium. For example, *Br*LPS confers very high resistance to cationic bactericidal peptides and make *Brucella* a poor activator of the complement system [Barquero-Calvo *et al.*, 2007]. Moreover, the endotoxin component of the *Br*LPS, the lipid A, has several biochemical features (e.g. diaminoglucose and long chain (C28) fatty acids) that avoid activation of strong inflammatory responses in infected hosts [Iriarte M, 2004].

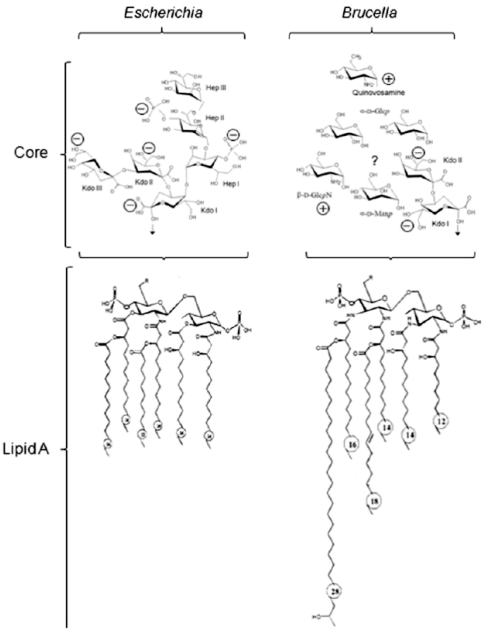


Figure |22 Model of *Escherichia* and *Brucella* lipid A and core oligosaccharide moieties. The lipd A of *Brucella* is characterized by diaminoglucoses and long chain (C28) fatty acids. The *O* side-chain of *Brucella* is a linear homopolymer of β-1,2-linked-4,6-dideoxy-4-formamido-β-D-mannopyranosyl subunits averaging between 96 and 100 subunits in length in comparaison to average of about 40 subunits per chain in *E. coli Bru*LPS present also a non-canonical lipid A. Interestingly, the differential sensitivity of *Escherichia*, and *Brucella* to cationic peptides is linked to the different LPSs properties such as a differentially charged core oligosaccharide. Adapted from [Barquero-Calvo *et al.*, 2009].

7.5. *Brucella* and the innate immune response

7.5.1. Brucella and the TLR signaling

Interaction of *Brucella* with TLR on cytokine-producing cells is likely to be an early event following the initial host infection, and an important step in shaping the subsequent immune response. In literature, conflicting studies have reported the impact of TLR on the control of *Brucella* infection. Although a general consensus is still lacking, there is sufficient evidence to conclude that *Bru*LPS, specifically the lipid A moiety, can stimulate through TLR4, whereas heat killed *Brucella* use additional components of the bacteria, including lipoproteins and DNA to stimulate additional TLR (see **Table 7** for a summary).

Table 6| Role of *B. abortus* components in stimulating through TLRs *in vivo* and *in vitro*

TLR evaluated	Stimulus	Sample evaluated	Relative change in cytokine secreted*			References
			TNF-α	IL-12	IL-6	
TLR4	HKBA	$TLR4^{mut}$ MØs	↓	↓		[Campos *et al.*, 2004]
	LPS	$TLR4^{mut}$ MØs	↓			[Giambartolomei *et al.*, 2004]
	LPS	$TLR4^{mut}$ MØs	↓	↓		[Campos *et al.*, 2004]
	LPS	THP-1 + anti-TLR4	↓		↓	[Giambartolomei *et al.*, 2004]
	HKBA	$TLR4^{mut}$ splenocytes	↔			[Huang *et al.*, 2003]
	HKBA	$TLR4^{mut}$ serum	↔	↔		[Huang *et al.*, 2003]
	HKBA	$TLR4^{mut}$ MØs	↔			[Giambartolomei *et al.*, 2004]
	HKBA	$TLR4^{-/-}$ DCs	↔	↔		[Huang *et al.*, 2003]
	HKBA	THP-1 + anti-TLR4	↔		↔	[Giambartolomei *et al.*, 2004]
	LPS	$TLR4^{mut}$ splenocytes	↔			[Huang *et al.*, 2003]
	Omp19	THP-1 + anti-TLR4	↔		↔	[Giambartolomei *et al.*, 2004]
TLR2	HKBA	$TLR2^{-/-}$ Splenocytes	↓			[Huang *et al.*, 2003]
	HKBA	$TLR2^{-/-}$ Serum	↓	↔		[Huang *et al.*, 2003]
	HKBA	$TLR2^{-/-}$ DCs	↓	↔		[Huang *et al.*, 2003]
	HKBA	THP-1 + anti-TLR2	↓		↓	[Giambartolomei *et al.*, 2004]
	Omp19	THP-1 + anti-TLR2	↓		↓	[Giambartolomei *et al.*, 2004]
	HKBA	$TLR2^{-/-}$ MØs	↔	↔		[Campos *et al.*, 2004]
	LPS	$TLR2^{-/-}$ MØs	↔	↔		[Campos *et al.*, 2004]
	LPS	THP-1 + anti-TLR2	↔		↔	[Giambartolomei *et al.*, 2004]
TLR9	*Brucella* DNA	$TLR9^{-/-}$ DCs		↓		[Huang *et al.*, 2005]
	HKBA	$TLR9^{-/-}$ DCs		↓		[Huang *et al.*, 2005]
	HKBA	$TLR9^{-/-}$ serum		↓		[Huang *et al.*, 2005]

*Note: ↔ denotes no change in amount of cytokine produced when compared to WT mice; ↓ denotes reduction in cytokine produced when compared to WT mice or without antibodies. Abbreviations: HKBA, heat-killed *B. abortus*; $TLR4^{mut}$ cells from C3H/HeJ mice; anti-TLR2/4, antibodies to TLR2/4.

TLR2

Although, the course of experimental brucellosis seems unaltered in TLR2 knockout mice [Campos *et al.*, 2004; Weiss *et al.*, 2005], the receptor was shown to be involved in the detection of heat-killed *B. abortus* [Huang *et al.*, 2003]. Furthermore, some authors reported that *Brucella* lipoproteins are potent triggers of proinflammatory cytokines such as IL-6 and IL-10 through TLR2, and proposed that *Brucella* stimulates the innate immune system and induces not only inflammatory but also immunomodulatory responses [Giambartolomei *et al.*, 2004]. Moreover, it has been reported that *Brucella* inhibits DCs maturation through the secretion of a TIR domain-containing protein that hampers the expression of proinflammatory genes such as *NF-κB* in a TLR2 dependant manner [Sengupta *et al.*; Salcedo *et al.*, 2008]. The direct consequence is a control by *Brucella* of inflammatory cytokine secretion and antigen presentation to T lymphocytes.

TLR4

Although *Brucella* is recognized by TLR4, this receptor is activated only at very high concentration of *Br*LPS [Weiss *et al.*, 2005]. Indeed, it has been shown that a 5000 fold higher concentration of *Bru*LPS is required compared to *Salmonella* LPS to induce equivalent level of cytokine. In experimental infection model, the role of TLR4 is the subject of many discussions. As some authors impute a role to the receptor in the control of infection with virulent strains of *Brucella* [Campos *et al.*, 2004], others report an absence of impact of its deletion in experimentally infected mice as well as in cell-line infection models [Weiss *et al.*, 2005; Barquero-Calvo *et al.*, 2007]. These discrepancies have been explained by differences in *Brucella* and mice strains used (e.g. *B. abortus* versus *B. melitensis*, *Brucella* resistant versus sensitive mice) and in variation of bacterial doses injected. Nevertheless, the influence of this TLR during the course of infection is still a matter of concern [Barquero-Calvo *et al.*, 2007].

TLR9

The impact of TLR9 during the course of the infection remains undetermined. However, a report provides evidence that bacterial DNA in the context of heat-killed *B. abortus* engages TLR9 in DCs, resulting in a T_H1-like cytokine response [Huang *et al.*, 2005]. This observation was based on the findings that heat-killed *B. abortus* induction of IL-12p40 was: (1) abolished in DC from TLR9 knockout mice, (2) blocked by suppressive oligodeoxynucleotides, and abrogated by DNase or methylation of the DNA from heat-killed *Brucella* [Cole *et al.*].

Myd88

The molecule Myd88 has been clearly demonstrated as being implicated for the control of *Brucella* replication in mice and cell lines, suggesting that some signaling through receptors that use Myd88 is required to control brucellosis, mainly once adaptive immunity has taken place [Huang *et al.*, 2003; Weiss *et al.*, 2005]. In absence of Myd88, the bacterial load present in the spleen is at least 100 fold more important at the peak of infection. Furthermore, inflammation and therefore splenic weight is considerably reduced in the Myd88 knockout mice, indicating major perturbation in the establishment of immune responses [Weiss *et al.*, 2005].

7.5.2. Brucella *and the DC activation*

In addition to stealth strategies, *Brucella* has also developed active mechanisms to interfere with innate immunity and specifically with the maturation of DCs. As for *L. monocytogenes* (see section 6.1.3) or *M. tuberculosis* (see section 6.2.4), *Brucella* survive and replicate in human and murine DCs [Billard *et al.*, 2005]. When infected with *Brucella,* these cells fail to become fully activated and are not able to connect the innate information to T-cell. In the case of DCs prepared from human monocytes and infected with *B. suis*, IL-12 and TNF-α secretion is strongly induced when outer membrane protein (OMP) 25 is missing, indicating that *Brucella* is able to control immune responses in such context [Billard *et al.*, 2007].

Brucella has also been reported to interfere with the maturation of DCs by producing a Tir receptor domain-containing protein designated TcpB or Btp1 [Sengupta *et al.*; Salcedo *et al.*, 2008]. When expressed in eukaryotic cells, it has been shown that such protein blocks TLR2 and TLR4 -mediated induction of the proinflammatory gene *NF-κB* expression through its capacity to elicit the targeted degradation of the TLR signaling adaptor protein MAL/TIRAP [Sengupta *et al.*]. Considering the importance of DCs in the development of host immune responses, it is feasible to propose that the capacity of *Brucella* to hinder maturation of these APCs allows this bacterium to circumvent the triggering of adaptive mechanisms. Concomitantly, as is the case in M∅s, it is also possible that DCs serve as safe havens to prevent exposure of the *Brucella* to components of the intracellular bactericidal arsenal and act as vehicles for the dissemination of these bacteria in the host. Importantly, these cells have been mainly used *in vitro*, and no evidences for such roles have been reported during the course of infection.

7.5.3. Brucella *and the inhibition of Ag presentation*

The strategies that allow *Brucella* to survive for prolonged periods inside MØs in the face of vigorous immune responses are not completely understood. However, it has been demonstrated that *B. abortus* LPS, despite its low endotoxic activity, acts as a downregulator of T-cell activation in murine peritoneal MØs, impairing MHC class II restricted Ag presentation by at least two possible mechanisms [Forestier *et al.*, 2000] [Barrionuevo *et al.*, 2008]:

- Sequestration of MHC class II molecules inside LPS macrodomains [Forestier *et al.*, 1999; Forestier *et al.*, 2000]. The *O* side-chain of *Bru*LPS is poorly degraded by the infected cells and forms complexes with components of the MHC class II machinery of the phagocytes, which interferes with their Ag-processing capacity.

- Outer membrane proteins (Omp) mediated down-modulation of the expression of MHC class II molecules. *B. abortus* Omp19 stimulates MØs via TLR2 to produce IL-6 [Giambartolomei *et al.*, 2004]. Under some circumstances, this cytokine has been involved in mediating the inhibition of IFN-γ induced MHC class II expression and Ag presentation induced by heat killed *B. abortus* and purified Omp19 [Barrionuevo *et al.*, 2008]. This observation suggests that IL-6 has inhibitory effects on MØs functions.

7.5.4. Brucella *as a T_H1 inducer*

As for many other diseases due to an intracellular bacterium, IFN-γ is a crucial element of the immune system implicated in brucellosis. Characterized by a sustained inability to control infections, IFN-γ deficient mice are unable to eliminate *Brucella* and die approximately 6 weeks post-infection [Murphy *et al.*, 2001]. Concomitantly, administration of recombinant IFN-γ enhances mice resistance, resulting in a significant decrease in the number of viable bacteria [Stevens *et al.*, 1992]. In murine MØs, the activation by IFN-γ generally does not greatly enhance killing during the first hours after infection [Jones and Winter, 1992]. Rather, it inhibits an increase in the number of intracellular *Brucella* at around 24 hours post-infection, which corresponds to the point at which bacteria have reached their replicative niche. Moreover, if the addition of IFN-γ is delayed until that point, IFN-γ has no effect on bacterial expansion [Jiang and Baldwin, 1993]. This suggests that the IFN-γ activation prevents the *Brucella* from

establishing its replicative niche, and thus, if the brucellosome is already established, IFN-γ becomes useless. One of the major functions of this cytokine in *Brucella* immunity is the stimulation of bactericidal activity mechanisms in phagocytic cells, including the production of RNSs and ROSs. Some authors have reported that gp91^{phox} knockout mice as well as iNOS knockout mice are both susceptible to *Brucella abortus* infection even though iNOS deficiency more drastically affects the resistance of the mice, suggesting a predominant role of RNSs in *Brucella* killing [Ko *et al.*, 2002]. Although production of IFN-γ has mainly been attributed to $CD4^+$ and $CD8^+$ T cell, a role for NKs cells in protective immunity to brucellosis has not been substantiated experimentally. Moreover, it is important to note that the respective contribution of T cells to *Brucella* control is controversial (reviewed in [Ko and Splitter, 2003]).

7.5.5. Other cytokines involved in Brucella *infection*

At the time of the initial host infection, the interaction of *Brucella* with M∅s and DCs can crucially influence the nature of the subsequent immune response according to the induction of either proinflammatory cytokines, such as IL-12 and TNF-α, or anti-inflammatory cytokines, such as IL-10. These cytokines promote or inhibit, respectively, the production or function of the anti-*brucella* cytokine IFN-γ.

IL-12

IL-12 has been shown to have a role in protective immunity to brucellosis *in vivo*, but only in a limited number of studies. *In vivo* depletion of IL-12 with antibodies decreases control of the pathogen in experimental mice models [Zhan and Cheers, 1995; Sathiyaseelan *et al.*, 2006]. Also, IL-12p40 deficient mice did not clear the infection with virulent *Brucella* strains but, nevertheless, survived at least for 4 weeks post-infection [Ko *et al.*, 2002]. Reciprocally, administering recombinant IL-12 reduces CFU of virulent *B. abortus* strains in BALB/c mice suggesting that the endogeneously produced level of IL-12 is suboptimal in this mouse strain [Sathiyaseelan *et al.*, 2006]. Interestingly, the results of adding or depleting IL-12 parallel the outcomes of infection when IFN-γ is depleted or supplemented thereby indicating the likely and logical connection between the two cytokines. Moreover, the reduction in *Brucella* CFU in BALB/c mice in the first week after infection is optimized when IL-18 is combined with IL-12 [Pasquali *et al.*, 2002]. This is explained by the fact that IL-18 acts synergistically with IL-12 to stimulate IFN-γ production by T cells. Finally, it has been shown that recombinant IL-12 required IFN-γ to be effective in reducing bacterial load in BALB/c mice because the administration of anti-IFN-γ

antibodies reversed the protective effect gained by administering recombinant IL-12 [Sathiyaseelan *et al.*, 2006].

TNF-α

TNF-α and IL-12 are key mediators of cellular immunity, forming a loop that ultimately results in IFN-γ production. Based on neutralization of TNF-α with antibodies, it has been demonstrated that endogenously produced TNF-α was required for maximal killing of brucellae by MØs during the first 24 hours after infection [Jiang *et al.*, 1993]. Interestingly, during *B. suis* infection of human monocytes, the production of this cytokine is abrogated [Caron *et al.*, 1994]. The inhibition of TNF-α production has been attributed to *Brucella* OMP 25 [Jubier-Maurin *et al.*, 2001] and is suggested to be a key to the parasitism of MØs by *B. suis*. The importance of TNF-α for the control of *B. abortus* has also been demonstrated *in vivo*. It has been showed that mice lacking the receptors for TNF are deficient in IL-12 production following infection with *B. abortus* but surprisingly not IFN-γ [Zhan *et al.*, 1996; Zhan and Cheers, 1998]. However, despite IFN-γ production in the TNFR deficient mice, there were fewer MØs in the spleen and thus a decreased splenomegaly and lack of RNS production by the MØs.

IL-10

IL-10 is an anti-inflammatory cytokine that affects both IL-12 production, as well as the ability of cells to respond to IL-12 [Aste-Amezaga *et al.*, 1998], by decreasing expression of the IL-12 receptor, IL-12Rβ2 [Wu *et al.*, 1997]. As noticed above, the interaction of *Brucella* with TLR2 on MØs can result in their production of IL-10 [Giambartolomei *et al.*, 2004]. It has been found that IL-10 decreases IFN-γ production *in vitro* when BALB/c splenocytes are stimulated with heat-killed bacteria [Fernandes and Baldwin, 1995], and Huang *et al.* found that IL-10 inhibits the ability of *B. abortus* LPS to stimulate BALB/c splenocytes to produce IL-12 and IFN-γ [Huang *et al.*, 1999]. *In vivo* studies showed that IL-10 negatively affects control of brucellosis in the susceptible BALB/c mice [Fernandes and Baldwin, 1995], increasing CFU by 10 to 100 fold, whereas, it has no effect in resistant C57BL/6 [Zhan and Cheers, 1998].

7.6. *The Mouse and* Brucella*: a forced marriage*

As for many other intracellular pathogens, the relationship of the innate immune system with *Brucella* has been mostly studied in the mouse experimental model. Albeit very complex and far from being mastered, this model presents the great advantage to more closely approximate the biologically relevant situation of human disease. It is important to note that many different species and strains of *Brucella*, as well as hosts (generally inbred strains of mice), are employed in the various studies, and therefore, the relative importance of immune system components in protection may vary accordingly.

Although most natural infections with *Brucella* spp. are acquired following ingestion or aerosols inspiration, most laboratory studies characterizing immune responses to this pathogen use either intravenous or intraperitoneal inoculation to initiate infection. So, most studies of infection with this pathogen focus on immune responses to systemic infection. By contrast, "natural" routes of infection are less well characterized but are increasingly becoming the focus of new investigation.

7.6.1. Balb/c versus C57BL/6

BALB/c and C57BL/6 mice are often used as the models for susceptibility and resistance to *Brucella* infections [Araya *et al.*, 1989; Fernandes *et al.*, 1996]. Both are able to eliminate strains but differ in their propensity for M1M∅s versus M2M∅s, which are distinguished by their cytokine production profiles (see section 3.2.2) [Mills *et al.*, 2000] [Okamoto *et al.*, 2009]. However, *in vitro* studies show that there is no difference in the innate abilities of M∅s from BALB/ c and C57BL/6 mice to control replication of intracellular *Brucella* stains under any of several experimental conditions evaluated [Sathiyaseelan *et al.*, 2000]. In light of this, and because control of *Brucella* infections in M∅s is known to be influenced by endogenously produced cytokines (ultimately, IFN-γ, as discussed above), it is likely that the differences between these mouse strains find an explanation in the cytokines pole respectively produced that may affect the production or action of IFN-γ in the host and, subsequently, M∅s control of the organism.

7.6.2. Histopathology in mice infected with Brucella

In mice, systemic inoculation of *Brucella* results in dissemination and persistence of the bacterium in organs of the reticuloendothelial system [Braude, 1951; Enright *et al.*, 1990].

In the spleen of infected animals, virulent *B. abortus* strains assume a plateau phase during the first 6 weeks post-infection and thereafter are eliminated very slowly over a period exceeding 6 months. At weeks 1 and 2 post-infection, histological changes in this organ occur and are characterized by recruitment of innate immune cells including monocytes, and the development of small granulomas structures. This phenomenon is associated with a manifest splenomegaly. Later, a profound depletion of periarteriolar lymphoid tissue marks the white pulp of the spleen and is followed by a slow repopulation of this zone. At weeks 5 and 6 post-infection, numbers and sizes of splenic granulomas start to decrease and the bacterial load become lower [Enright *et al.*, 1990].

7.6.3. The T4SS is required for microgranuloma formation in the spleen

As for *M. tuberculosis* and *L. monocytogenes*, virulent *Brucella* spp. are associated with the formation of focal granulomatous lesions in the spleen, liver and lymphoid tissues of both humans and rodents, starting at 2 to 3 weeks post-infection [Fabyan, 1912; Enright *et al.*, 1990]. The mechanisms underlying the formation of *Brucella* granulomas as well as the nature of the cells composing the structure are largely undetermined. However, histopathologic and morphometric analyses of spleen tissue from mice susceptible to *B. abortus virB* mutant proliferation revealed that the T4SS was essential to establish the granulomatous response [Rolan *et al.*, 2009]. Among the possible explanations, the VirB T4SS was shown crucial to elicit proinflammatory responses in the spleen after *B. abortus* infection [Roux *et al.*, 2007]. Indeed, comparison of transcriptional profiles of the host response to infection with wild type and *virB* mutant *Brucella* strains revealed that the T4SS was required to trigger a proinflammatory response, demonstrating the importance of such system during the early steps of infection.

7.6.4. *Dissemination of a lighted* Brucella

Recently, bioluminescent *B. melitensis* strains were developed to permit the study of pathogenic dissemination of *Brucella* in mice [Rajashekara *et al.*, 2005] | Fig 23. By day 1 post-infection, C57BL/6 mice presented a strong signal at the site of intra-peritoneal injection suggesting replication of the bacteria in large numbers in the peritoneum. In addition, signals were detected in areas of inguinal lymph nodes, livers, and spleen suggesting systemic spread. Interestingly, bioluminescence was readily observed in the salivary glands and testes by day 1 post-infection, indicating a preference of *Brucella* for these regions. At day 7, very little bioluminescence in the peritoneum, liver, and spleen of infected C57BL/6 mice was observed. By day 14, most of the signal disappeared indicating bacterial clearance consistent with these mice being relatively resistant to *Brucella* infection. However, weak or occasional bioluminescence was observed in the tail, extremities, and submandible regions even 21 days p.i., suggesting a preference of *Brucella* for joint rich locations in the later stages of infection [Rajashekara *et al.*, 2005].

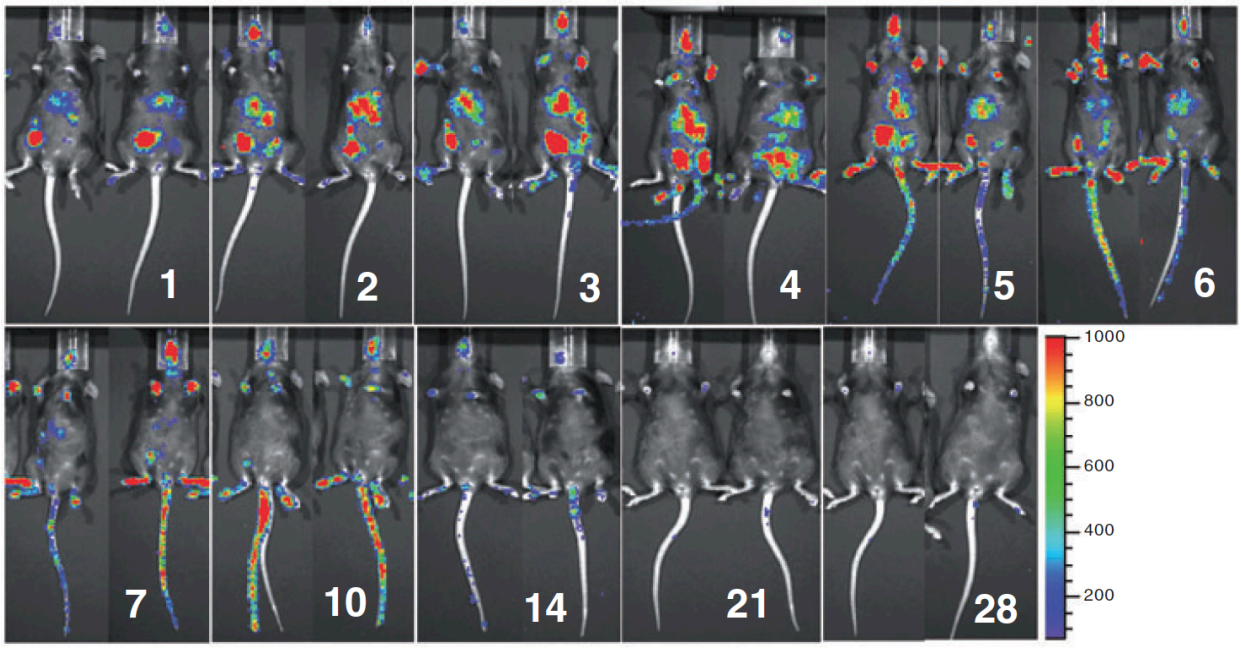


Figure | 23 Bioluminescent imaging of *Brucella* in infected C57BL/6 mice.
Distribution of a bioluminescent *B. melitensis* strain following intraperitoneal inoculation of 1 x 10^7 colony forming units. Mice were imaged with a 10 minutes exposure for 4 weeks. Numbers at the bottom indicate days post infection. Rainbow scale represents approximate photon counts. We can observe that the pathogen is firstly found in location corresponding to the spleen, liver, and in the head area. Over the course of infection, the bacteria reach the testis and finally persist in the extremities. [Rajashekara *et al.*, 2005]

OBJECTIVES

Brucella spp. are facultative intracellular cocobacilli that cause economically important infections in food animals as well as chronic disease with severe clinical manifestations in human. Among the factors that contribute to the continued growth of brucellosis as a global health problem is the ability of *Brucella* to persist despite the development of host immune responses. The chronicity of infection results, on one hand, from the ability of *Brucella* to evade the bactericidal components of the immune system, and on the other hand from the crucial component of immunity that control the bacterial growth and thereby maintain this chronic infection state. Although extensive studies have provided major insights into *Brucella* pathogenesis, surprisingly little information is available detailing the cellular components, the transduction pathways, and the nature of the effector mechanisms of the immune system induced by *Brucella in vivo*. In addition, the precise phenotype of the cellular niche in which *Brucella* resides during the course of infection is also undetermined.

Therefore, the aims of the present work are:

- The characterization of the immune cellular actors and the *Brucella*-killing effector mechanisms of the mouse involved in the control of *B. melitensis* infection.

- The identification of TLRs and associated adaptor proteins implicated in the activation of these cells.

- The phenotypic analysis of the *Brucella*-infected cells during the early steps of infection in the spleen and liver of the mouse.

Within the body of this manuscript, attempts to reach these objectives will be presented in two distinct parts:

⇒ The characterization of the cellular immune response induced by *B. melitensis* infection and the evaluation of TLRs and their adaptor molecules contribution in this response.

⇒ The phenotypical description of *B. melitensis* infected cells in the spleen and liver of wild type and immunodeficient mice as well as the elucidation of the cellular nature of granulomas structure in these organs.

RESULTS

1. Part I:

1.1. Prologue

The recent advances in our understanding of innate and adaptive immune responses induced by infection with *B. melitensis* are not abundant but have shed light on fundamental aspects of the pathogenesis and have opened new fields of investigation. By the utilization of flow cytometry techniques to compare genetically distinct mouse lineages, the purpose of the present work was to characterize more deeply some of these aspects.

1.1.1. Characterization of the nature and cell surface phenotype of immune effector cells implicated in B. melitensis *infection.*

One of the crucial elements involved in *Brucella* immunity is IFN-γ. The importance of this cytokine is indubitable since IFN-γ deficient mice are among the rare animals unable to survive to infection with virulent strains of *Brucella* [Murphy *et al.*, 2001]. Its production results from the ability of *Brucella* components, including *Bru*LPS, to engage PRRs for the production of inflammatory mediators and the stimulation of *Brucella*-killing effector mechanisms in phagocytic cells (see introduction, section 7.5.4).

Although $CD4^+$ and $CD8^+$ T cells are clearly involved in the production of IFN-γ, their respective contribution to protective immune response against *Brucella* was controversial at the start of the current research project (reviewed in [Ko and Splitter, 2003]). Indeed, if transfer experiments showed an equal role for both T cell subsets, studies using knock out mice revealed a more important contribution for $CD8^+$ T cell activation in the control of the pathogen proliferation. However, at latter stages of infection, MHCII deficient mice have been found clinically more affected than β_2-microglobulin (deficient for $CD8^+$ T cell activation) deficient mice, suggesting a role for $CD4^+$ T cell subsets [Izadjoo *et al.*, 2000; Murphy *et al.*, 2001].

In addition, the cell surface phenotype of spleen cells that respond to IFN-γ by inducing *Brucella*-killing mechanisms such as ROSs and RNSs production was not yet investigated *in vivo* during the course of *B. melitensis* infection. A previous study reported that both ROSs deficient mice and RNSs deficient mice are susceptible to *Brucella* infection but that iNOS deficiency more drastically affects the resistance of the mouse, suggesting a more predominant role of RNSs in *Brucella* killing [Ko *et al.*, 2002]. The iNOS-producing cells populations are heterogeneous and distinct subsets have been discriminated on the basis of differential expression of various cell surface markers in several infectious models. However, little is known regarding iNOS-producing cells nature and recruitment in response to *Brucella* infection. This being so, one of the aim of this work was to characterized the main iNOS-producing cells recruited and activated during *B. melitensis* infection in all mouse compartments analyzed.

1.1.2. Analysis of the contribution of Tir domain-containing proteins in the control of brucellosis.

Experiments in genetically deficient mice showed that Myd88 adaptor protein is implicated in the resistance to *B. abortus* infection [Huang *et al.*, 2003; Weiss *et al.*, 2005]. However, contradictory results have been reported regarding the implication of TLR2 and TLR4 in the resistance to *Brucella.* TLR2 has been associated to heat-killed *B. abortus*-induced cytokines [Huang *et al.*, 2003] but not in protection to infection [Campos *et al.*, 2004; Weiss *et al.*, 2005]. TLR4 absence has been reported to reduce [Campos *et al.*, 2004] or to have no affect [Weiss *et al.*, 2005] on resistance to *B. abortus*. Furthermore, the impact of other TIR domain-containing proteins such as TLR9 or TRIF adaptor *in vivo* remained undetermined. To gain insight into the role of TLRs and their adaptor proteins during the course of *B. melitensis* infection, we compared the ability of mice genetically deficient for TLR2, 4 and 9 as well as Myd88 and TRIF to develop immune response against this pathogen.

1.1.3. Comparative study of Balb/c and C57BL/6 mice infected with B. melitensis.

Balb/c mice present the well-known reduced capacity to mount a T_H1 response and are described as being more drastically affected by *Brucella* invasion when compared with its black counterpart, the C57BL/6 mouse. Experiments that intended to understand this discrepancy have been realized *in vitro* and concluded that Balb/c susceptibility resulted from an interruption of INF-γ secretion by immune splenic cells that begins after the first week of

infection [Fernandes *et al.*, 1996; Murphy *et al.*, 2001]. To either confirm or refute this hypothesis *in vivo*, and to improve our knowledge on immune responses developed by these experimental models during an infectious process, we realized our experiments in both mouse lineages and compared the results obtained.

In summary, the aims of this part of the work were: (1) to identify the phenotype of INF-γ and iNOS producing cells during the course of *B. melitensis* infection; (2) to determine the ability of mice genetically deficient for some TLRs and their adaptor molecules to induce the activation of these effector cells and [Cole *et al.*] to compare the nature and kinetic of the immune cells recruited by Balb/c and C57BL/6 mice during *B. melitensis* infection.

1.2. Manuscript

MyD88-Dependent Activation of B220$^-$CD11b$^+$LY-6C$^+$ Dendritic Cells during *Brucella melitensis* Infection[1]

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IFN-γ is a key cytokine controlling *Brucella* infection. One of its major function is the stimulation of *Brucella*-killing effector mechanisms, such as inducible NO synthase (iNOS)/NOS2 activity, in phagocytic cells. In this study, an attempt to identify the main cellular components of the immune response induced by *Brucella melitensis* in vivo is made. IFN-γ and iNOS protein were analyzed intracellularly using flow cytometry in chronically infected mice. Although TCRβ^+CD4$^+$ cells were the predominant source of IFN-γ in the spleen, we also identified CD11b$^+$LY-6C$^+$LY-6G$^-$MHC-II$^+$ cells as the main iNOS-producing cells in the spleen and the peritoneal cavity. These cells appear similar to inflammatory dendritic cells recently described in the mouse model of *Listeria monocytogenes* infection and human psoriasis: the TNF/iNOS-producing dendritic cells. Using genetically deficient mice, we demonstrated that the induction of iNOS and IFN-γ-producing cells due to *Brucella* infection required TLR4 and TLR9 stimulation coupled to Myd88-dependent signaling pathways. The unique role of MyD88 was confirmed by the lack of impact of Toll-IL-1R domain-containing adaptor inducing IFN-β deficiency. The reduction of IFN-γ^+ and iNOS$^+$ cell frequency observed in MyD88-, TLR4-, and TLR9-deficient mice correlated with a proportional lack of *Brucella* growth control. Taken together, our results provide new insight into how immune responses fight *Brucella* infection. *The Journal of Immunology,* 2007, 178: 5182–5191.

Brucella organisms are facultative intracellular Gram-negative coccobacilli that infect humans and animals. They are causative agents of a worldwide zoonosis. Acute human brucellosis is characterized by undulating fever, which, if untreated, may result in localization of bacteria in various tissues leading to chronic disease with serious clinical manifestations, such as orchitis, osteoarthritis, spondylitis, endocarditis, and several neurological disorders (1–4). *Brucella melitensis* is the most frequent cause of human brucellosis (5).

Brucella resides and replicates in a vacuolar compartment within macrophages of the infected host (6–8). As for other intracellular microbial pathogens, IFN-γ, a Th1 cytokine produced by activated NK, CD4$^+$ T cells, and CD8$^+$ T cells, contributes to control the infection (9–11). One of its major functions is the stimulation of *Brucella*-killing effector mechanisms, such as the production of a reactive nitrogen intermediate (RNI)[4] and a reactive oxygen intermediate (ROI) in phagocytic cells (12, 13). A previous study (14) has reported that gp91$^{phox-/-}$ mice and iNOS/NOS2$^{-/-}$ mice are both susceptible to *Brucella abortus* infection but that inducible NO synthase (iNOS) deficiency more drastically affects the resistance, suggesting a predominant role of RNI in *Brucella* killing. BALB/c mice are less able to control *Brucella* infections than C57BL/6 mice, having an ~10-fold increase in bacteria in their spleens during the plateau phase of the infection which occurs between 1 and 6 wk postinfection (15, 16). It has been suggested that BALB/c susceptibility is due to a cessation of IFN-γ production that begins after the first week of infection. This has been mainly demonstrated by measuring in vitro IFN-γ secretion by spleen cells isolated from infected mice and cultivated in the presence of *Brucella* Ag (15, 11). Despite progresses in mouse models of brucellosis, much remains unknown regarding cellular components of the innate and adaptive immune response induced by *B. melitensis* infection. In particular, the nature and cell surface phenotype of innate immune effectors implicated in *Brucella* control are largely undetermined.

The initial host defense to infection is stimulated by pathogen-associated molecular patterns (PAMPs) which are common to different groups of pathogens. PAMPs are known to be recognized by a battery of germline-encoded host receptors which rapidly detect and signal microbial infections. Over the past few years, the TLR family has emerged as a major group of signaling receptors for PAMPs (17). Mammalian TLRs comprise a large family consisting of at least 11 members. TLRs detect multiple PAMPs, including

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[4] Abbreviations used in this paper: RNI, reactive nitrogen intermediate; ROI, reactive oxygen intermediate; iNOS, inducible NO synthase; PAMP, pathogen-associated molecular pattern; TIR, Toll-IL-1R; TRIF, TIR domain-containing adaptor inducing IFN-β; DC, dendritic cell.

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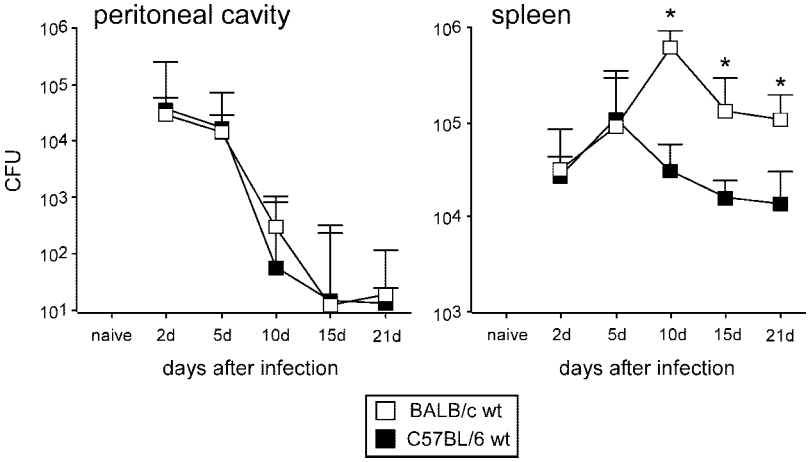


FIGURE 1. BALB/c and C57BL/6 mice infected with *B. melitensis* 16M. Wild-type C57BL/6 and BALB/c mice (eight per group) were injected i.p. with PBS alone or 4×10^4 CFU of *B. melitensis* in PBS as indicated in the figure. At the selected time, mice were sacrificed. Peritoneal exudates and spleens were harvested. Data represent the CFU per organs. Significant differences in relation to C57BL/6 mice are denoted by an asterisk (*) for $p < 0.05$.

LPS—the major glycolipidic component of Gram-negative bacteria (detected by TLR4), bacterial lipoproteins, and lipoteichoic acids (detected by TLR2), and the unmethylated CpG DNA of bacteria (detected by TLR9). TLRs recruit adaptor molecules that subsequently activate downstream signaling pathways and NF-κB. Four adaptor molecules have been identified: MyD88 (18), Toll-IL-1R domain-containing adaptor protein (TIRAP), Toll-IL-1R domain-containing adaptor inducing IFN-β (TRIF) (19), and Toll-IL-1R domain-containing adaptor molecule-2 (TICAM-2). Although the role of the various adaptors in the activation of specific signaling pathways still remains controversial, recent studies (17, 20) suggest that Toll-IL-1R domain-containing adaptor protein/MyD88 and TRIF-related adaptor molecule/TRIF regulate independent pathways.

TLR2 (21–23), TLR4 (21–23), TLR9 (24), and MyD88 (21, 22) have been found to be involved in the activation of immune response by *B. abortus*. Although the role of the MyD88 adaptor has been well-established (21, 22), several studies (21–23) have reported contradictory results about the roles of TLR2 and TLR4. Furthermore, the impact of TLR9 and TRIF adaptor deficiency in the course of infection remains undetermined. In this work, our aim is to characterize the main cellular components of the innate and adaptive immune response induced by mice infected with *B. melitensis* and to determine which TLRs and adaptor molecules are involved in the activation of these cells.

Materials and Methods

Mice and reagents

Six- to 8-wk-old female BALB/c and C57BL/6 mice were purchased from Harlan. TLR2$^{-/-}$ (25), TLR4$^{-/-}$ (26), and TLR2,4$^{-/-}$ mice with the BALB/c background and TLR9$^{-/-}$ (27), MyD88$^{-/-}$ (18), TRIF$^{-/-}$ (Ref. 19; B. Beutler, The Scripps Research Institute, La Jolla, CA) mice on the C57BL/6 background were bred in the animal facility of the Free University of Brussels (ULB). The maintenance and care of mice complied with the guidelines of the ULB Ethic Committee for the use of laboratory animals.

B. melitensis strain 16M (Biotype1; American Type Culture Collection (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 medium (Luria-Bertani broth with double quantity of yeast extract) to stationary phase was washed twice in PBS ($3500 \times g$, 10 min) before use in the inoculation of mice as previously described (28).

Mouse infection

Mice were injected i.p. with 4×10^4 CFU of *B. melitensis* in 500 μl of PBS. Control animals were injected with the same volume of PBS alone. The infectious doses were validated by plating serial dilutions of the inoculums.

Harvest of spleen and peritoneal exudates

At selected time intervals, mice were sacrificed by cervical dislocation. Immediately after being killed, mice were peritoneally washed with 10 ml of ice-cold HBSS and the spleen harvested. Before cytofluorometric analysis, spleens were cut in very small pieces and incubated (37°C, 30 min) into HBSS (Invitrogen Life Technologies) medium containing 4000 U/ml collagenase IV (Roche) and 0.1 mg/ml DNase I fraction IX (Sigma-Aldrich Chimie SARL). This procedure was performed in all experiments of this work.

Bacteria titers in spleens and peritoneal exudates of infected mice

Spleen cells or peritoneal exudates were recovered in PBS/0.1% Triton X-100 (Sigma-Aldrich). We performed successive serial dilutions in PBS to get the most accurate bacterial count and we plated them onto 2YT medium plates. CFU were counted after 3 days of culture at 37°C.

Statistical analysis

ANOVA I was used for data analysis after testing the homogeneity of variance (Bartlett test). Average comparisons were performed by pairwise Scheffe's test. Error bars represent the 95% interval of confidence of the mean (computed from residual mean square and Student's *t*, 0.95).

Cytofluorometric analysis

Peritoneal cells and spleen cells were first incubated in saturating doses of 2.4G2 (a rat anti-mouse FcR mAb; ATCC) for 10 min to prevent Ab binding to FcR. A total of $3–5 \times 10^6$ cells were stained with various fluorescent mAbs combinations in 200 μl of PBS, 0.5% BSA, 0.02% NaN3 (FACS buffer), and further collected on a FACSCalibur cytofluorometer (BD Biosciences). We purchased the following mAbs from BD Pharmingen: fluorescein (FITC)-coupled M1/70 (anti-CD11b), H57-597 (anti-TCRβ), PE-coupled M5/114.15.2 (anti-IA/IE), HL3 (anti-CD11c), AL-21 (anti-LY-6C), 1A8 (anti-LY-6G), RM4-5 (anti-CD4), 53-6.7 (anti-CD8α). Cells were gated according to size and scatter to eliminate dead cells and debris from analysis.

Intracellular cytokine staining

Peritoneal and spleen cells were incubated for 4 h in RPMI 1640, 5% FCS with 10 μg/ml Golgi Plug (BD Pharmingen) at 37°C, 5% CO_2. 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) containing one of the following intracellular staining mAbs: allophycocyanin-coupled XMG1.2 (anti-IFN-γ), allophycocyanin-coupled MP6-XT22 (anti-TNF-α), purified M-19 (anti-NOS_2; Santa Cruz Biotechnology) stained with Alexa Fluor 647 goat anti-rabbit (Molecular Probes). After final fixation in PBS/1% PFA, the cells were analyzed on a FACSCalibur cytofluorometer. No signal was detectable with isotype controls.

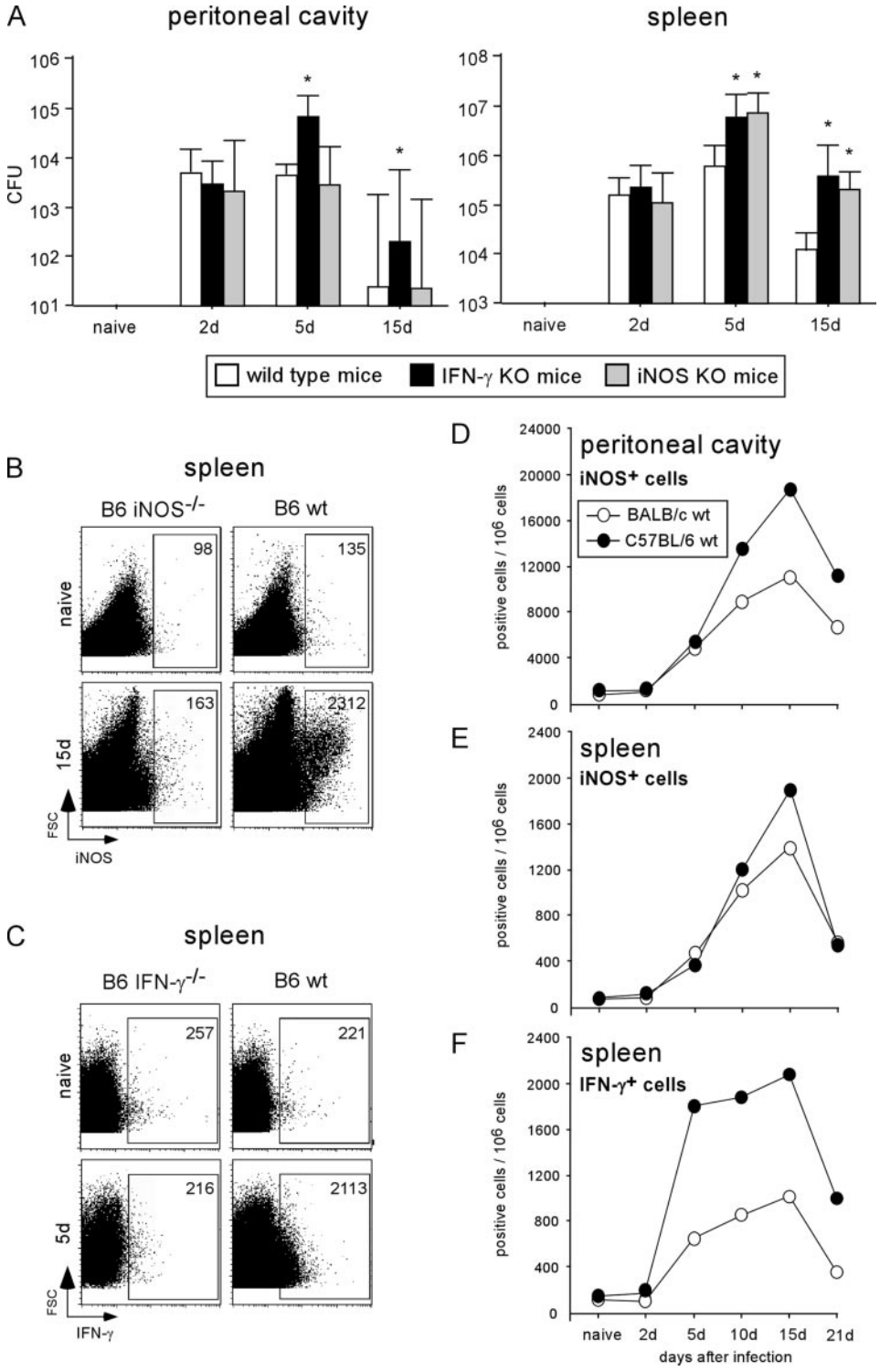


FIGURE 2. IFN-γ and iNOS are implicated in the control of *B. melitensis* infection. Wild-type, IFN-$\gamma^{-/-}$, iNOS$^{-/-}$ C57BL/6 mice (eight per group) were injected i.p. with PBS or 4×10^4 CFU of *B. melitensis*, as specified in the figure. Mice were sacrificed at selected time after treatment and peritoneal exudates and spleens harvested. *A*, Data represent the CFU per organs. These results are representative of two independent experiments. Significant differences in relation to wild-type mice are denoted by an asterisk (*) for $p < 0.05$. *B–F*, Pooled peritoneal cells and spleen cells from indicated group of mice were stained with allophycocyanin-labeled anti-IFN-γ mAb or anti-iNOS/NOS2 mAb stained with Alexa Fluor 647 Goat anti-rabbit. In each case, 10^6 cells per group were collected on a FACSCalibur cytofluorometer. Data represent the number of positive cells per million of acquired total cells. These results are representative of three independent experiments.

Results

Analysis of C57BL/6 and BALB/c mice infected with B. melitensis *16M*

C57BL/6 and BALB/c mice were infected with 4×10^4 CFU of *B. melitensis* (strain 16M) by i.p. inoculation. Bacteria titers inside peritoneal cavity and spleen were used to evaluate infection (Fig. 1). As illustrated (Fig. 1 and data not shown), infection peaks in peritoneal cavity and in spleen occurred at 2 and 5 days, respectively. This was followed by a rapid decrease in the peritoneal cavity against a substantial persistent level in the spleen. BALB/c mice susceptibility is therefore confirmed in our experimental model (Fig. 1) with marked susceptibility mainly observed during the plateau phase of infection in spleen. Meanwhile, both C57BL/6 and BALB/c mice could control early infection development with the same efficacy, as evidenced by the similar kinetic of bacteria titer inside the peritoneal cavity and the spleen during 5 days after infection (Fig. 1).

IFN-γ (9–11) and iNOS (11, 14) have been implicated in resistance to *B. abortus* infection. Using IFN-$\gamma^{-/-}$ and iNOS$^{-/-}$ C57BL/6 mice, we showed that IFN-γ and iNOS are implicated

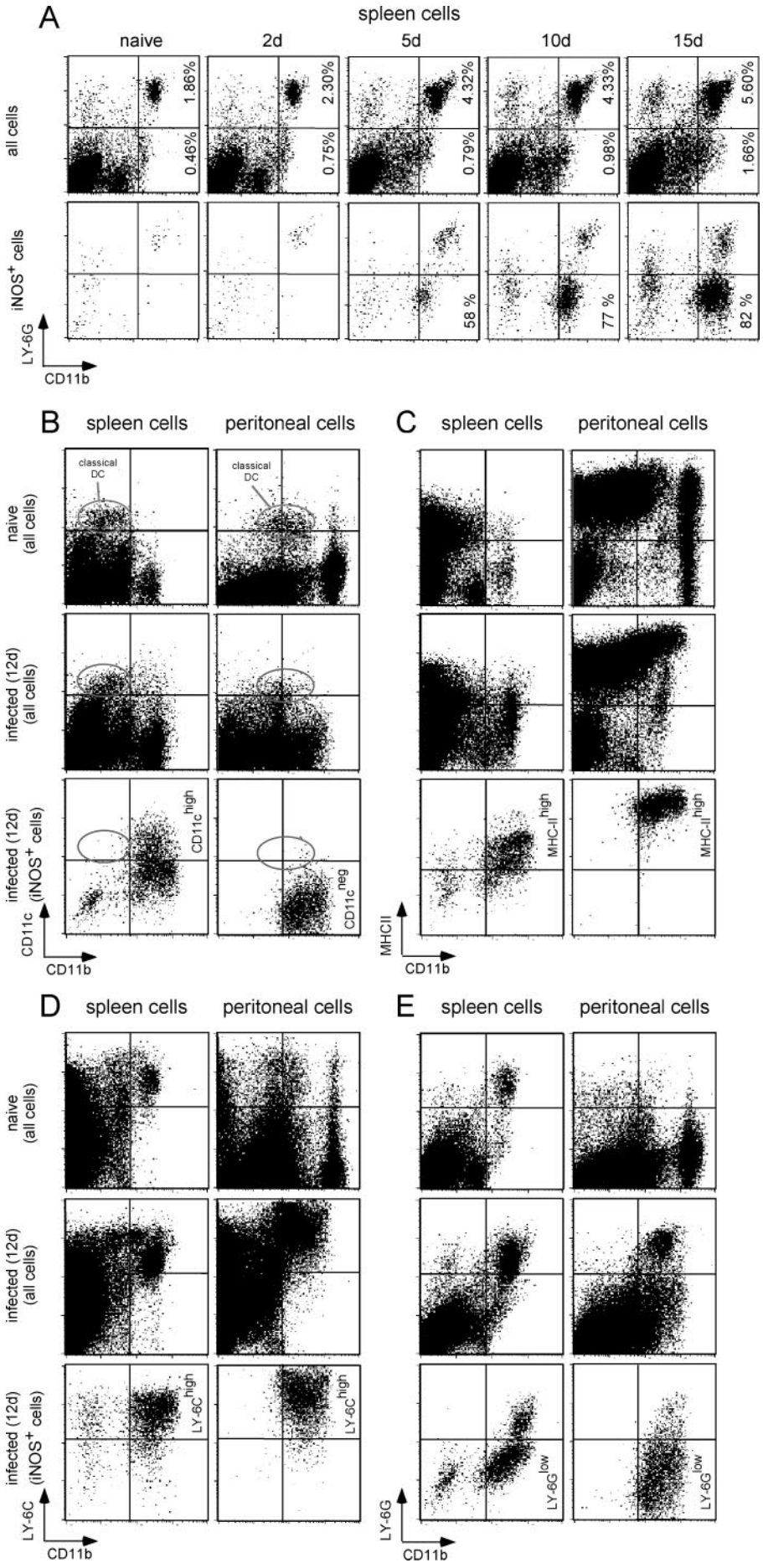


FIGURE 3. CD11b$^+$ LY-6C$^+$ cells are the main iNOS-producing cells during *B. melitensis* infection. Wild-type C57BL/6 mice (five per group) were injected i.p. with PBS or 4×10^4 CFU of *B. melitensis*, as specified. At the indicated time after treatment, mice were sacrificed and peritoneal exudates and spleens harvested. *A*, Pooled spleen cells were incubated with FITC-coupled anti-CD11b, PE-coupled anti-LY-6G, and anti-iNOS/NOS2 mAb stained with Alexa Fluor 647 goat anti-rabbit. Number indicates the percentage of cells in the selected quadrant. *B–E*, Pooled peritoneal cells an spleen cells from control or infected mice (as indicated) were stained with FITC-labeled anti-CD11b, anti-iNOS/NOS2 mAb stained with Alexa Fluor 647 goat anti-rabbit and with the following PE-coupled mAbs: anti-CD11c (*B*), anti-IA/IE (*C*), anti-LY-6C (*D*), and anti-LY-6G (*E*). These results are representative of five independent experiments.

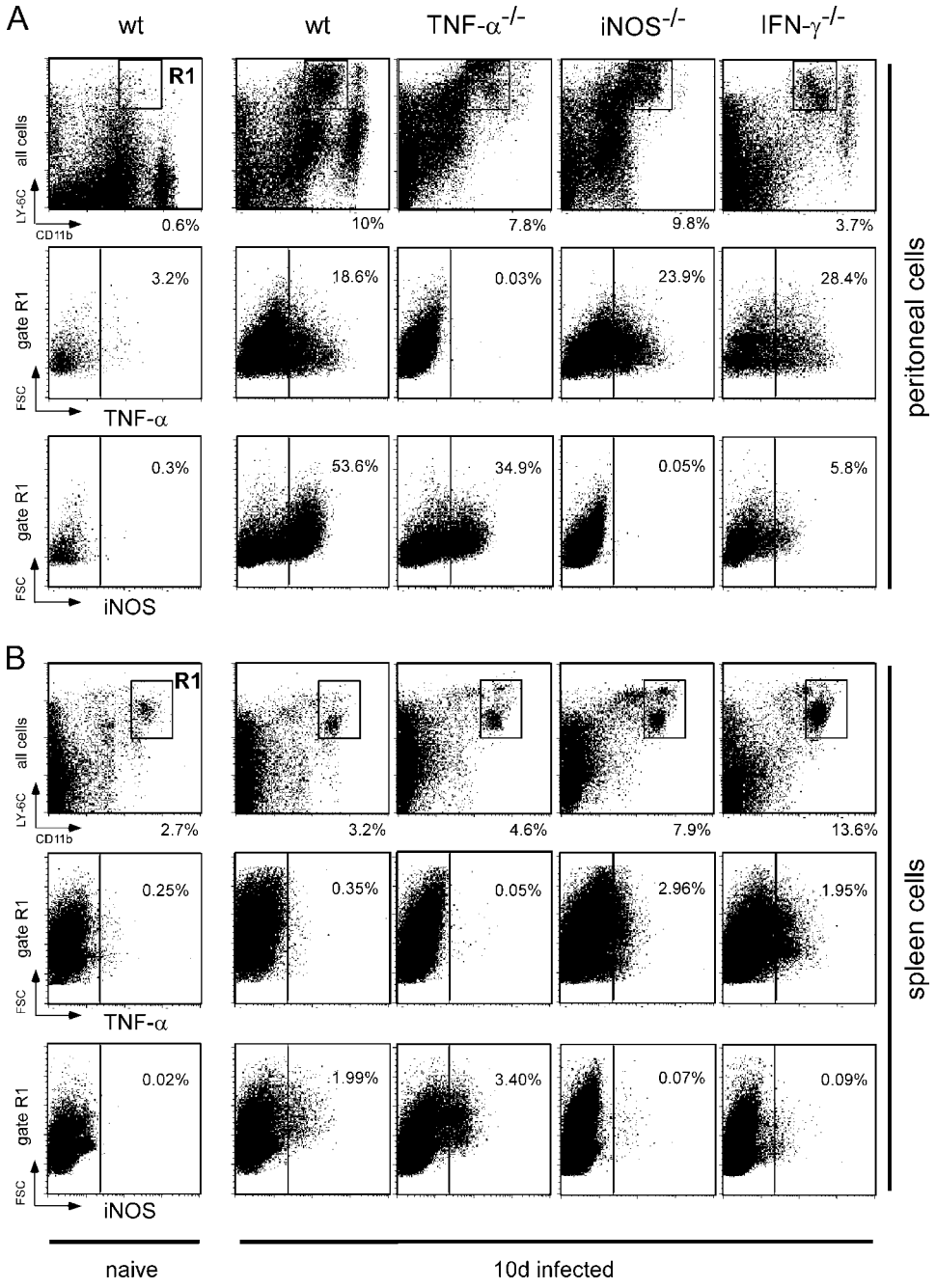


FIGURE 4. Frequency of iNOS$^+$ cells is dependent of IFN-γ. Wild-type, TNF-$\alpha^{-/-}$, iNOS$^{-/-}$, and IFN-$\gamma^{-/-}$ C57BL/6 mice (four per group) were injected i.p. with PBS or 4×10^4 CFU of *B. melitensis*, as specified in the figure. Mice were sacrificed 10 days after treatment and peritoneal cells (*A*) and spleen cells (*B*) harvested. Pooled cells in each group were stained with FITC-labeled anti-CD11b, PE-coupled anti-LY-6C, and with allophycocyanin-coupled anti-TNF-α or anti-iNOS/NOS2 mAb stained with Alexa Fluor 647 goat anti-rabbit. Number under the first line of plot in *A* and *B* indicate the percentage of cells in the R1 gate among the total cells acquired. Number in the second and third line of plot indicate the percentage of cells in the quadrant among the cells gated in R1. These results are representative of three independent experiments.

in the resistance to *B. melitensis* infection (Fig. 2*A*). At 5 days postinfection, we found 10-fold increase CFU in the spleen of infected IFN-$\gamma^{-/-}$ and iNOS$^{-/-}$ mice when compared with wild-type mice. In contrast, in the peritoneal compartment, while iNOS$^{-/-}$ and wild-type mice displayed similar bacteria levels at all test times, IFN-$\gamma^{-/-}$ mice displayed 10-fold increase CFU. These data suggest that both IFN-γ and iNOS may have a potential role in the control of *B. melitensis* 16M growth in the spleen whereas only IFN-γ plays a role in the peritoneal cavity.

Flow cytometric analysis of IFN-γ and iNOS cell frequency in peritoneal and splenic compartments in the course of *B. melitensis* infection was used in the clarification of BALB/c mice susceptibility. It is important to note that cells purified from naive and infected mice were not stimulated in vitro before intracellular staining. Thus, iNOS and IFN-γ detected in these cells derive only from natural in vivo activation during the course of infection. Naive and infected iNOS$^{-/-}$ and IFN-$\gamma^{-/-}$ C57BL/6 mice were used as internal negative control in these experiments. As expected, significant iNOS and IFN-γ staining were only observed in infected wild-type C57BL/6 mice (Fig. 2, *B* and *C*). We observed that the frequency of iNOS$^+$ cells increased slowly during infection of wild-type C57BL/6 mice and peak at 10–15 days in the peritoneal cavity and spleen (Fig. 2, *D* and *E*). IFN-γ^+ cells were not detected in the peritoneal cavity (data not shown); meanwhile, an impact of this cytokine was observed on the resistance against the bacterial invasion (Fig. 2*A*). Thus, it is possible that IFN-γ produced in another compartment, such as the spleen, mediated resistance in the peritoneal cavity. In the spleen of C57BL/6 mice, the frequency of IFN-γ^+ cells reached its plateau phase between 5 and 15 days postinfection (Fig. 2*F*). Surprisingly, a similar observation

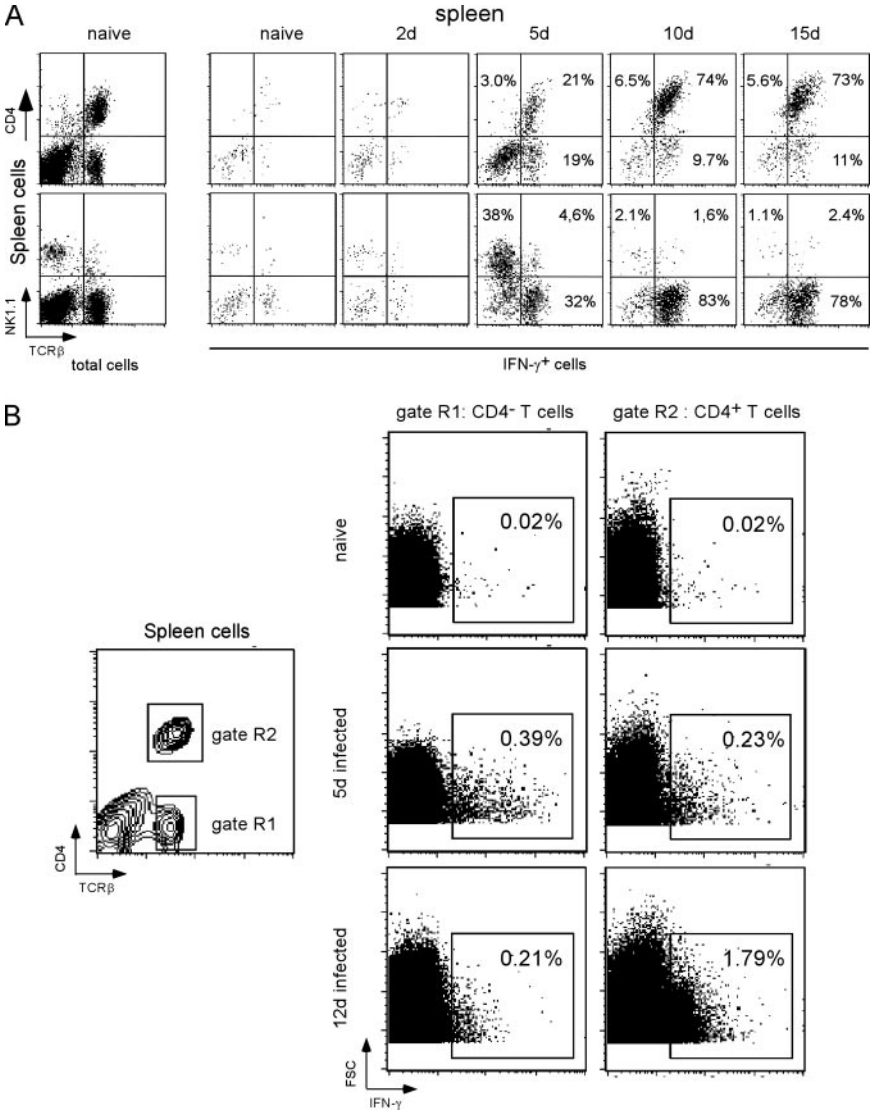


FIGURE 5. CD4$^+$ T cells are the main IFN-γ-producing splenic cells at the later stage of *B. melitensis* infection. Wild-type C57BL/6 mice (five per group) were injected i.p. with PBS or 4×10^4 CFU of *B. melitensis*, as specified in the figure. Mice were sacrificed at selected time after treatment and spleen cells were harvested. Pooled spleen cells in each group were stained with FITC-labeled anti-TCRβ, allophycocyanin-coupled anti-IFN-γ and with one of the following PE-coupled mAb: anti-CD4 or anti-NK1.1. Number indicates the percentage of cells in the selected quadrant (*A*) or gate (*B*). These results are representative of four independent experiments.

was found for both iNOS- and IFN-γ-producing cells in infected BALB/c mice (Fig. 2, *D–F*). However, the frequency of iNOS$^+$ cells and mostly IFN-γ^+ cells is lower in BALB/c mice. Thus, we found that BALB/c mice kept the ability to produce IFN-γ during the plateau phase of the infection. However, the frequency of IFN-γ^+ cells is weak and can explain the lack of resistance of these mice. Cell surface phenotypes of IFN-γ^+ and iNOS$^+$ cells during *B. melitensis* infection in C57BL/6 and BALB/c mice were later characterized.

CD11b^{high}LY-6C^{high}LY-6G^{low}MHC-II^{high} cells are the main iNOS-producing cells during B. melitensis *infection*

B. melitensis infection induces a recruitment of CD11b^{high}LY-6G^{high} cells (granulocyte) and CD11b^{high}LY-6G^{low} cells in the spleen of infected C57BL/6 mice (Fig. 3*A*). Both populations contain iNOS$^+$ cells, but the CD11b^{high}LY-6G^{low} population appears as the major iNOS-producing cells at 10–15 days of infection. Further analysis of their cell surface phenotype by flow cytometry showed that these later cells display CD11c^{high}MHC-II^{high}, LY-6C^{high} and LY-6C^{low} (Fig. 3, *B–E*, respectively). INOS$^+$ cells in the peritoneal cavity expressed a very similar phenotype with the exception of CD11c which appeared negative (Fig. 3*B*). Similar results were obtained in infected BALB/c mice (data not shown).

The majority of iNOS$^+$ cells from both the peritoneal cavity and the spleen expressed CD11b^{high}LY-6C^{high}LY-6G^{low} and MHC-II^{high} cell surface markers similar to inflammatory dendritic cells (DC) recently described during the course of *Listeria monocytogenes* infection in mouse (28–31) and in human psoriasis (32). Because these cells have been described to produce TNF-α (28–31) and TNF-α has been associated to the control of *Brucella* infection (33), we investigated the ability of CD11b^{high}LY-6C^{high} cells to produce TNF-α during *B. melitensis* infection (Fig. 4). Infected TNF-$\alpha^{-/-}$ mice were used as negative control for TNF-α staining in these experiments. In wild-type C57BL/6 mice, a large fraction of CD11b^{high}LY-6C^{high} peritoneal cells appeared TNF-α positive. In contrast, splenic CD11b^{high}LY-6C^{high} cells were almost TNF-α negative.

It is well-known that IFN-γ positively regulate iNOS production in experimental bacterial infectious model (12, 13) and mediate resistance to *Brucella* infection (Refs. 9–11 and Fig. 2*A*). We therefore analyzed the impact of IFN-γ deficiency on the activation of CD11b^{high}LY-6C^{high} cell population during *B. melitensis* infection. As shown in Fig. 4, IFN-γ deficiency strongly decreased iNOS$^+$ cell frequency in peritoneal cavity and spleen. In contrast, TNF-α^+ cells frequency was enhanced in peritoneal cavity and in spleen.

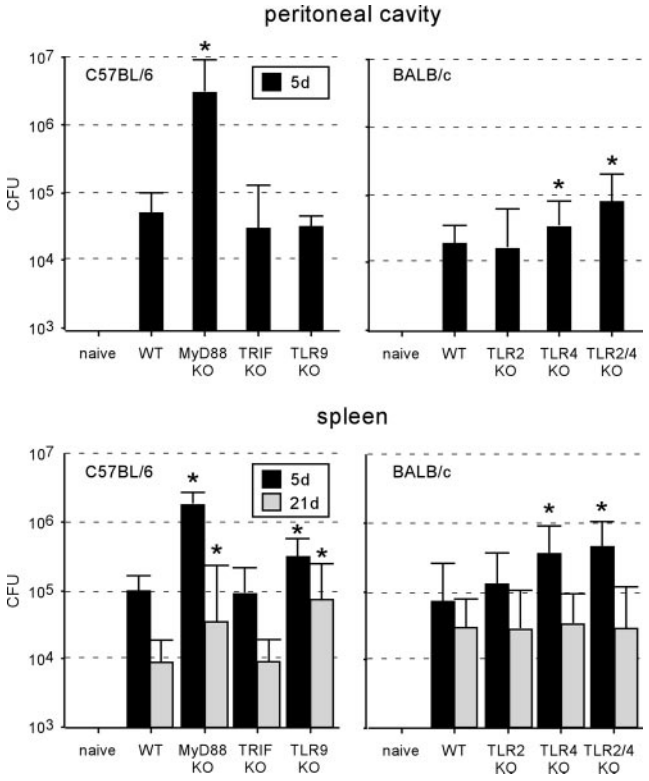


FIGURE 6. TLR-4, TLR-9, and MyD88, but not TLR-2 and TRIF, are implicated in the control of *B. melitensis* infection. Wild-type, MyD88$^{-/-}$, TRIF$^{-/-}$, TLR9$^{-/-}$ C57BL/6 mice and wild-type, TLR2$^{-/-}$, TLR4$^{-/-}$, TLR2/4$^{-/-}$ BALB/c mice (eight per group) were injected i.p. with PBS or 4×10^4 CFU of *B. melitensis*, as specified in the figure. Mice were sacrificed at selected time after treatment and peritoneal exudates and spleens harvested. Data represent the CFU per organs. These results are representative of four independent experiments. Significant differences in relation to wild-type mice are denoted by an asterisk (*) for $p < 0.05$.

Globally, these results suggest that 1) a fraction of the splenic CD11b^{high}LY-6C^{high} cell population can produce both TNF-α and iNOS during *Brucella* infection, 2) iNOS synthesis is strictly dependent on IFN-γ, and 3) iNOS and TNF-α production can be independently regulated.

CD4$^+$ T cells are the main IFN-γ-producing splenic cells at the later stage of B. melitensis *infection*

IFN-γ appears to be produced by distinct cells during the course of infection. In the early course (5 days), IFN-γ is synthesized mainly by NK cells (TCRβ^-NK1.1$^+$ cells, 38% of producing cells), CD4$^+$ T cells (TCRβ^+CD4$^+$ cells, 21%) and CD8$^+$ T cells (TCRβ^+CD4$^-$ cells, 19%) (Fig. 5*A*). Later on (10 days and above), CD4$^+$ T cells become the major producing cells (74%). IFN-γ^+ cells display a similar phenotype during the course of infection in BALB/c mice (data not shown). It is interesting to observe that the frequency of IFN-γ^+ cells at 5 days among CD4$^+$ T cells and CD8$^+$ T cells appear to be very close (0.23 and 0.39%, respectively) (Fig. 5*B*). However, at 12 days, only the CD4$^+$ T cells frequency increase to 1.79%.

TLR-4, TLR-9 and MyD88, but not TLR-2 and TRIF, are implicated in the control of brucellosis

To gain insight into the role of TLRs and their adaptor proteins during *Brucella* infection, we compared in the same experiment the susceptibility of mice genetically deficient for these molecules. Mice were infected and bacteria count of bacteria examined at 5 and 21 days in peritoneal exudates and spleen (Fig. 6). We observed that TLR4 and TLR9 deficiency moderately affected the resistance of mice to infection. This was mostly apparent in the spleen and less in the peritoneal cavity. TLR2 deficiency, alone or in combination with TLR4 deficiency, has no effect. MyD88- but not TRIF-deficient mice displayed higher susceptibility.

In the same experiment, we analyzed the impact of TLRs and adaptor protein deficiency on the activation of iNOS and IFN-γ-producing cells (Fig. 7). As expected, deficiencies that affect resistance to infection proportionally reduce the frequency of effectors cells observed in peritoneal exudates and spleen. MyD88 and TLR4 deficiency strongly affects the frequency of iNOS$^+$ and IFN-γ^+ cells at 5 and 21 days in both compartments tested. In contrast, TLR-9 deficiency reduces iNOS$^+$ cell frequency only in peritoneal cavity.

Discussion

IFN-γ is a key cytokine regulating the protective immune response against *Brucella*. This has been demonstrated by showing that administering of rIFN-γ enhances mice resistance resulting in a 10-fold decrease in the number of bacteria (9). Also, it has been shown that depleting IFN-γ by the administration of mAbs which neutralize IFN-γ (10) or by the use of IFN-$\gamma^{-/-}$ mice (Ref. 11 and data not shown) results in a 10-fold increase in the number of bacteria. Here, for the first time, we characterized by flow cytometry the cell surface phenotype of spleen cells producing IFN-γ in vivo during the course of *Brucella* infection. At the peak of infection (5 days), IFN-γ is produced mainly by TCRβ^-NK1.1$^+$ NK cells, TCRβ^+CD4$^+$ T cells, and TCRβ^+CD8$^+$ T cells. During the plateau phase of infection (10–21 days), CD4$^+$ T cells become the major producing cells (74% of IFN-γ^+ cells).

The respective contribution of CD4$^+$ T cells and CD8$^+$ T cells to protective immune response against *Brucella* is controversial (reviewed in Ref. 34). Transfer experiments showed an equal rule for both subsets. In contrast, β_2-microglobulin$^{-/-}$ mice (deficient for CD8$^+$ T cell activation) appeared more susceptible that MHC-II$^{-/-}$ mice (deficient for CD4$^+$ T cell activation) suggesting that CD8$^+$ T cells play a crucial role in protection. However, it is well-known that β_2-microglobulin$^{-/-}$ mice are deficient for both MHC-I and CD1d Ag-presentation pathways rendering complex the interpretation of the results in this model. The relatively equal role of NK, CD4$^+$, and CD8$^+$ T cells in IFN-γ secretion at the peak of infection could explain the results of transfer experiments. In addition, the predominant role of CD4$^+$ T cells in IFN-γ secretion during the plateau phase of infection could explain why RAG1 (35) but not β_2-microglobulin-deficient mice (11) and NK-depleted mice (36) drastically fail to control *Brucella* infection.

Experiments in genetically deficient mice have showed that MyD88 adaptor protein is implicated in the resistance to *B. abortus* infection (21, 22). We demonstrated that MyD88 deficiency strongly reduces the frequency of IFN-γ^+ cells at all time of *B. melitensis* infection. The unique role of MyD88-dependent signaling pathways is confirmed by the fact that TRIF-deficient mice do not have a reduced frequency of IFN-γ^+ cells and display normal resistance to infection. Identification of TLRs coupled to MyD88 and detecting *B. melitensis* infection appear complex. Contradictory results have been reported concerning the implication of TLR2 and TLR4 in resistance to *B. abortus* infection. TLR2 has been involved in heat-killed *B. abortus*-induced TNF-α (22) but not in the resistance to infection (21, 23). TLR4 deficiency has been reported to reduce (23) or have no affect (21) on resistance in

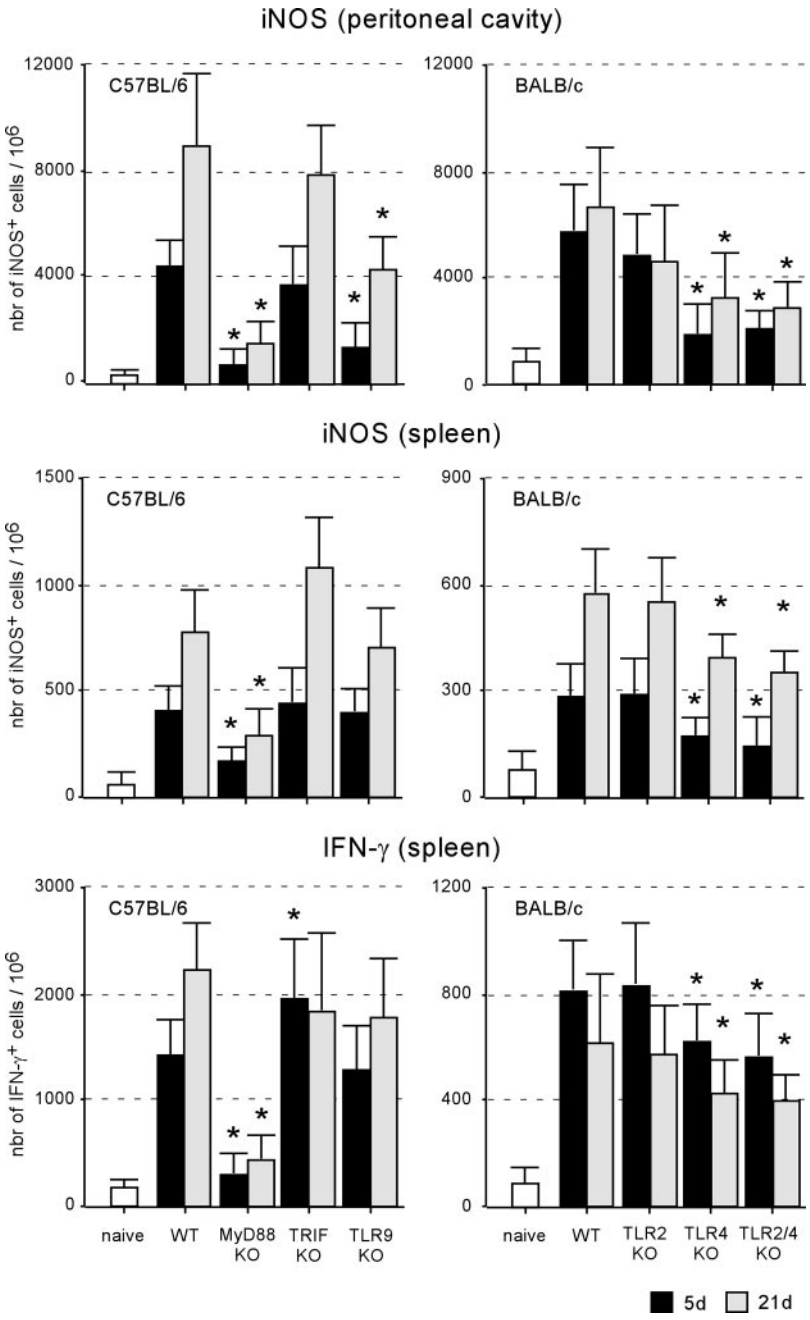


FIGURE 7. TLR-4, TLR-9, and MyD88, but not TLR-2 and TRIF, regulate frequency of iNOS$^+$ and IFN-γ^+ cell during *B. melitensis* infection. Wild-type, MyD88$^{-/-}$, TRIF$^{-/-}$, TLR9$^{-/-}$ C57BL/6 mice and wild-type, TLR2$^{-/-}$, TLR4$^{-/-}$, TLR2/4$^{-/-}$ BALB/c mice (five per group) were injected i.p. with PBS or 4×10^4 CFU of *B. melitensis*, as specified in the figure. Mice were sacrificed at selected time after treatment and peritoneal exudates and spleens harvested. Cells of each animals were stained with allophycocyanin-labeled anti-IFN-γ or anti-iNOS/NOS2 mAb stained with Alexa Fluor 647 goat anti-rabbit. Data represent the number of positive cells per 10^6 total cells acquired. These results are representative of three independent experiments. Significant differences in relation to wild-type mice are denoted by an asterisk (*) for $p < 0.05$.

B. abortus. In our experimental model of *B. melitensis* infection, we found that TLR4, but not TLR2, significantly decrease resistance to infection. The reasons for these discrepancies are not known but could be due to the different strain of *Brucella* used. In addition, we reported for the first time that TLR9 deficiency also reduces resistance. This result is in agreement with previous work (24) showing the role of TLR9 in the induction of inflammatory cytokines by heat-killed *B. abortus*. Collectively, our data suggest that both TLR4 and TLR9 cooperate in the immune system to detect *B. melitensis* infection. A logical consequence of multiple TLR signaling is that the reduction of IFN-γ^+ cell frequency is moderately or not affected by the deficiency of TLR4 or TLR9 alone.

In various experimental models of infection by intracellular pathogen, a major function of IFN-γ is the stimulation of killing effector mechanisms, such as the production of RNI and ROI in phagocytic cells (12, 13). A previous study (14) reported that both gp91$^{phox-/-}$ mice and iNOS/NOS2$^{-/-}$ mice are susceptible to *B. abortus* infection but that iNOS deficiency more drastically affects the resistance, suggesting a predominant role of RNI in *Brucella* killing. Based on these data, we selected iNOS as a marker of protective innate immune response against *Brucella*. We showed that IFN-γ positively regulates the frequency of iNOS$^+$ cells in the peritoneal cavity and spleen. Infected IFN-$\gamma^{-/-}$ mice display a 10-fold reduction of iNOS$^+$ cells frequency. In contrast, TNF-α deficiency moderately affects this frequency during *Brucella* infection. This suggests a key role of CD4$^+$ T cells, the main IFN-γ^+ cells, in the regulation of *Brucella*-killing effector mechanisms.

Characterization of cell surface marker expressed by peritoneal and splenic iNOS$^+$ cells showed that the great majority of these cells expressed the following phenotype: CD11b^{high}LY-6C^{high}LY-6G^{low}MHC-II^{high}. They are negative for T cell (TCRβ, CD8α), B cell (IgD, IgM, B220), mastocyte (c-*kit*), and NK (NK1.1) cell

surface markers (data not shown). Splenic iNOS$^+$ cells also express CD11c and thus display a phenotype very similar to inflammatory DC recently described during *L. monocytogenes* infection in mice (28–31) and psoriasis in human (32). These cells, termed TNF/iNOS-producing DC by the authors, appear as a major inflammatory and effectors cell type in these pathologies. In our infectious model, peritoneal iNOS$^+$ cells are CD11c negative but the absence of CD11c expression on DC is frequent in nonlymphoid organs (37). Their very similar cell surface phenotype suggests that they might be related to splenic inflammatory DC.

DC populations (38) are highly heterogeneous and distinct subsets have been discriminated on the basis of differential expression of various cell surface markers and specialized functions. However, relatively little is known about DC recruitment and function in response to pathogen infection. In addition to B220$^-$LY-6C$^-$ NK1.1$^-$ "classical" DC, various specialized DC subsets have been recently defined. Plasmacytoid DC, expressing B220$^+$CD11b$^-$ LY-6C$^+$NK1.1$^-$ markers, are specialized in IFN-α production and antiviral response (39). NK DC, expressing CD11b$^-$DX5$^+$ NK1.1$^+$ markers, displayed both cytotoxicity and APC functions (40). The implication of B220$^-$CD11b$^+$LY-6C$^+$NK1.1$^-$ iNOS$^+$ DC in both *Listeria* (28–31) and *Brucella* infection (this study) allows us to propose that these cells can be major effectors in murine Th1 immune response against intracellular bacteria. Moreover, as CD11c$^+$CD11b$^+$LY-6C$^+$ DC isolated during *Listeria* infection display APC function in vitro (30), it is possible that these cells also play an important regulatory role by stimulating and programming T cell responses during these infections. Interestingly, their recent finding in human psoriasis (32) suggests that they can also play an important role in human Th1 immune response. As demonstrated in the *Listeria* model (31), we also showed that inflammatory DC activation during *Brucella* infection is strongly dependent on MyD88-signaling pathways. In contrast, TRIF pathways do not appear to be required. As for IFN-γ^+ cell, we observed a reproducible but weak impact of TLR-4 and TLR-9 deficiency on iNOS$^+$ cell frequencies.

Finally, BALB/c mice, previously described as susceptible to *Brucella* infection (15, 16), display few differences with C57BL/6 mice. The kinetic and the type of IFN-γ and iNOS cells producing seem similar in both types of mice. Only the frequency of these cells is reduced. This suggests that the immune response developed by C57BL/6 and BALB/c mice differ only by its intensity. This might be explained by the well-known reduced ability of BALB/c mice to mount Th1 response.

In summary, our study confirms the predominant role of MyD88 adaptor molecule in the development of protective immune response against *Brucella*. We showed that MyD88-dependent signaling pathways appear strictly necessary to the development of IFN-γ^+ and iNOS$^+$ cells during infection. We also demonstrated that B220$^-$CD11b$^+$LY-6C$^+$ DC are recruited and activated during infection and constitute the main iNOS-producing cells in all compartments analyzed. Taken together, our results provide new insight into how innate and adaptive immune response fight *Brucella* infection.

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Disclosures

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2. Part II:

2.1. Prologue

Despite the numerous studies carried out to upgrade our knowledge on the relationship between *Brucella* and the immune system, few elements have allowed a significant breakthrough in the field of brucellosis over the past ten years. One of the reasons that may explain this fact can be the extensive use of similar approaches to describe the effector mechanisms induced by *Brucella* infection. If studies of knock out mice immune responses by cytometry techniques have been useful to uncover the great avenues of *Brucella* pathogenesis, this approach is today insufficient to dissect the precise immune effectors mechanisms elaborated by the host to control *B. melitensis* infection. Indeed, even if we know that *Brucella* is an intracellular pathogen inducing a strong T_H1 response, this bacterium, in contrast to other intracellular pathogens like *L. monocytogenes*, does not irreversibly affect the health status of the majority of mice genetically deficient for immune proteins such as Myd88 or IL-12. This observation illustrates that the immune responses of the mouse interfering with *Brucella* proliferation are complex and necessitate new methods to be further characterized.

This is the perspective from which we decided to use *in situ* techniques for studying the relationship between *B. melitensis* and the innate immune system of the mouse.

2.1.1. In situ *characterization of immune effectors mechanisms deployed against* B. melitensis.

During the first part of the thesis, we discovered that the main iNOS-producing cells in the infected spleen presented a cell surface phenotype similar to the inflammatory TipDCs. Effector cells implicated in the fight against intracellular pathogens need to be immediately adjacent to infected cells in a manner as to avoid a potential detrimental impact of the immune response on host self-tissues. To better understand the existing link between *B. melitensis* and TipDCs, and to characterize more deeply the effector mechanisms deployed by the immune system during the infection, we used immunohistofluorescence to study the local immune environment developed around *B. melitensis* infected cells in wild type and immunodeficient mice.

2.1.2. *Phenotypical description of* B. melitensis *infected cells in the spleen and liver of wild type and immunodeficient mice.*

Being defined as an intracellular pathogen, *Brucella* can replicate in professional phagocytes such as MØs, DCs, and PMNs as well as non-professional phagocytes including epithelial, fibroblastic and trophoblastic cells, in the context of cell line cultures. *In vivo*, however, the role of such *Brucella* "reservoir" during the course of infection is largely undetermined. If MØs and trophoblastic cells have been clearly associated with *Brucella* infection in the natural hosts, little has been demonstrated regarding the contribution of other potential cellular niche for *Brucella in vivo*. During this last part of the work, we described the surface phenotype of cells targeted by *B. melitensis* in the spleen and liver of wild type and immunodeficient mice during the five first days of infection.

2.1.3. *Analysis of the impact of* B. melitensis *infection on the behavior of DCs.*

B. melitensis is described as a furtive pathogen able to interfere with DCs maturation. This ability has been used to explain the capacity of *Brucella* to impede the development of an effective anti-*Brucella* immune response and thereby to provoke long-lasting infection in its host. However, the negative impact of *Brucella* on the maturation of these APCs has only been shown *in-vitro* or during an experimental infection of ligated ileal loops of calves [Salcedo *et al.*, 2008]. To shed light on the impact of *B. melitensis* on DC subset maturation, we analyzed the behavior of these cells during the first steps of infection in the spleen of infected mice, by immunohistofluorescence and flow cytometry techniques.

In summary, the aims of this part of the work were: (1) to identify innate immune effector mechanisms associated with *B. melitenis* infection, *in situ*; (2) to determine the phenotypical nature of *Brucella* infected cells in the spleen during the first stages of infection [Cole *et al.*] to characterize the impact of *B. melitensis* infection on the maturation of DCs.

IMPORTANT REMARK

The following pages contain the preliminary manuscript describing the results obtained during the second part of the thesis. We insist on the fact that it does not correspond to the final version of the document.

In situ characterization of local immune environment developed around *Brucella melitensis* infected cells in resistant and susceptible mice.

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ABSTRACT

Brucella organisms are facultative intracellular Gram-negative bacteria that chronically infect humans and animals causing Brucellosis, one of the most frequent zoonosis in the world displaying a high morbidity. *Brucella* are able to infect and multiply in a large range of cell lines *in vitro*, however the cells supporting bacterial growth *in vivo* as well as serving as "reservoir" during the infection are completely unknown. In order to identify these, we developed a *Brucella melitensis* strain stably expressing mCherry fluorescent tracer. This new tool has allowed us to phenotype the infected cells in the spleens and livers, two major sites of *B. melitensis* growth. In both tissues, the majority of infected cells express the F4/80 myeloid marker. In wild type mice, infection leads to granuloma development in spleen and liver. These structures are mainly composed of $CD11b^+$ $CD11c^+$ $MHC\text{-}II^+$ cells expressing iNOS/NOS2 enzyme. These latter are similar to iNOS-producing inflammatory DC (iNOS-DC) observed in the mouse models of *Listeria monocytogenes* and *Leishmania major* infection. Analysis of genetically deficient mouse strains reveals that granuloma formation is affected in absence of MyD88 adapter molecule. Taken together, our results suggest that iNOS-DC represent a major class of Th1-regulated effector cells critical for host defense to *Brucella* bacteria.

INTRODUCTION

Brucella organisms (α-proteobacteria) are facultative intracellular Gram-negative coccobacilli that infect humans and animals (goat, sheep, swine …). Animal infection leads to abortion and infertility. Brucellosis is mainly transmitted to humans through the ingestion of raw milk or non-pasteurized cheese contaminated with *Brucella.* It is characterized by undulating fever which, if untreated, may 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 commonest zoonotic disease worldwide with more than 500 000 new cases annually (4).

Brucella strains are highly infectious via oral and aerosol route and difficult to treat with antibiotics. No safe and effective vaccine is available to prevent human infection. This combination of characteristics has led to the inclusion of *Brucella* strains on lists of category B pathogens considered to confer risks 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 bacteria dissemination following intraperitoneal inoculation in mice (Rajashekara, 2005). Results confirmed that intraperitoneal mice infection parallels human infection and identify major site of bacteria growth, such as spleen and liver, during early chronic phase (1-5 days) of infection. However, the cells supporting bacterial growth *in vivo* and serving as "reservoir" remain completely unknown. In order to identify these, we developed a *B. melitensis* strain stably expressing mCherry fluorescent tracer (mCherry-Br). This new tool has allowed us to phenotype the infected cells in the spleens and livers of infected mice.

Despite progresses in mouse models of brucellosis, much remains unknown regarding cellular components of the innate and adaptive immune response induced by *B. melitensis* infection. We and other have shown that IFN-γ producing $CD4^+$ T cells (9-13) and inducible Nitric Oxide Synthase (iNOS/NOS2)-producing inflammatory dendritic cells (DC) (9) are major components of protective immune response against *B. melitensis*. Activation of these cells implicate Toll Like Receptor (TLR)4 and TLR9 coupled to MyD88 adaptor protein (9). However, little understanding exists regarding how these cells are positioned *in situ* such that they can initiate cellular interactions to control infection. Several experimental

models of infection by intracellular bacteria such as *Mycobacteria tuberculosis* and *Listeria monocytogenes* have showed 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. 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 granulomic structure. Chronic granulomatous inflammation has been reported in spleen and liver from natural host, human and mice infected by *Brucella* bacteria (ref). However, their importance in the control of *Brucella* growth, their ellulaur ccomposition and the signalling pathways implicated in their formation are completely unknown. Using mcherry- expressing *Brucella* (mcherry Br), we characterized the narure of granuloma in the spleen liver of the mouse. We observed the formation of $iNOS^+$ granuloma structure surrounding infected cells in these organs. We identified inflammatory DC ($CD11b^+$ $CD11c^+$ $Ly\text{-}6G^-$ $MHC\text{-}II^+$) and activated monocytes ($CD11b^+$ $CD11c^-$ $Ly\text{-}6G^-$ $MHC\text{-}II^+$) as the major cells constituting granuloma lesion in both tissues. In addition, we observed that MyD88 deficiency affect the cellular composition of granuloma and reduce its ability to control bacteria dissemination.

RESULTS

Construction of mCherry stably expressing *Brucella melitensis* 16M.

In order to detect infected cells on tissue sections by fluorescent microscopy, we constructed a constitutive fluorescent strain of *B. melitensis*. The mCherry protein, a previously described rapidly maturing variant of the red fluorescent protein DsRed (14), was cloned into the suicide vector *pKSoriT-bla-kan* downstream a strong *Brucella* spp. promoter named *PsojA* (15). This promoter is described as controlling the expression of the protein translocase SecE. 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).

Fluorescence of mCherry-expressing *Brucella* (mCherry-Br) was well detectable by flow cytometry on bacteria (Figure S1.A) and by fluorescent microscopy on *in vitro* infected hamster fibroblastic BHK cell line (Figure S1.B). Wild type and mChery-Br strains displayed similar growth curve *in vitro* (data not shown) suggesting that mCherry gene insertion does not affect ability of *B. melitensis* to proliferate. In addition, following intraperitoneal inoculation of $4x10^4$ CFU, mCherry-Br was able to persist for a long time in resistant C57BL/6 mice (Figure S1.C). At 5 days post infection, bacteria counts in spleen from mCherry-Br infected mice were reduced of approximately 0.5 log when compared to wild type bacteria infected mice. However, at 25 and 42 days post infection, bacteria count of spleen from both groups appeared similar suggesting that insertion of mCherry tracer affect weakly bacteria virulence.

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

At low dose of infection (10^4 to 10^6 CFU) in C57BL/6 and BALB/c wild type mice, *B. melitensis* peaked at 5 days post infection with modest CFU count (between 10^5-10^6 CFU / gr of spleen (9)) rending its visualization on tissue sections very difficult. Dose experiments demonstrated that 10^6 CFU / gr of tissue constitute the detection limit with < 0.1 infected cells by observation surface (200x230 µm) in tissue section of 5µm thickness with 63x objective (data not shown). Thus, to practically visualize mCherry-Br infection *in vivo* several days after inoculation, we chose to inject 10^8 CFU of bacteria in wild type mice. At this dose, mCherry-Br infected wild type C57BL/6 and BALB/c mice did not present any mortality and spleens displayed in average 10^6 CFU 10 minutes after intraperitoneal inoculation and maintained a level of 10^7-10^8 CFU between 1 to 5 days post infection

(Figure S2.A), rending possible the observation of mCherry-Br infected cells *in situ* during this time (Figure S2.B). At 12 days, spleens from wild type mice displayed less of 10^5-10^6 CFU and bacteria become untraceable by microscopic analysis.

In spleen, first *Brucella* infected cells are mainly F4/80$^+$ red pulp macrophages and MOMA-1$^+$ marginal zone macrophages.

Following intraperitoneal inoculation of 10^8 CFU in resistant C57BL/6 mice, mCherry-Br was detectable in the spleen by microscopic analysis by 10 minutes post infection. At this time, mCherry-Br located mainly in marginal zone (m.z.) and red pulp (r.p.) of infected spleen (data not shown). The number of bacteria exponentially increased during the first three hours (Figure S2.A) but bacteria remained always located in m.z. and r.p (Figure 1.A). Only rare infected cells were observed in splenic white pulp area (w.p.). Further quantitative analyses of the phenotype of infected cells (Figure 1.B and Figure 2.A-B) showed that first infected spleen cells were mainly (~80%) macrophages displaying a F4/80$^+$ CD11b$^-$ phenotype and (~20%) marginal zone macrophages expressing MOMA-1 marker and localized in r.p. and m.z., respectively. Between 3 and 6 hours after inoculation, the frequency of infected MOMA-1$^+$ cells decreased strongly and CD11c$^+$ DC became infected (~30% of total infected cells at 6 hours). Similar results were obtained in BALB/c mice (data not shown).

Using a polyclonal antibody specific of *Brucella* (homemade), we showed that specific *Brucella* staining closely colocalized with mCherry-Br signal (Figure S3) suggesting that all *Brucella* infected cells are well detected *in situ* using mCherry-Br. Confocal analysis in Z-stack demonstrated that bacteria localize the cytoplasm of F4/80$^+$ red pulp macrophages (Figure S4.A-B). This was also true for mCherry-expressing VirB-defective *Brucella* mutant (Figure S4.C). The VirB mutant display a strongly reduced ability to infect cells *in vitro* (16) although it is able to persist several days *in vivo* (17). It has been hypothesizing that this *in vivo* persistence could be due to extracellular multiplication (18). Our observations are not in favor of such hypothesis as VirB mutant and wild type bacterium localize within the same cell phenotype in infected spleen at all times tested (data not shown).

Control of *Brucella* infection in spleen appears linked to iNOS$^+$ granuloma formation.

The phenotype of infected spleen cells present a drastic evolution between day 1 and 5 post infection. At day 1, ~80% of infected cells are CD11b$^-$ F4/80$^+$ red pulp macrophages (Figure 2.A-B). A fraction of these cells are also MHC-II$^+$ (Figure 2.B and Figure 3.A). Between 1 and 3 days, CD11b$^+$ Ly-6G$^-$ infected monocytes appear progressively (Figure 2.B and Figure S5). At day 5, infected monocytes (CD11b$^+$ Ly-6G$^-$ MHC-II$^+$) constitute ~30-40% of infected cells (Figure 2.B) and are mainly found clustered in granuloma structures. In granuloma, these cells are CD11b$^+$ F4/80$^+$ Ly-6G$^-$ MHC-II$^+$ (Figure 3.B and Figure 4) and a large fraction is also CD11c$^+$, suggesting that these cells are inflammatory DC. These latters were detected by flow cytometry in previous reports and identified as the main iNOS producing cells during *Brucella* infection (9). In agreement with this hypothesis, we observed *in situ* that CD11b$^+$ and CD11c$^+$ cells located in granuloma are iNOS$^+$ (Figure 4). mCherry-Br were detected in these granuloma cells. It is important to note that B cells (B220$^+$ cells), T cells (CD90$^+$ cells) or granulocytes (Ly-6G$^+$ cells) never appeared infected during the course of infection in spleen (Figure 2.A and Figure 4A). Thus, infection seems always limited to myeloid cells.

In summary, establishment of infection at day 5 in spleen appear associated to monocyte recruitment and building of granuloma structure. These latter seem to participate to bacteria containment or elimination as these structures contain most of the iNOS-producing cells.

Infected cells in resistant C57BL/6 mice and susceptible BALB/c mice appear similar.

It has been frequently reported that BALB/c mice display higher bacterial load in all organs compared to C57BL/6 mice during *Brucella* infection (9,19). The reasons of this susceptibility are not clear but have been associated to lower frequency of IFN-γ-secreting CD4$^+$ cells and iNOS-producing cells in these mice (9). In order to determine if enhanced susceptibility of BALB/c mice could be associated to the persistence of bacteria in a distinct cell reservoir, we further compared infected cells in C57BL/6 and BALB/c mice 5 days after infection. Semi-quantitative analyses of the phenotype of infected cells (Figure 5.A) showed that they are globally similar in C57BL/6 and BALB/c mice. In addition, as observed for C57BL/6 mice, the large majority of bacteria and infected cells locate mainly in splenic r.p. and m.z. in BALB/c mice (Figure 5.B-C). However, the frequency of infected cells locate in

w.p. is clearly higher in BALB/c mice when compared to C57BL/6 mice. In accordance with bacteria CFU counts observed in spleens (Figure S2.A), the average number of bacteria per cells observed by microscopic analysis in BALB/c and C57BL/6 mice is similar at day 1, yet this number is higher at 5 days in BALB/c mice (Figure 5.D). Further analysis of the spatial distribution of infected cells in spleen (Figure 5.E-F) showed that highly infected cells (>20 bacteria / cell) locate in w.p. and are more frequent in BALB/c mice. In conclusion, phenotype of infected cells appeared relatively similar in both strains of mice. However, higher CFU counts observed in infected BALB/c mice seems to be associated to higher colonization of w.p. cells. These later frequently expressed MOMA-1 marker (typical of Marginal Zone macrophages) or $CD11c^+$ $CD11b^-$ $F4/80^-$ phenotype.

***Brucella* infection induces dendritic cells migration and maturation in spleen.**

Brucella bacteria have been frequently considered as "silent pathogens" able to actively escape the immune response by their ability to furtively growth in cells (20). This hypothesis is mainly based on the fact that *Brucella* LPS is a weak activator of macrophage and DC when compared to conventional LPS from *Escherichia coli* (21). In our study, we have observed that $CD11c^+$ dendritic cells are infected by mCherry-Br during the first 24 hours of infection (Figure 1.B and Figure 2.A). Thus, we investigated if at our dose of infection (10^8 CFU) *Brucella* induces DC activation *in vivo*. Microscopic analysis (Figure S6) of spleen section showed that $CD11c^+$ DCs relocate massively in w.p. T cell area 24 hours post inoculation. At 72 hours, DCs were mainly found in m.z. and their number appeared slowly reduced compared to control mice.

Flow cytometry analyses (Figure S7 A-B) demonstrated that DC migration observed at 24 hours *in situ* is associated to DC maturation as shown by cell surface upregulation of MHC-II and CD86 costimulatory molecule expression on $CD11c^+$ cells. Flow cytometry also confirmed that DC migration and maturation is followed by a transitory reduction of DC number, presumably by apoptosis as observed in other models (21). Notably Heat Killed (HK) *Brucella* are also able to induce DC maturation (Figure S7.B) demonstrating that infection is not required to induce this phenomenon suggesting that pathogen-associated molecular patterns derived from *Brucella* are sufficient. However, HK *Escherichia coli* are able to induced DC maturation at ten fold lower dose (Figure 7.B)

Formation of granuloma in spleen is altered in absence of MyD88 adaptor molecule.

In order to gain insight about the immune mechanisms controlling bacteria growth in spleen *in vivo*, we investigated, using genetically deficient mice, the impact of MyD88 adapter molecule on the phenotype of infected cells with a special focus on the ability of immune system to build granuloma structures. Previously, we have identified MyD88 adaptor molecule as a key element of the immune system, which detects *Brucella* infection and induces effector mechanisms such as iNOS expression (9). Thus, we compared the ability of wild type and MyD88$^{-/-}$ C57BL/6 mice to develop iNOS^{+} granuloma in spleen 5 day after bacteria inoculation (Figure 6). iNOS$^{-/-}$ C57BL/6 mice were initially used as internal control. Both MyD88$^{-/-}$ and iNOS$^{-/-}$ mice displayed granuloma structures, mainly in red pulp. However, granuloma from MyD88-/- mice, identified as dense aggregate of CD11b^{+} cells appear reduced in size (Figure 6) and frequently contain high number of Ly-6G^{+} cells (data not shown), presumably granulocyte cells. Importantly, granuloma seems fail to control bacteria growth and dissemination as mcherry-Br signal is strongly enhanced in these mice and that the great majority of infected cells appears outside of granuloma (Figure 6). As expected, iNOS staining was absent in iNOS$^{-/-}$ mice and strongly reduced in MyD88$^{-/-}$ mice (Figure 6).

Flow cytometry analyses (Figure 7) confirmed previous observations from low dose ($5x10^4$ CFU) infectious model (9). As expected, frequency of CD4^{+} IFN-γ^{+} was normal in iNOS$^{-/-}$ mice and reduced in MyD88$^{-/-}$ mice when compared to infected wild type mice (Figure 7.A-B). Similarly, frequency of CD11b^{+} CD11c^{+} iNOS^{+} inflammatory DC was partially reduced in MyD88$^{-/-}$ infected mice. However, frequency of CD11b^{+} CD11c^{+} cells appeared similar in infected wild type, iNOS$^{-/-}$ and MyD88$^{-/-}$ mice, suggesting that inflammatory DC are well recruited in these mice, yet iNOS induction is defective.

In MyD88$^{-/-}$ and iNOS$^{-/-}$ mice, absence of iNOS production correlated to enhanced CFU count (Figure 8). It is interesting to note that in MyD88$^{-/-}$ mice, at day 5 post inoculation, in contrast with wild type mice, higher number of infected cells located in white pulp and displayed mainly CD11c and MOMA-1 markers (Figure 9.B and C). These w.p. CD11c^{+} infected cells were CD11b^{-}, F4/80^{-} and iNOS^{-} (data not shown) and consequently appeared completely distinct from inflammatory DC observed in wild type mice.

Granuloma formation is observed in liver from *Brucella* infected mice.

As granuloma formation seems to be an important step in the control of *Brucella* infection in spleen, we investigated the phenotype of infected cells in liver, another important site of bacterial growth. At 5 day post inoculation, microscopic analyses showed that, as observed in spleen, liver from infected C57BL/6 and BALB/c wild type mice displayed granuloma formation composed of $CD11b^+$ $iNOS^+$ cells (Figure 10.A). Granuloma structures frequently surrounded the portal space and contained the majority of infected cells. As in spleen, granuloma cells also expressed CD11c, F4/80 and MHC-II markers (Figure S8), suggesting that granuloma are composed of activated monocytes and inflammatory DC. Importantly, we never observed bacteria inside hepathocyte or Ly-$6G^+$ granulocytes suggesting that, like in spleen, infection is limited to myeloid cells.

$iNOS^{-/-}$ and $MyD88^{-/-}$ infected mice, which presented enhanced CFU count in liver (Figure S9), displayed very large granulomas (Figure 10.A) containing dense aggregates of Ly-$6G^+$ granulocytes (data not shown). As expected, $iNOS^{-/-}$ and $MyD88^{-/-}$ mice were negative or strongly reduced for iNOS staining, respectively (Figure 10.B).

DISCUSSION

Despite the numerous studies carried out to increase our knowledge on the relationship between *Brucella* and the immune system, few elements have allowed a significant breakthrough in the field of brucellosis over the past ten years. If studies of genetically deficient mice have been useful to uncover the great avenues of protective immune responses against *Brucella* infection, this approach appears insufficient to dissect the precise immune effectors mechanisms elaborated by the host to fight *B. melitensis*. In contrast to other well studied intracellular bacteria, like *L. monocytogenes*, *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 (9), IL-12p40 (10) and iNOS (10). These observations suggest that effector mechanisms of immune responses interfering with *Brucella* proliferation are complex and necessitate new approach to be further characterized. This is the perspective from which we decided to use *in situ* microscopic techniques for studying the local immune environment developed around *B. melitensis* infected cells.

Brucella has been initially described as an intracellular bacteria able to replicate in professional phagocytes such as macrophages (22), DCs, (23) and granulocytes as well as non-professional phagocytes (24) including epithelial, fibroblastic and trophoblastic cells, in the context of cell line cultures. *In vivo*, however, the identity of *Brucella* "reservoir" cells during the course of infection is largely undetermined. If macrophages (25) and trophoblastic cells have been clearly associated with *Brucella* infection in the natural hosts, little has been demonstrated regarding the contribution of other potential cellular niche for *Brucella in vivo*. In this study, using a mCherry-expressing bacterium we demonstrated for the first time that *Brucella* present a very restrictive cellular infection spectrum as the majority of infected cells in spleen and liver of wild type mice identified belong to myeloid lineage. Granulocytes, B cells, T cells, fibroblasts and hepatocytes did not appear significantly infected in both tissues. This result may explained the frequent absence of impact of virulence factors inactivation *in vivo* that have been identified using *in vitro* cell line models. In the complex microenvironment of the host and in physiological cell reservoir many of these factors may have little or no relevance.

Our study brought to light the complexity and the dynamism of the cellular environment of the pathogen during the course of infection. In spleen, first infected cells are marginal zone (MOMA-1^+) and red pulp ($F4/80^+$) macrophages followed by DCs. We hypothesized that infection-mediated inflammation is responsible of subsequent recruitment of $CD11b^+$ $Ly\text{-}6G^-$ monocytes. Our data suggest that these cells are rapidly infected and progressively mature to form complex granuloma structure constituted of a mix of activated MHC-II^+ $CD11b^+$ $CD11c^-$ $F4/80^+$ mature monocytes and MHC-II^+ $CD11b^+$ $CD11c^+$ $F4/80^+$ iNOS-producing inflammatory DC surrounded by $CD90.2^+$ T cells. The presence of phenotypically similar structures in the liver and spleen of infected animals support the hypothesis of granulomas as the "spearhead" of the immune response against *Brucella*. In accord, correlation between susceptibility of $MyD88^{-/-}$ mice and alterated granuloma structure in these mice suggested a causal link between granulomas and *Brucella* growth control. However, the precise role of granuloma during *Brucella* infection remains unclear and further investigations are needed.

Transient infection of $CD11c^+$ cells during the early stages of infection in the spleen lead us to characterize the impact of *Brucella* infection on the maturation of conventional DCs. Flow cytometry and *in situ* stainings showed that the infection with *B. melitensis* provoked the migration of DCs in the T cell area of white pulp and their maturation during the first 24 hours of infection, followed, latter, by a gradual disappearance. By using heat-killed (HK) *B. melitensis* whole extracts, we observed that the maturation process was dose dependent and necessitated very high concentration of *Brucella* antigens. Comparisons with results obtained in this study or in the literature (21) using HK *Escherichia coli* showed that 10 times more HK *Brucella* than HK *E. coli* were needed to trigger DC maturation. Again, these results are in harmony with the stealthy strategy attributed to *Brucella* bacteria. Interestingly, histological analysis indicated that none of $CD11c^+$ cells migrating to T cell area were infected by *B. melitensis* (data not shown), suggesting that *Brucella* infection could impair DC migration. The relationship between *Brucella* and DCs seems complex and necessitates further experiments to be elucidated. However, the preliminary results obtained here suggest that *Brucella* can actively affect DCs natural behavior *in vivo*.

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

MATERIAL AND METHODS

Mice and reagents

Genetically deficient mice in C57BL/6 background: MyD88$^{-/-}$ (26) were obtained from Dr. S. Akira (Osaka University, Japan), iNOS$^{-/-}$ mice (27) from Dr. G. Lauvau (Université de Nice-Sophia Antipolis, France), 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 the Free University of Brussels (ULB, Belgium). The maintenance and care of mice complied with the guidelines of the ULB Ethic Committee for the use of laboratory animals.

B. melitensis strain 16M (Biotype1, ATCC 23456) was isolated from an infected goat and 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.) before use in mice inoculation as previously described (28).

Construction of a mcherry-producing B. melitensis strain.

The mCherry protein, a previously described rapidly maturing variant of the red fluorescent protein DsRed (Shaner, 2004), was cloned into the suicide vector pKSoriT-bla-kan downstream a strong Brucella spp. promoter named PsojA (Kohler, 2002). This promoter is described as controlling the expression of the protein translocase SecE.

The vector was constructed as follows: the mcherry coding sequence was amplified by PCR from pRSET-B-mcherry (Shaner, 2004 #1044) with the mcherry-up and mcherry-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 (Tibor, 2002)) 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 PsojA-up and PsojA-down primers and ligated into pGemT-Easy (Promega) to generate pGEM-T-SojA. The PsojA fragment was then excised from pGEM-T-PsojA by NotI/XbaI double restriction and subsequently inserted into NotI/XbaI-cut pKSoriT-bla-mcherry to generate pKSoriT-bla-PsojA-mcherry. Finally, the aphA4 cassette (a

promoterless kanamycin resistance gene (Dozot, 2006)) was excised from pUC4aphA4 with SalI, and subsequently cloned into the XhoI site of pKSoriT-bla-PsojA-mcherry to generate plasmid pKSoriT-bla-kan-PsojA-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 PsojA 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 (28).

Mouse infection

Mice were injected intraperitonealy (i.p.) with indicated dose of *B. melitensis* in 500 µl of PBS. Control animals were injected with the same volume of PBS alone. The infectious doses were validated by plating serial dilutions of the inoculums.

Harvest of spleen and liver

At selected time intervals, mice were sacrificed by cervical dislocation. Immediately after being killed, spleen and liver were collected for cytofluorometric and microscopic analyses.. Before cytofluorometric analysis, spleens were cut in very small pieces and incubated (37C, 30 min) into HBSS (Gibco) medium containing 4000 U/ml of collagenase IV (Roche) and 0.1 mg/ml of DNase I fraction IX (Sigma-Aldrich Chimie SARL, Lyon, France).

Bacteria titers in spleens and livers of infected mice

Spleens or 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 culture at 37°C.

Cytofluorometric analysis

Spleen were harvested and digested 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 0.5% BSA 0.02% NaN3 (FACS buffer) for 10 minutes on ice to prevent antibody binding to Fc receptor. 3-5x10^6 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), Phycoerythrin (PE)-coupled HL3 (anti-CD11c), RM4-5 (anti-CD4). 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 (29). 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 (1X 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 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 at -80°C, and cryostat sections (10µm) 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 cytofluorometric analysis is not use for immunofluorescence microscopy), biotin-coupled HL3 (anti-CD11c, BD Biosciences), 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 under a Zeiss fluorescent inverted microscope (Axiovert 200) equipped with high resolution monochrome camera (AxioCam HR, Zeiss). Images were acquired with 10x or 63x objective at maximal camera resolution. Measurement of colocalization between two staining was realized using Colocalization module from AxioVision program (Zeiss). When images were treated with Colocalization module, double positive surface was stained in white, gray or yellow (as indicated in Figure).

Confocal analysis

Confocal analysis were performed with LSM510 NLO multiphoton confocal microscope fitted on an Axiovert M200 inverted microscope equipped with C-Apochromat 40x/1.2 N.A. water immersion objectives (Zeiss, Iena, Germany). Optical sections of 1µm thick, 568 x 568 pixels, were collected sequentially for each fluorochrome.

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.

FIGURE LEGENDS

Figure 1. Red pulp localization of *B. melitensis* at the onset of infection. Wild-type C57BL/6 mice were injected i.p. with PBS or 1 x 10^8 CFU of mcherry-Br. Mice were sacrificed at selected time and the spleens were collected and examined by immunohistofluorescence. **A**, Positioning of mcherry-Br in the spleen of mice at 3 and 24 hours post infection. **B**, Immunoflurescence analysis of F4/80, MOMA-1, Ly-6G, and CD11c expressing cells and mcherry-Br. The inset areas in the middle 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. r.p.: red pulp; w.p.: white pulp; m.z.: marginal zone. Data are representative of at least 3 independent experiments.

Figure 2. 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 1 x 10^8 CFU of *mcherry-Br*. Mice were sacrificed at selected time and the spleens were collected and examined by immunohistofluorescence. **A**, **B**, Percentage of mcherry-Br that colocalize with C90.2-, B220-, Ly6G-, MOMA-1-, F4/80-, CD11c-, MHCII-, iNOS-, CD11b-expressing cells at the selected time. The bars are the mean±SD from at least 3 spleen sections per spleen from 5 mice.

Figure 3. Characterization of F4/80 infected cells in the spleen. Wild-type C57BL/6 mice were injected i.p. with PBS or 1 x 10^8 CFU of mcherry-Br. Mice were sacrificed at selected time and the spleens were collected and examined by immunohistofluorescence. **A**, Immunoflurescence analysis of F4/80, MHC-II, and actin expression in spleen of mice before, 1, and 5 days 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 the spleen of mice before and 5 days after mcherry-Br infection. Scale bar= 50 and 200 μm, as indicated. r.p.: red pulp; w.p.: white pulp. Data are representative of at least 3 independent experiments.

Figure 4. Phenotypical description of *B. melitensis* infected granulomas in the spleen. Wild-type C57BL/6 mice were injected i.p. with PBS or 1 x 10^8 CFU of mcherry-Br. Mice were sacrificed at 5 days post infection and the 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. 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.

Figure 5. Comparison of phenotype and spatial distribution of infected cells in spleen of 57BL/6 and BALB/c mice. Wild-type C57BL/6 and BALB/c mice were injected i.p. with PBS or 1 x 10^8 CFU of mcherry-Br. Mice were sacrificed at selected time and the spleens were collected and examined by immunohistofluorescence. **A**, Comparative analysis of the percentage of mcherry-Br that colocalize with C90.2-, B220-, Ly6G-, MOMA-1-, F4/80-, CD11c-, MHCII-, iNOS-, CD11b-expressing cells **B**, **C**, The percentage of bacteria and infected cells localized in the red plulp and MZ, or in the white pulp. **D**, The average number of bacteria per splenic cell at 2 and 5 days post infection. **E-F**, The comparative analysis of the bacterial load per cell present in the spleen, red pulp, or white pulp. The bars are the mean±SD from at least 3 spleen sections per spleen from 5 mice.

Figure 6. $CD11b^+$ granuloma in the spleen of wild type and genetically deficient infected mice. Location by immunohistofluorescence of $CD11b^{hi}$ and iNOS expressing cells in spleen from control and 5 days mcherry-Br infected wild-type, $iNOS^{-/-}$ and $MyD88^{-/-}$ C57BL/6 as well as BALB/c mice. Mice were injected i.p. with PBS (control) or 1 x 10^8 CFU of mcherry-Br. 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.

Figure 7. IFN-γ- and iNOS-producing cells following *B. melitensis* infection. Wild-type, $iNOS^{-/-}$ and $MyD88^{-/-}$ C57BL/6 as well as BALB/c mice (4 per group) were injected i.p. with PBS or 1 x 10^8 CFU of *B. melitensis*. Mice were sacrificed 5 days post-infection and the 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 IFN- γ expression. IFN-γ^+ cells in each group were then analyzed for CD4 and CD3ε expression. Number indicates the percentage of cells in the selected quadrant. **B**, Number of CD3ε^+ CD4$^+$ IFN-γ^+ cells per 10^6 spleen cells acquired. **C**, 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. **D**, Number of, CD11b$^+$ CD11c$^+$ iNOS$^+$ cells per 10^6 spleen cells acquired. Each data represents the value obtained from an individual spleen and the data are pooled from 2 independent experiments.

Figure 8. Characterization of the bacterial load in the spleen of iNOS and MyD88 deficient mice. Wild type, MyD88$^{-/-}$ and iNOS$^{-/-}$ C57BL/6 mice (6 per groups) were inoculated i.p. with 1 x 10^8 CFU of mcherry-Br. 5 days post infection, the CFU per spleen were calculated. These results are pooled data from three independent experiments. The bars are the median.

Figure 9. *B. melitenis* infect CD11c$^+$ or MOMA-1$^+$ cells in the marginal zone and white pulp of MyD88$^{-/-}$ mice. Wild-type and MyD88$^{-/-}$ C57BL/6 mice were injected i.p. with PBS or 1 x 10^8 CFU of mcherry-Br. Mice were sacrificed 5 days post infection and the spleens were collected and examined by immunohistofluorescence. **A**, Positioning of mcherry-Br and CD11c^{hi} expressing cells in the spleen of wild type and MyD88$^{-/-}$ mice. In the upper panel, yellow circles indicate the presence of the bacterium. **B**, **C**, Higher magnification view. Colocalization analysis of CD11c^{hi} (B) and MOMA-1^{hi}(C) -expressing cells and mcherry-Br in the designated mice. 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. Data are representative of at least 3 independent experiments.

Figure 10. CD11b$^+$ granuloma in the liver of wild type and genetically deficient mice. Liver immunohistofluorescence of positioning of CD11b (**A**) and CD11b iNOS co-expressing cells (**B**) before and 5 days after mcherry-Br infection in wild-type, iNOS$^{-/-}$ and MyD88$^{-/-}$ C57BL/6 as well as BALB/c mice. Mice were injected i.p. with PBS or 1 x 10^8 CFU of mcherry-Br. Central panels of A are higher magnification view. 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.

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Characterization of the mcherry-expressing fluorescent strain of *B. melitensis* 16M (mcherry-Br). A, Flow cytometric analysis of the wild type strain of *B. melitensis* 16M and mcherry-Br. **B**, Infection of hamster fibroblastic cells BHK with the wild type strain of *B. melitensis* 16M and mcherry-Br. The LPS of both bacteria is labeled green and the mcherry-Br strain appears in red. **C**, Virulent curve of the wild type strain of *B. melitensis* 16M and mcherry-Br. Groups of six mice were inoculated i.p. with 4 x 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.

Figure S2. Time course of mcherry-Br infection and number of infected spleen cells by surface unit. A, Wild time C57BL/6 and BALB/c mice (6 per groups) were inoculated i.p. with 1 x 10^8 CFU of mcherry-Br. At the indicated times the 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 1 and 5 days post infection. A unit surface is defined arbitrary as an area of 0,037 mm^2. The bars are the mean±SD from at least 3 spleen sections per spleen from 5 mice.

Figure S3. **Colocalization of *B. melitensis* and *Brucella* Antigens *in vivo*.** Colocalization analysis of F4/80 expressing cells, Brucella antigens and mcherry-Br before and 6 hours days after mcherry-Br infection in wild-type C57BL/6 mice. Mice were injected i.p. with PBS or 1 x 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.

Figure S4. *In vivo* intracellular localization of *Brucella* determined by confocal microscopy. Wild-type C57BL/6 mice were injected i.p. with PBS or 1 x 10^8 CFU of mcherry-Br. Mice were sacrificed at 1 day post infection 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.

Figure S5. *B. melitensis* infects LY-6G$^-$/CD11b$^+$ during the course of infection. Colocalization assay of CD11b, Ly-6G expressing cells and mcherry-Br, 5 days after infection in wild-type C57BL/6 mice. Mice were injected i.p. with PBS or 1 x 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.

Figure S6. *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, 24 and 72 hours after mcherry-Br inoculation. Wild-type C57BL/6 mice were injected i.p. with PBS or 1 x 10^8 CFU of mcherry-Br. Mice were sacrificed at selected time and the 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.

Figure S7. *B. melitensis* infection induces the DC maturation. C57BL/6 mice were injected i.p. with, 1 x 10^8 CFU of live *B. melitenis* and various doses (see Figure) of heat killed (HK) *B. melitensis* and *Escherichia coli* O18:K1. Mice were sacrificed at selected time and the 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 CD11c expression. $CD11c^{hi}$ cells in each group were then analyzed for CD8α expression combined to CD4, MHC-II and CD86 expression. Number indicates the percentage of positive cells per 10^6 spleen cells acquired for the specified marker. **B**, Comparative analysis of $CD11c^{hi}$/MHC-II^+ and $CD11c^{hi}$/$CD86^+$ cells. The bars are the median. Data are representative of at least 3 independent experiments.

Figure S8. Phenotypical description of *Brucella* infected granulomas in the liver. Wild-type C57BL/6 mice were injected i.p. with PBS or 1 x 10^8 CFU of mcherry-Br. Mice were sacrificed at 5 days post infection and the livers were collected and examined by immunohistofluorescence. **A**, Immunofluorescence analysis of Ly-6G, CD11b, CD11c, CD90, 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 Figure B indicate the percentage of colocalizing cells in the upper panel. Panels are color-coded with the text for the antigen or mcherry-Bmei examined as well as the colocalization. Scale bar= 50 μm, as indicated. Data are representative of at least 3 independent experiments.

Figure S9. Analysis of the bacterial load of the liver in wild type and genetically deficient mice. Wild-type, $iNOS^{-/-}$ and $MyD88^{-/-}$ C57BL/6 as well as BALB/c mice (4-6 per group) were inoculated i.p. with 1 x 10^8 CFU. 5 days post infection, the CFU per gr of liver were calculated. Data are pooled from 3 independent experiments.

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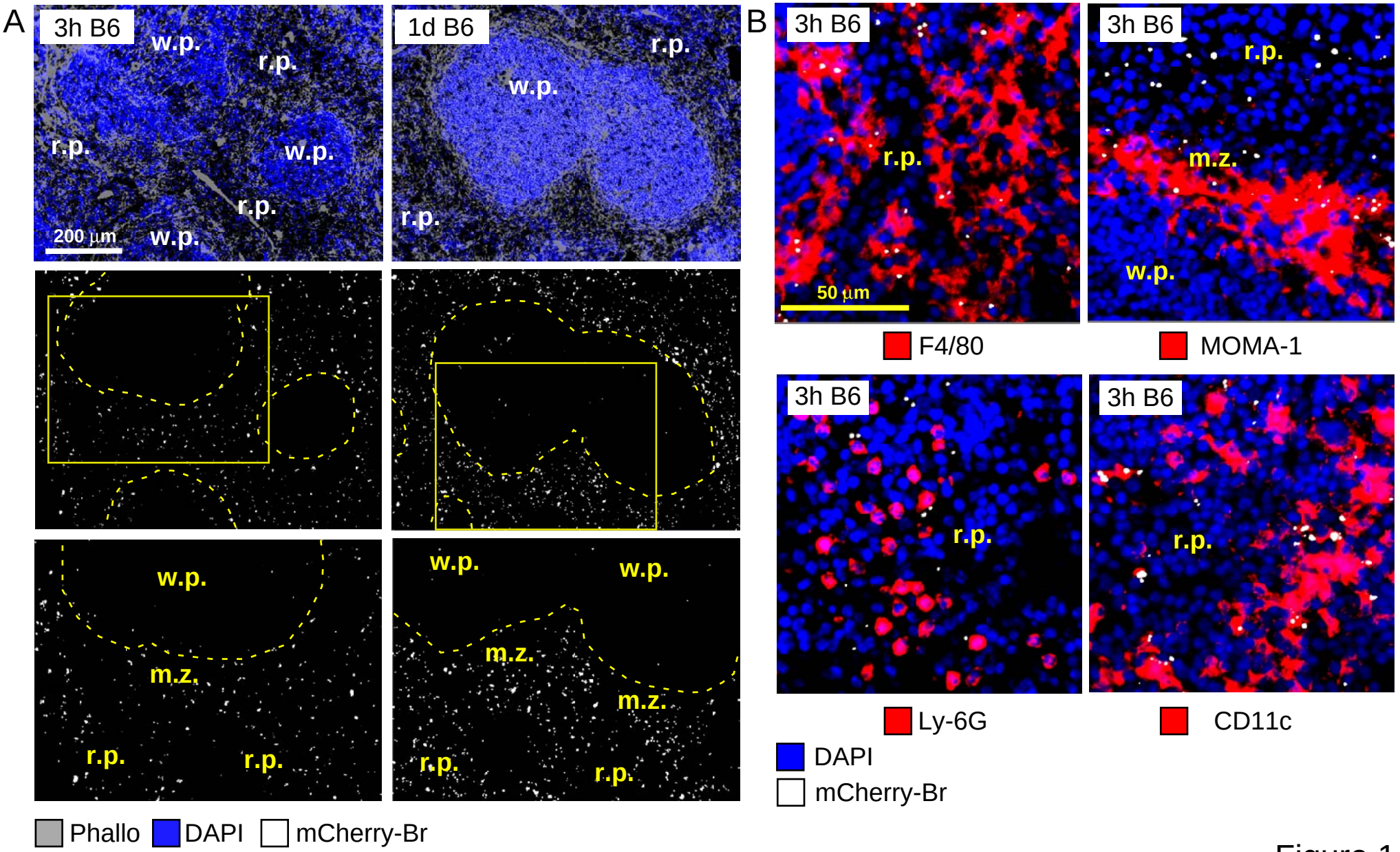

A
3h B6
w.p.
r.p.
r.p.
w.p.
r.p.
200 µm
w.p.
1d B6
r.p.
w.p.
r.p.
w.p.
m.z.
r.p.
r.p.
w.p.
w.p.
m.z.
m.z.
r.p.
r.p.
Phallo
DAPI
mCherry-Br
B
3h B6
r.p.
50 µm
3h B6
r.p.
m.z.
w.p.
F4/80
MOMA-1
3h B6
r.p.
3h B6
r.p.
Ly-6G
CD11c
DAPI
mCherry-Br

Figure 1

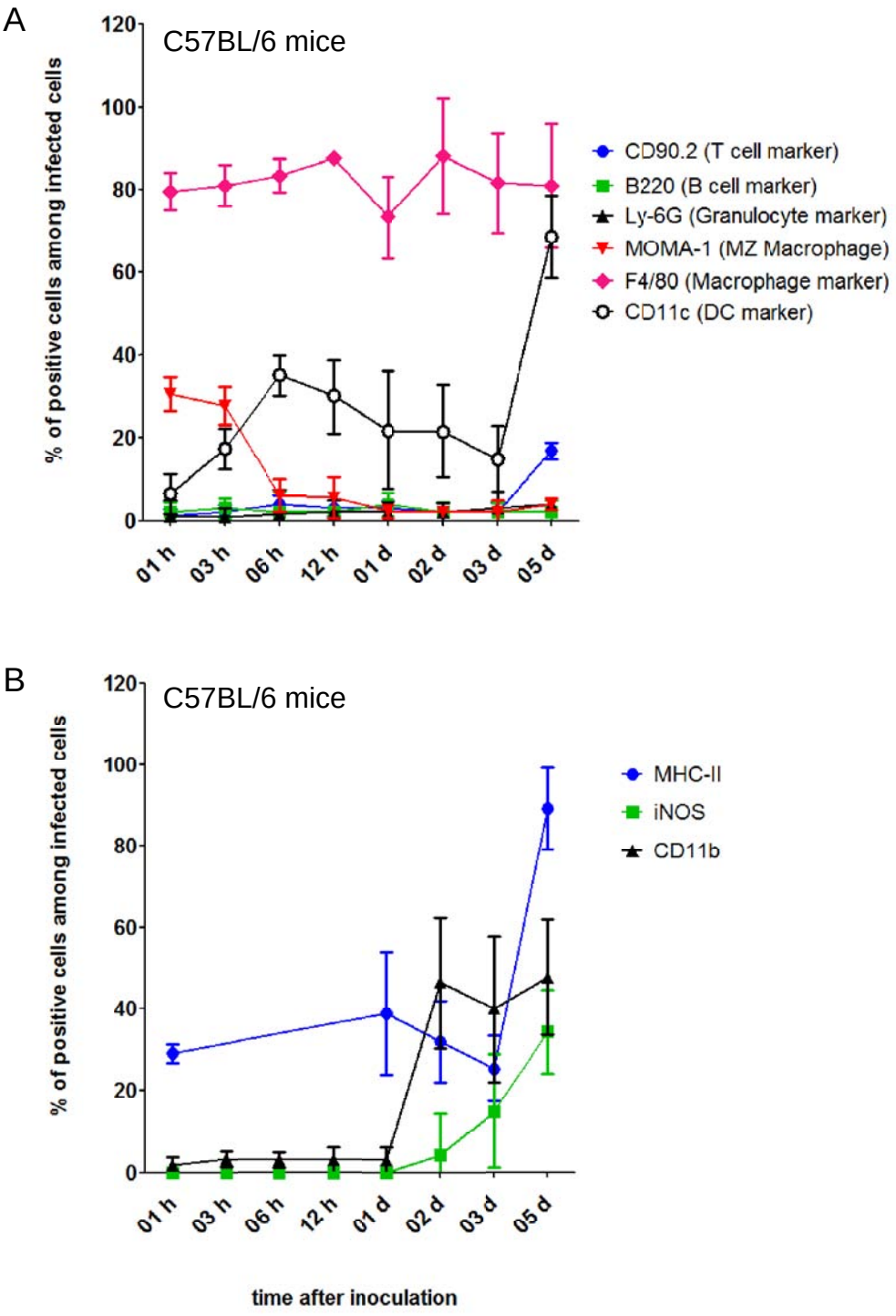

A
C57BL/6 mice
% of positive cells among infected cells
120
100
80
60
40
20
0
01 h
03 h
06 h
12 h
01 d
02 d
03 d
05 d
CD90.2 (T cell marker)
B220 (B cell marker)
Ly-6G (Granulocyte marker)
MOMA-1 (MZ Macrophage)
F4/80 (Macrophage marker)
CD11c (DC marker)
B
C57BL/6 mice
% of positive cells among infected cells
MHC-II
iNOS
CD11b
time after inoculation

Figure 2

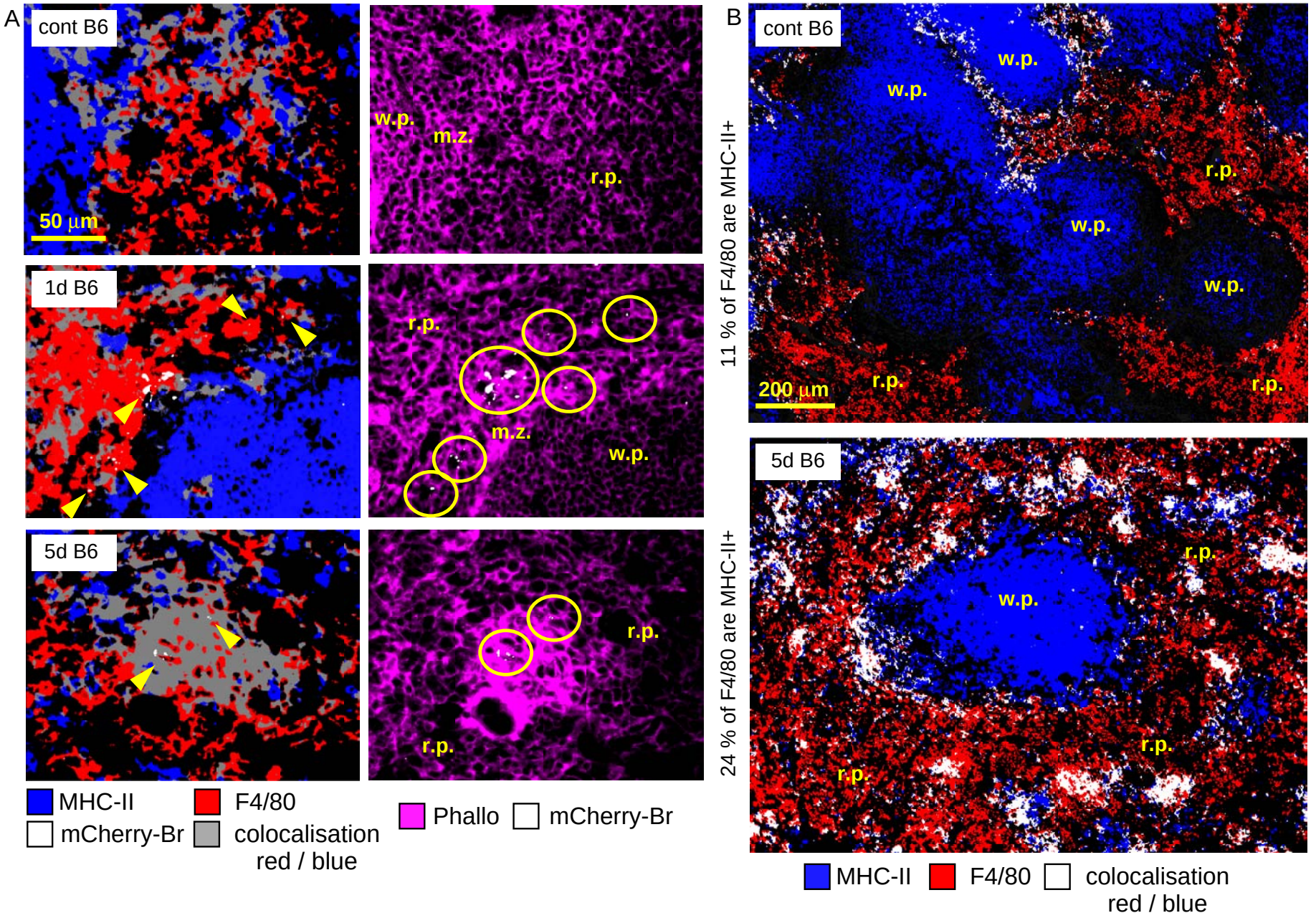

A
cont B6
50 µm
w.p.
m.z.
r.p.
1d B6
r.p.
m.z.
w.p.
5d B6
r.p.
r.p.
MHC-II
F4/80
mCherry-Br
colocalisation
red / blue
Phallo
mCherry-Br
B
cont B6
w.p.
w.p.
r.p.
w.p.
w.p.
200 µm
r.p.
r.p.
11 % of F4/80 are MHC-II+
5d B6
r.p.
w.p.
r.p.
r.p.
24 % of F4/80 are MHC-II+
MHC-II
F4/80
colocalisation
red / blue

Figure 3

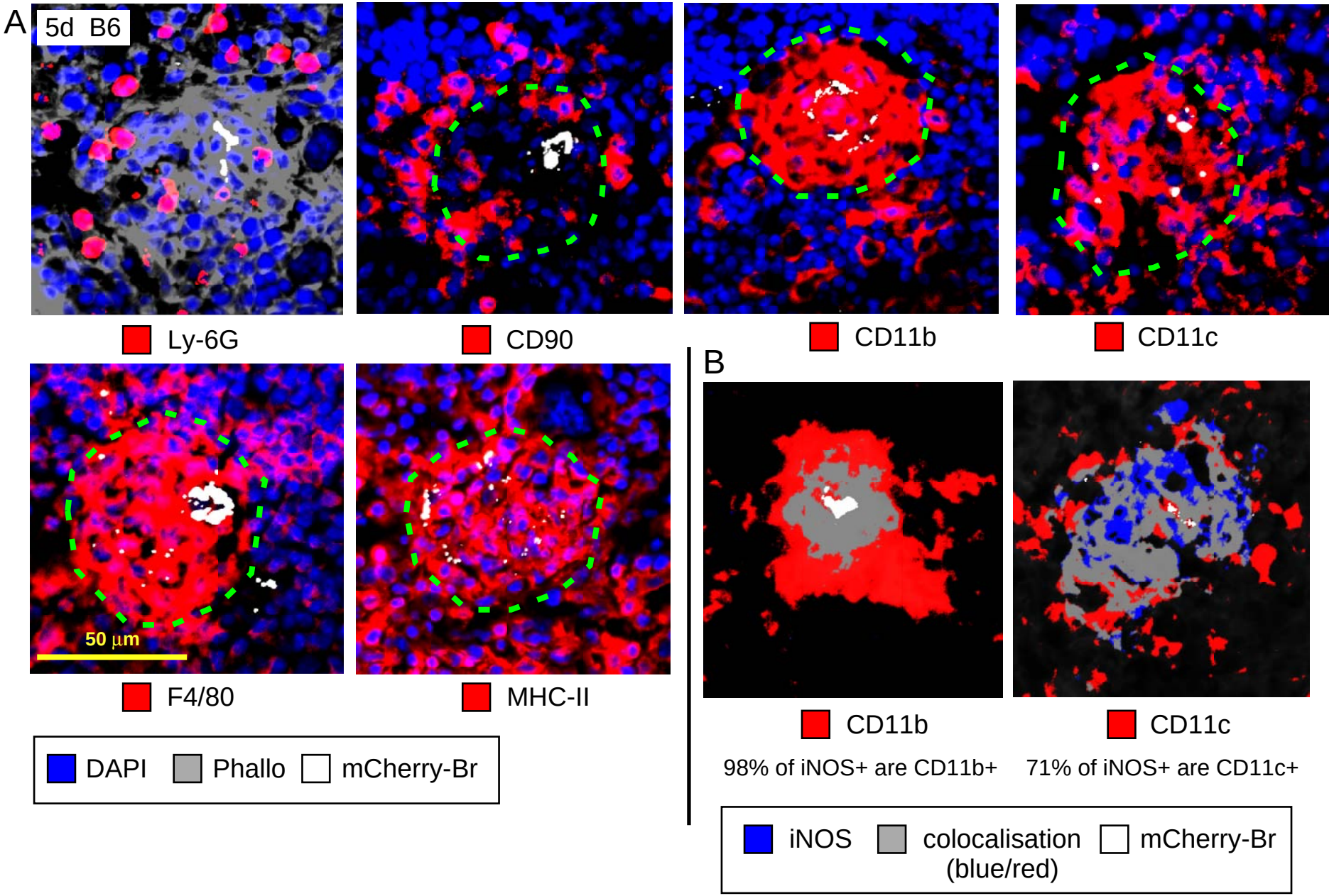

A
5d B6
Ly-6G
CD90
CD11b
CD11c
F4/80
MHC-II
50 µm
DAPI
Phallo
mCherry-Br
B
CD11b
CD11c
98% of iNOS+ are CD11b+
71% of iNOS+ are CD11c+
iNOS
colocalisation (blue/red)
mCherry-Br

Figure 4

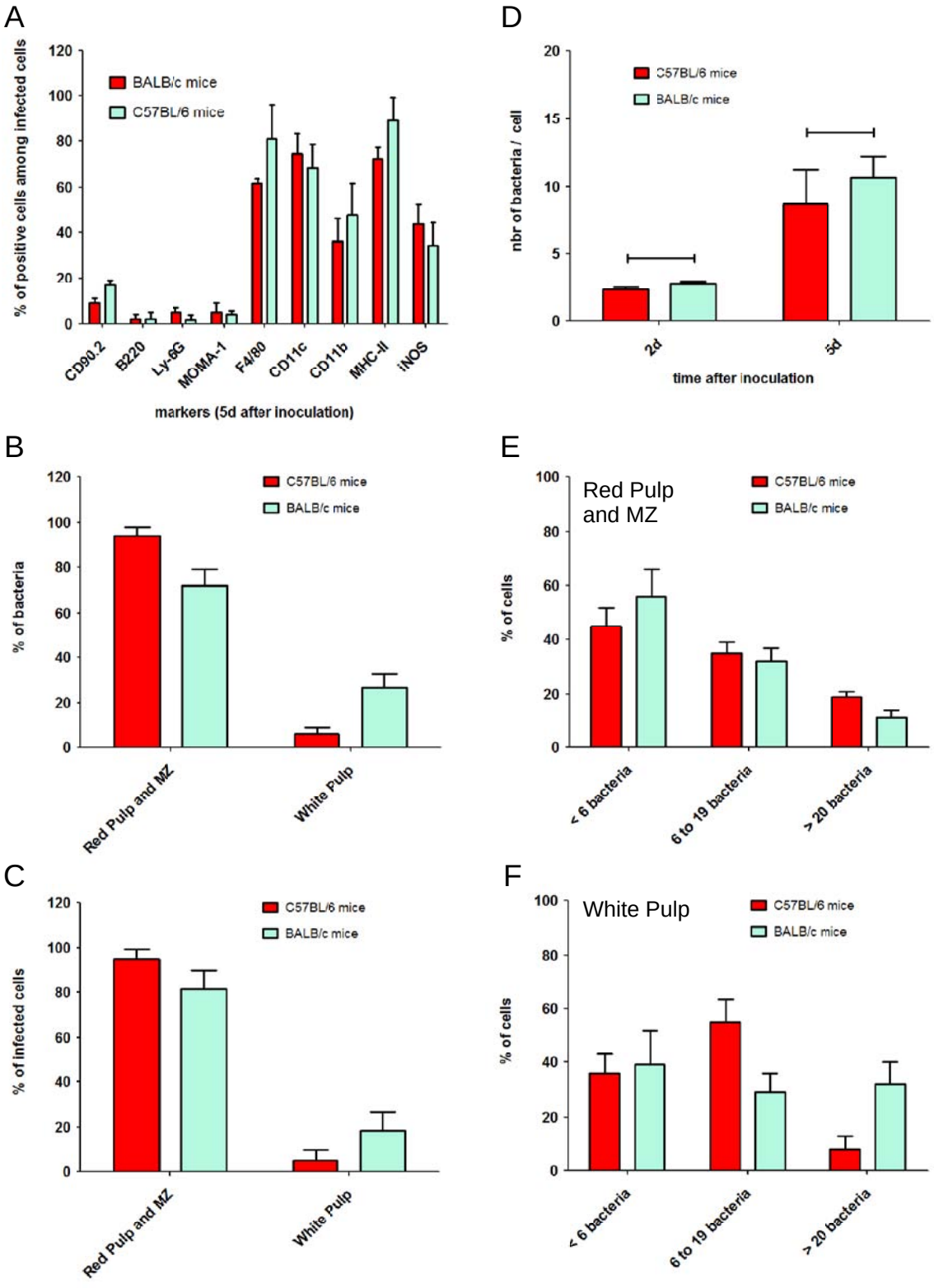

A
% of positive cells among infected cells
BALB/c mice
C57BL/6 mice
CD90.2
B220
Ly-6G
MOMA-1
F4/80
CD11c
CD11b
MHC-II
iNOS
markers (5d after inoculation)
D
nbr of bacteria / cell
C57BL/6 mice
BALB/c mice
2d
5d
time after inoculation
B
% of bacteria
C57BL/6 mice
BALB/c mice
Red Pulp and MZ
White Pulp
E
Red Pulp and MZ
% of cells
C57BL/6 mice
BALB/c mice
< 6 bacteria
6 to 19 bacteria
> 20 bacteria
C
% of infected cells
C57BL/6 mice
BALB/c mice
Red Pulp and MZ
White Pulp
F
White Pulp
% of cells
C57BL/6 mice
BALB/c mice
< 6 bacteria
6 to 19 bacteria
> 20 bacteria

Figure 5

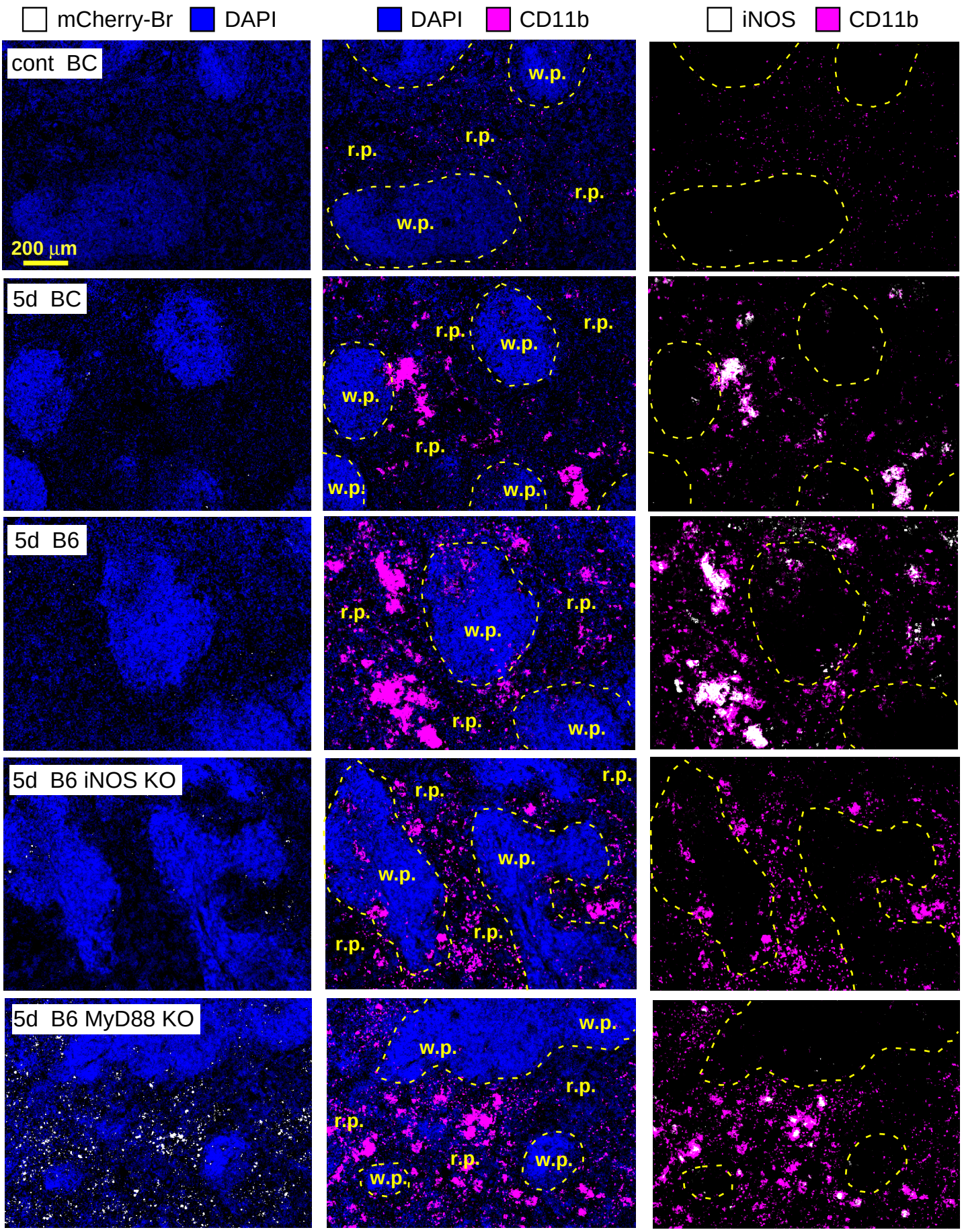

mCherry-Br
DAPI
DAPI
CD11b
iNOS
CD11b
cont BC
w.p.
r.p.
r.p.
r.p.
w.p.
200 μm
5d BC
r.p.
r.p.
w.p.
w.p.
r.p.
w.p.
w.p.
5d B6
r.p.
r.p.
w.p.
r.p.
w.p.
5d B6 iNOS KO
r.p.
r.p.
w.p.
w.p.
r.p.
r.p.
5d B6 MyD88 KO
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w.p.
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r.p.
w.p.
w.p.

Figure 6

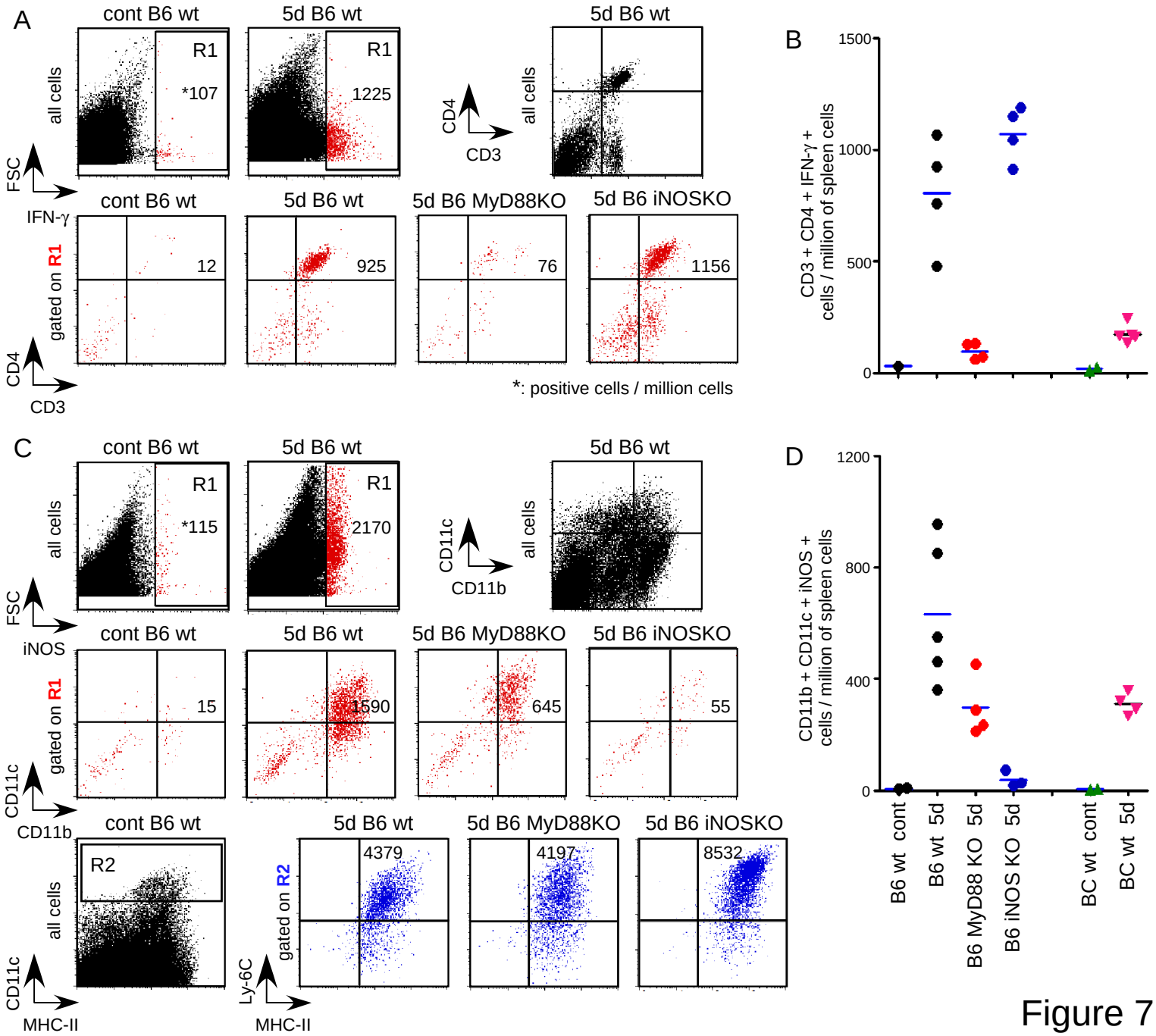

A
cont B6 wt
5d B6 wt
R1
*107
1225
all cells
FSC
CD4
CD3
IFN-γ
gated on R1
12
925
5d B6 MyD88KO
76
5d B6 iNOSKO
1156
*: positive cells / million cells
B
CD3 + CD4 + IFN-γ + cells / million of spleen cells
1500
1000
500
0
C
R1
*115
2170
CD11c
CD11b
iNOS
15
1590
645
55
R2
4379
4197
8532
Ly-6C
MHC-II
D
CD11b + CD11c + iNOS + cells / million of spleen cells
1200
800
400
0
B6 wt cont
B6 wt 5d
B6 MyD88 KO 5d
B6 iNOS KO 5d
BC wt cont
BC wt 5d

Figure 7

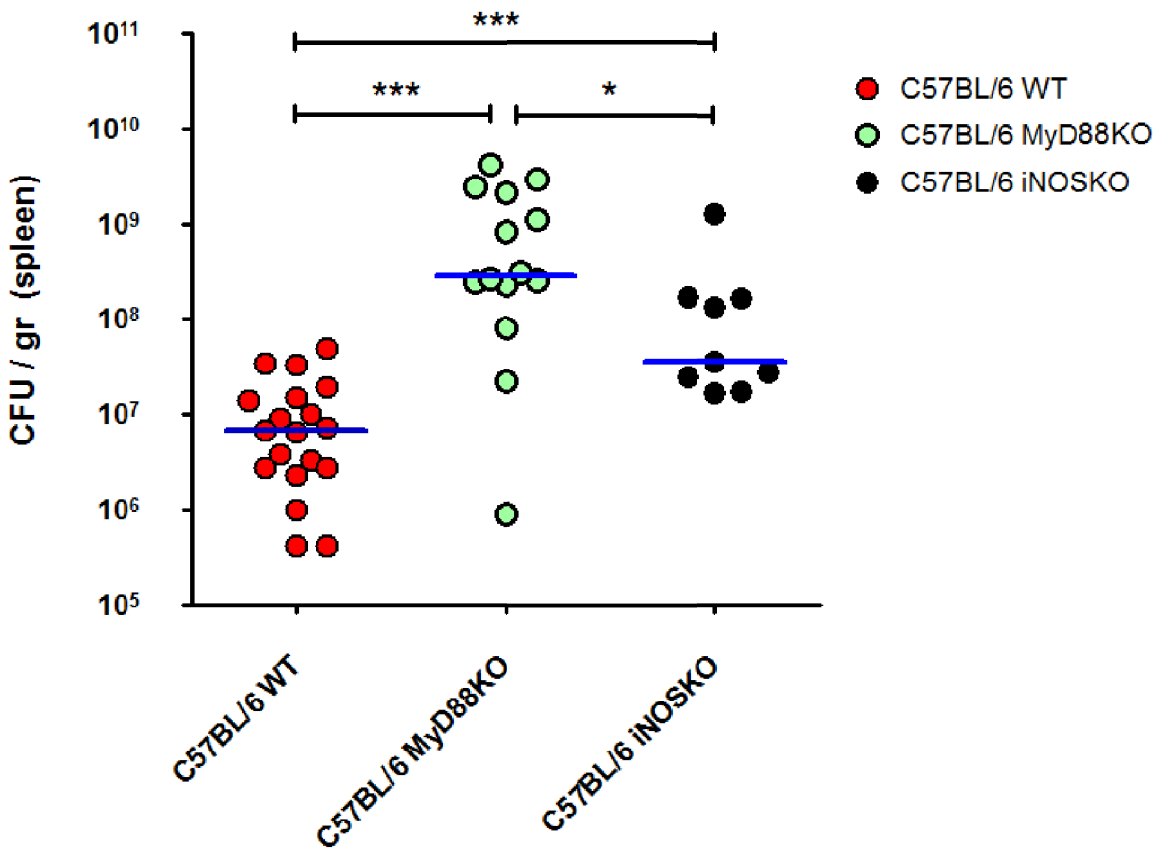

***
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*
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
CFU / gr (spleen)
C57BL/6 WT
C57BL/6 MyD88KO
C57BL/6 iNOSKO
C57BL/6 WT
C57BL/6 MyD88KO
C57BL/6 iNOSKO

Figure 8

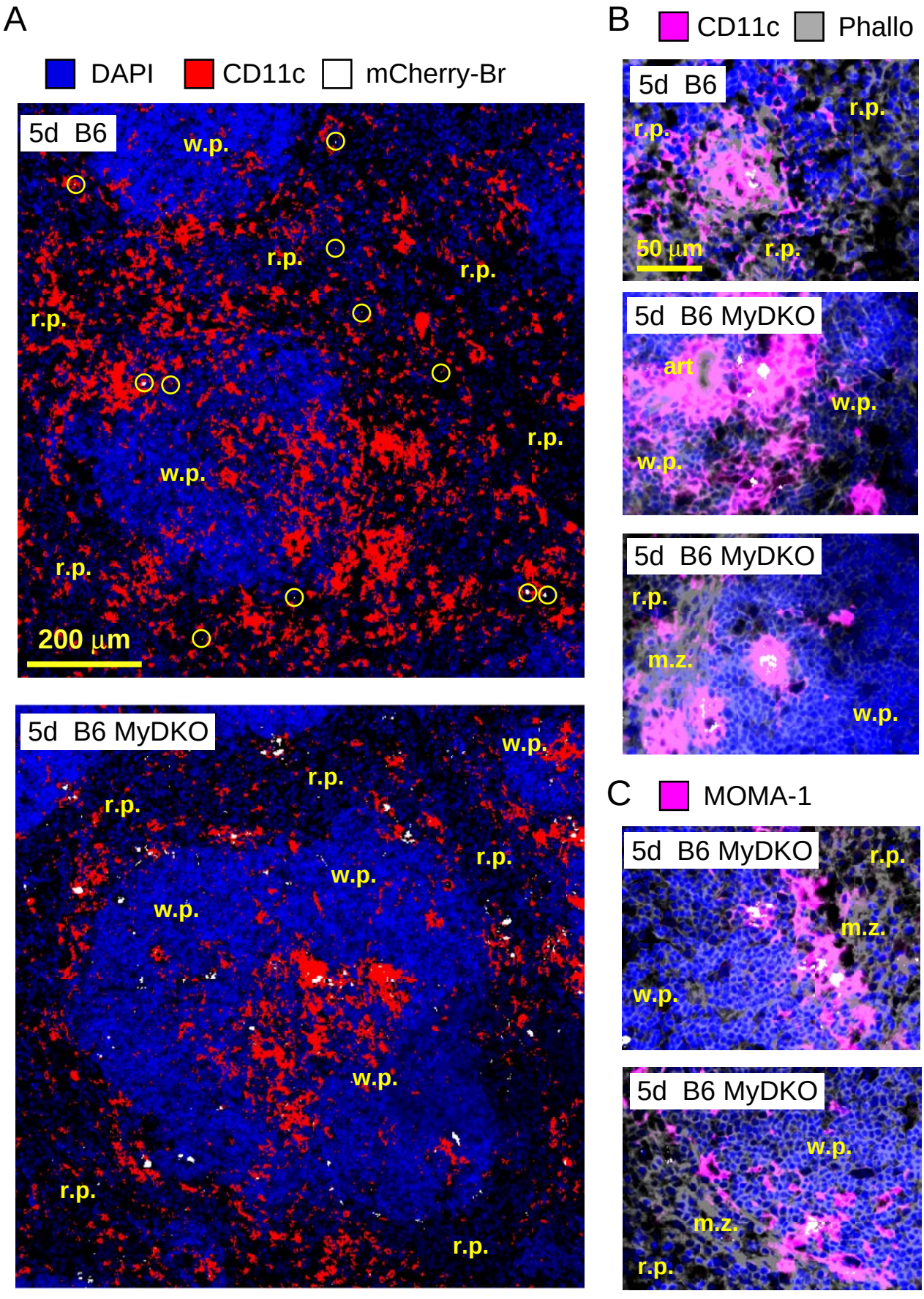

A
DAPI
CD11c
mCherry-Br
5d B6
w.p.
r.p.
r.p.
r.p.
r.p.
w.p.
r.p.
200 μm
5d B6 MyDKO
w.p.
r.p.
r.p.
r.p.
w.p.
w.p.
w.p.
r.p.
r.p.
B
CD11c
Phallo
5d B6
r.p.
r.p.
50 μm
r.p.
5d B6 MyDKO
art
w.p.
w.p.
5d B6 MyDKO
r.p.
m.z.
w.p.
C
MOMA-1
5d B6 MyDKO
r.p.
m.z.
w.p.
5d B6 MyDKO
w.p.
m.z.
r.p.

Figure 9

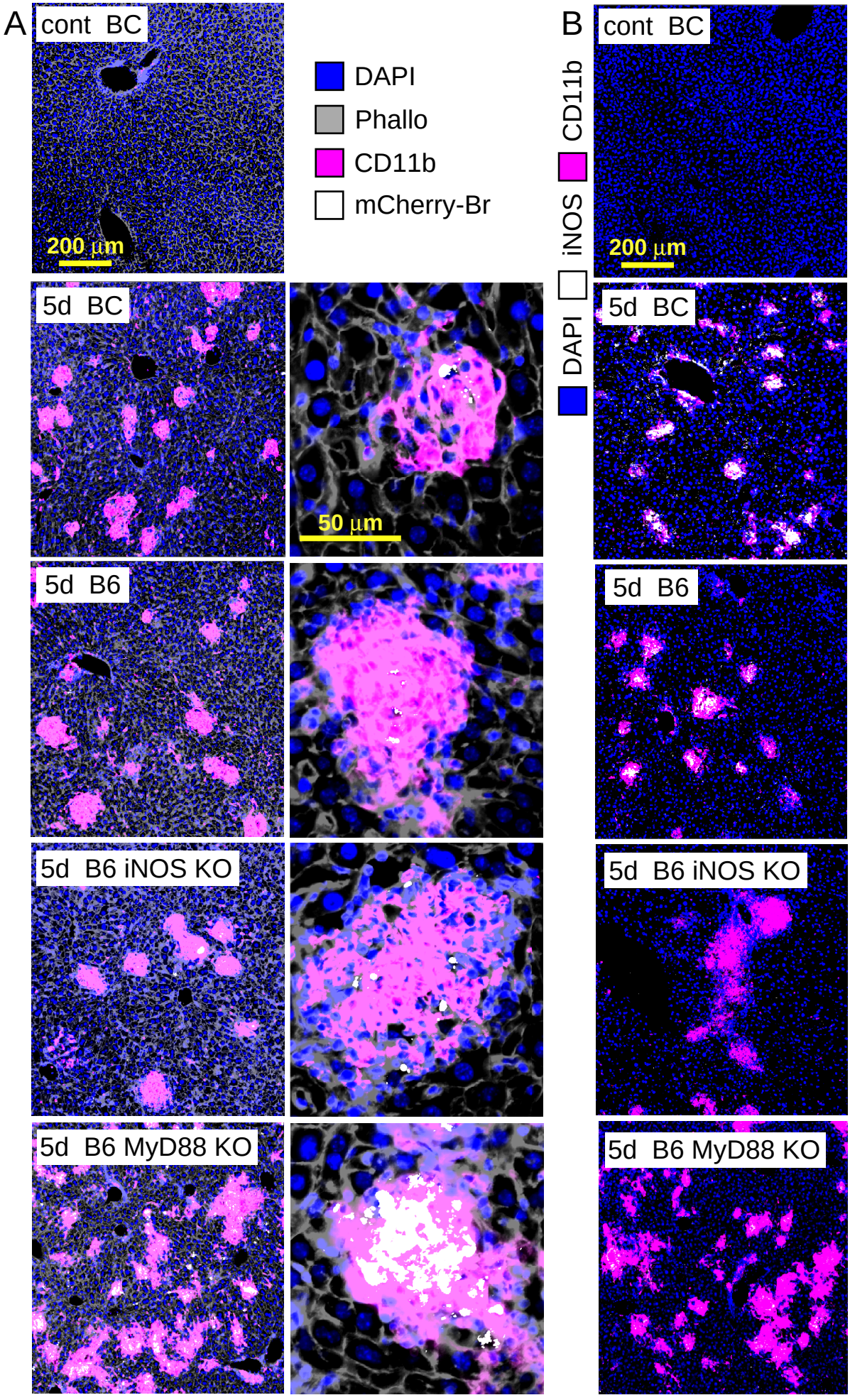

A
cont BC
200 μm
DAPI
Phallo
CD11b
mCherry-Br
5d BC
50 μm
5d B6
5d B6 iNOS KO
5d B6 MyD88 KO
B
cont BC
200 μm
CD11b
iNOS
DAPI
5d BC
5d B6
5d B6 iNOS KO
5d B6 MyD88 KO

Figure 10

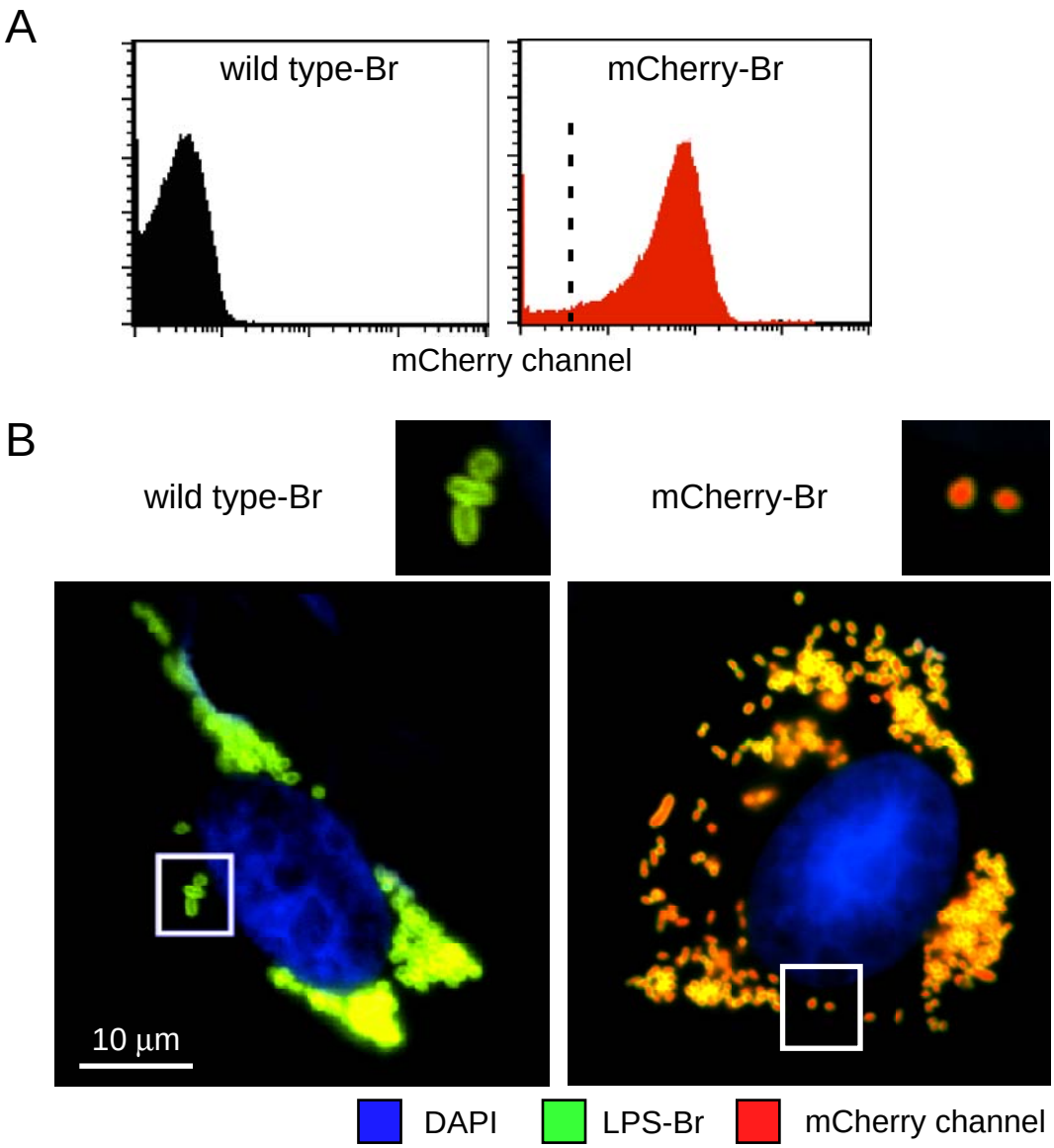

A
wild type-Br
mCherry-Br
mCherry channel
B
wild type-Br
mCherry-Br
10 µm
DAPI
LPS-Br
mCherry channel
C
C57BL/6 mice
CFU per / gr (spleen)
10^7
10^6
10^5
10^4
10^3
10^2
10^1
*
ns
ns
5d
25d
42d
time post infection
wild type-Br
mCherry-Br

Figure S1

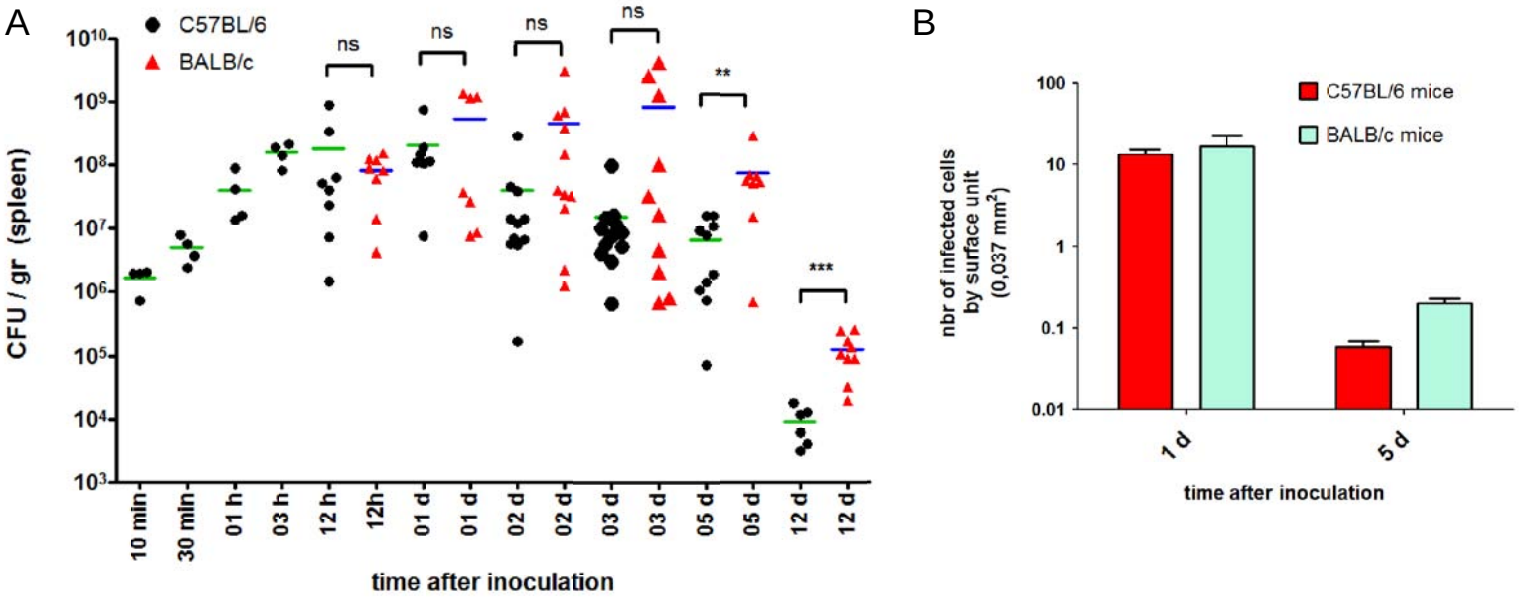

A
C57BL/6
BALB/c
ns
ns
ns
ns
**
***
CFU / gr (spleen)
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10 min
30 min
01 h
03 h
12 h
12h
01 d
01 d
02 d
02 d
03 d
03 d
05 d
05 d
12 d
12 d
time after inoculation
B
C57BL/6 mice
BALB/c mice
nbr of infected cells by surface unit (0,037 mm²)
100
10
1
0.1
0.01
1 d
5 d
time after inoculation

Figure S2

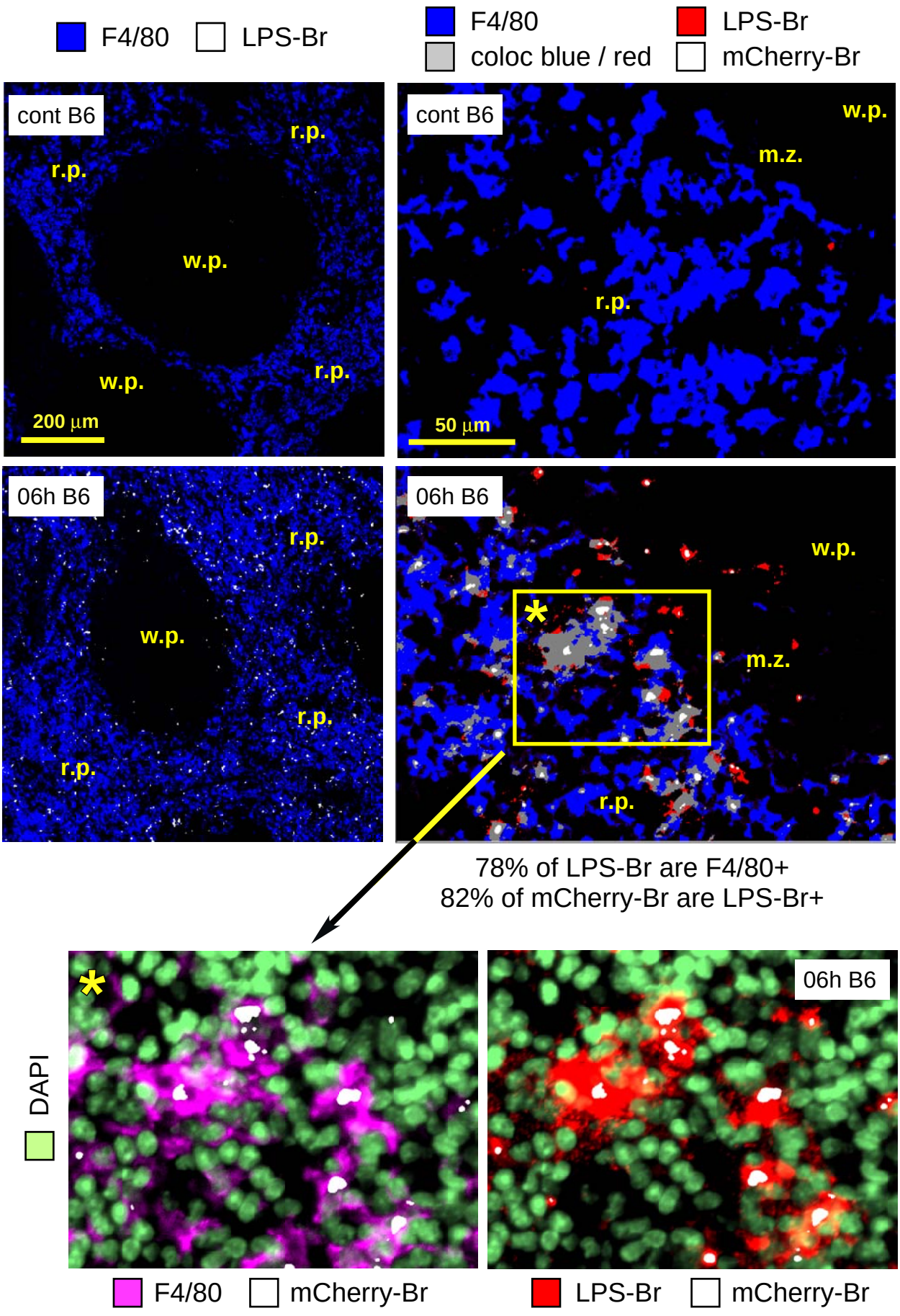

F4/80
LPS-Br
F4/80
coloc blue / red
LPS-Br
mCherry-Br
cont B6
r.p.
r.p.
w.p.
w.p.
r.p.
200 µm
cont B6
w.p.
m.z.
r.p.
50 µm
06h B6
r.p.
w.p.
r.p.
r.p.
06h B6
w.p.
*
m.z.
r.p.
78% of LPS-Br are F4/80+
82% of mCherry-Br are LPS-Br+
DAPI
*
06h B6
F4/80
mCherry-Br
LPS-Br
mCherry-Br

Figure S3

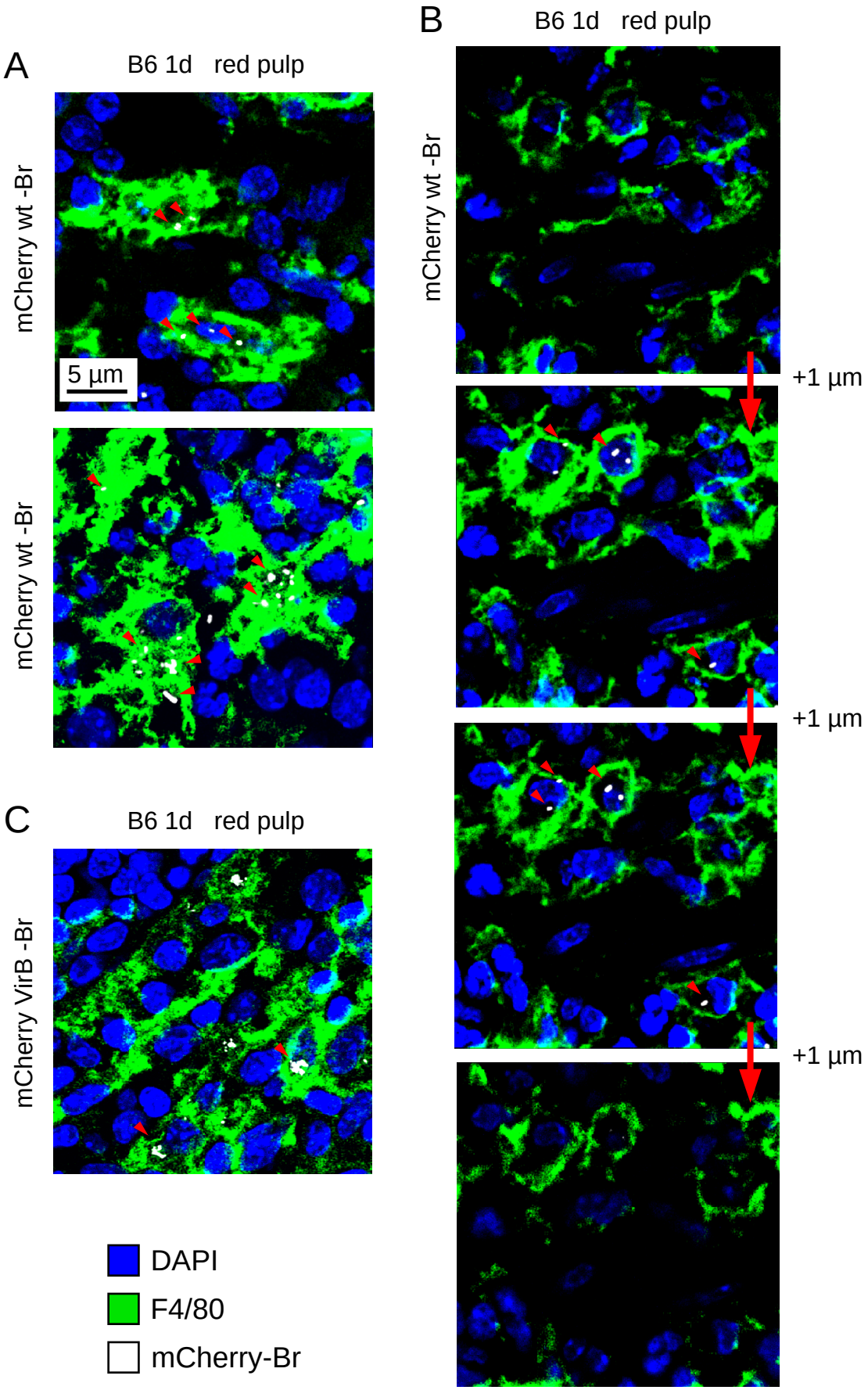

A
B6 1d red pulp
mCherry wt -Br
5 µm
mCherry wt -Br
B
B6 1d red pulp
mCherry wt -Br
+1 µm
+1 µm
+1 µm
C
B6 1d red pulp
mCherry VirB -Br
DAPI
F4/80
mCherry-Br

Figure S4

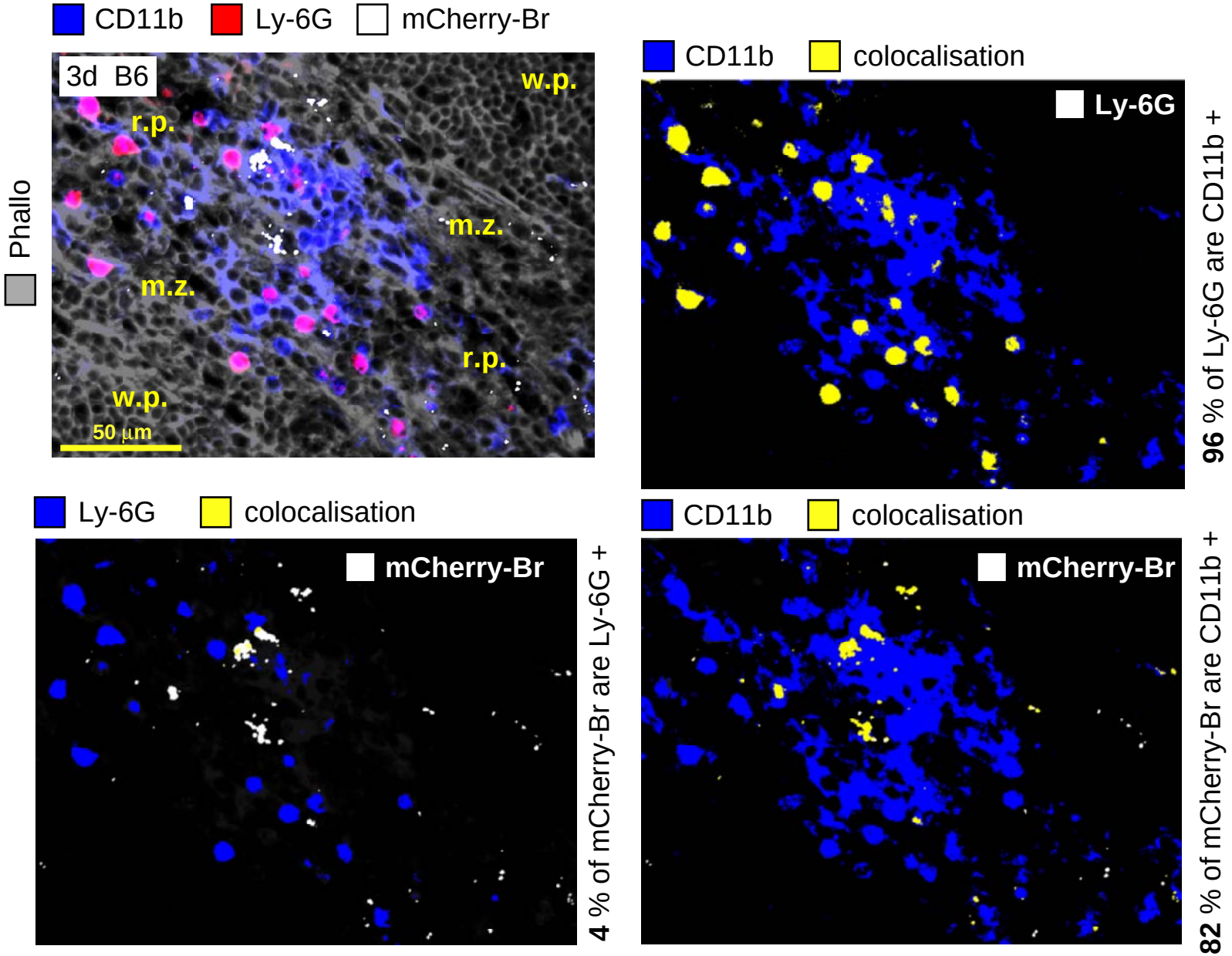

CD11b
Ly-6G
mCherry-Br
Phallo
3d B6
w.p.
r.p.
m.z.
m.z.
r.p.
w.p.
50 µm
CD11b
colocalisation
Ly-6G
96 % of Ly-6G are CD11b +
Ly-6G
colocalisation
mCherry-Br
4 % of mCherry-Br are Ly-6G +
CD11b
colocalisation
mCherry-Br
82 % of mCherry-Br are CD11b +

Figure S5

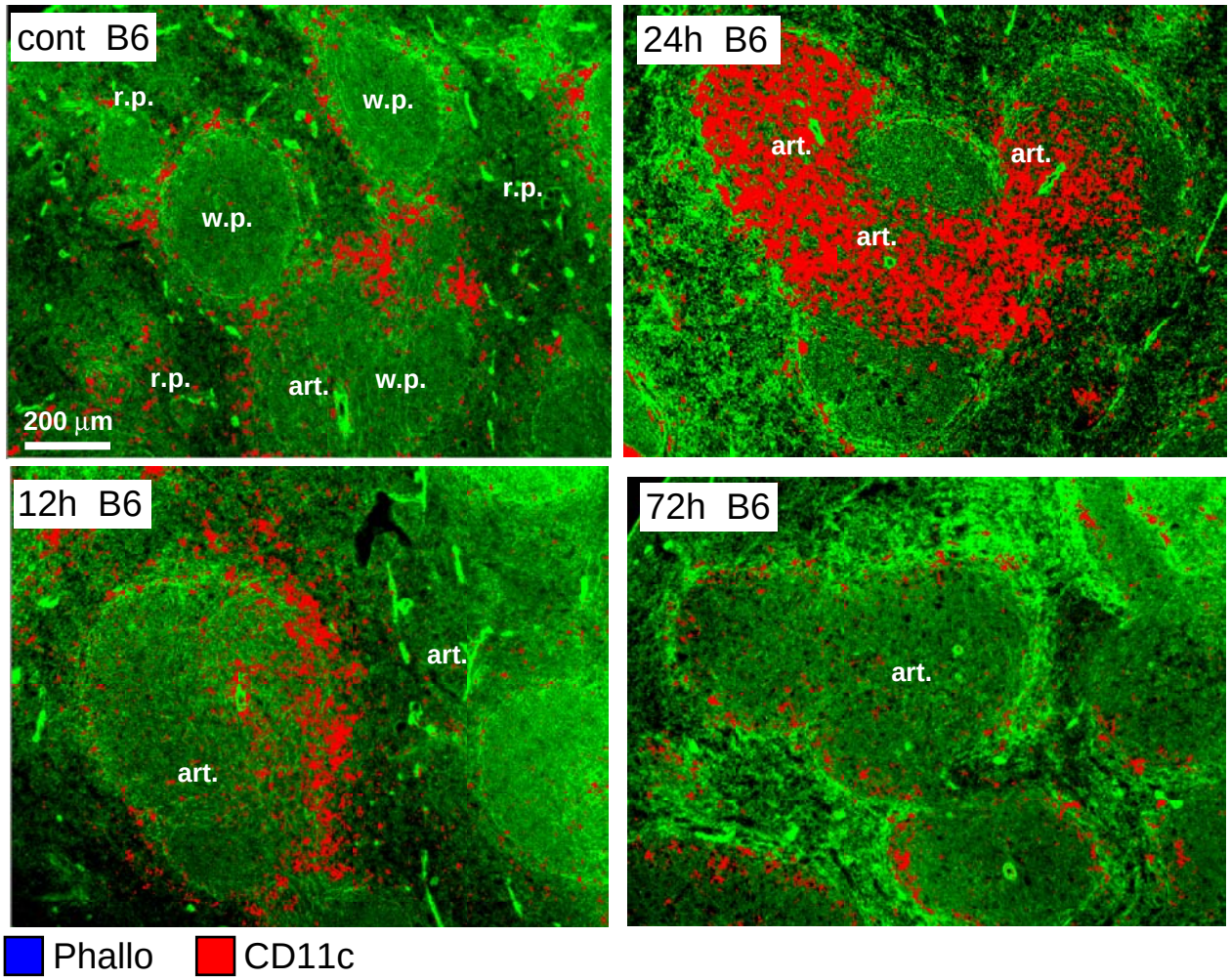

cont B6
r.p.
w.p.
r.p.
w.p.
r.p.
art.
w.p.
200 μm
24h B6
art.
art.
art.
12h B6
art.
art.
72h B6
art.
Phallo
CD11c

Figure S6

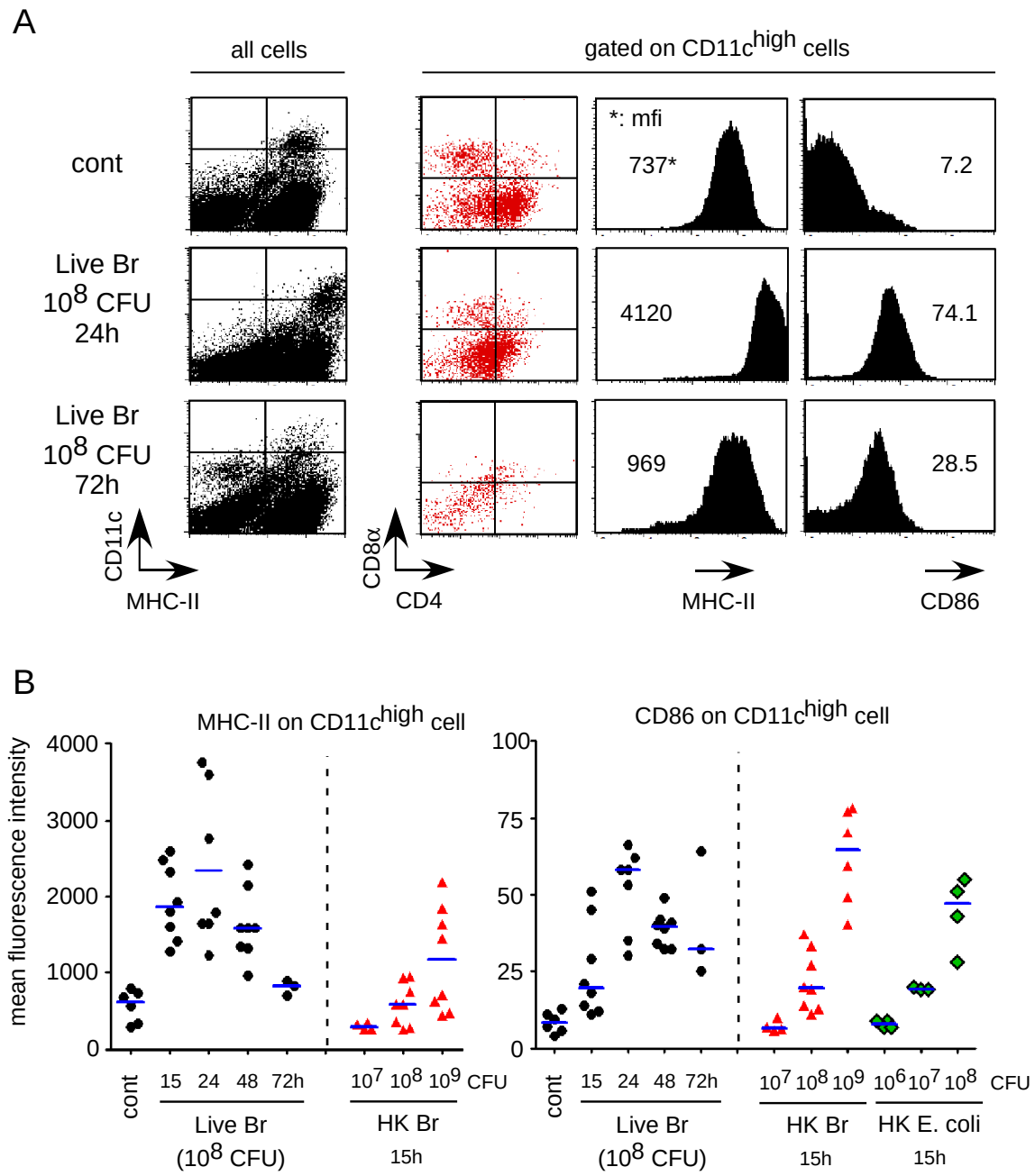

A
all cells
gated on CD11c^high cells
cont
Live Br 10^8 CFU 24h
Live Br 10^8 CFU 72h
*: mfi
737*
7.2
4120
74.1
969
28.5
CD11c
MHC-II
CD8α
CD4
MHC-II
CD86
B
MHC-II on CD11c^high cell
CD86 on CD11c^high cell
mean fluorescence intensity
4000
3000
2000
1000
0
100
75
50
25
0
cont
15 24 48 72h
Live Br (10^8 CFU)
10^7 10^8 10^9 CFU
HK Br 15h
10^6 10^7 10^8 CFU
HK E. coli 15h

Figure S7

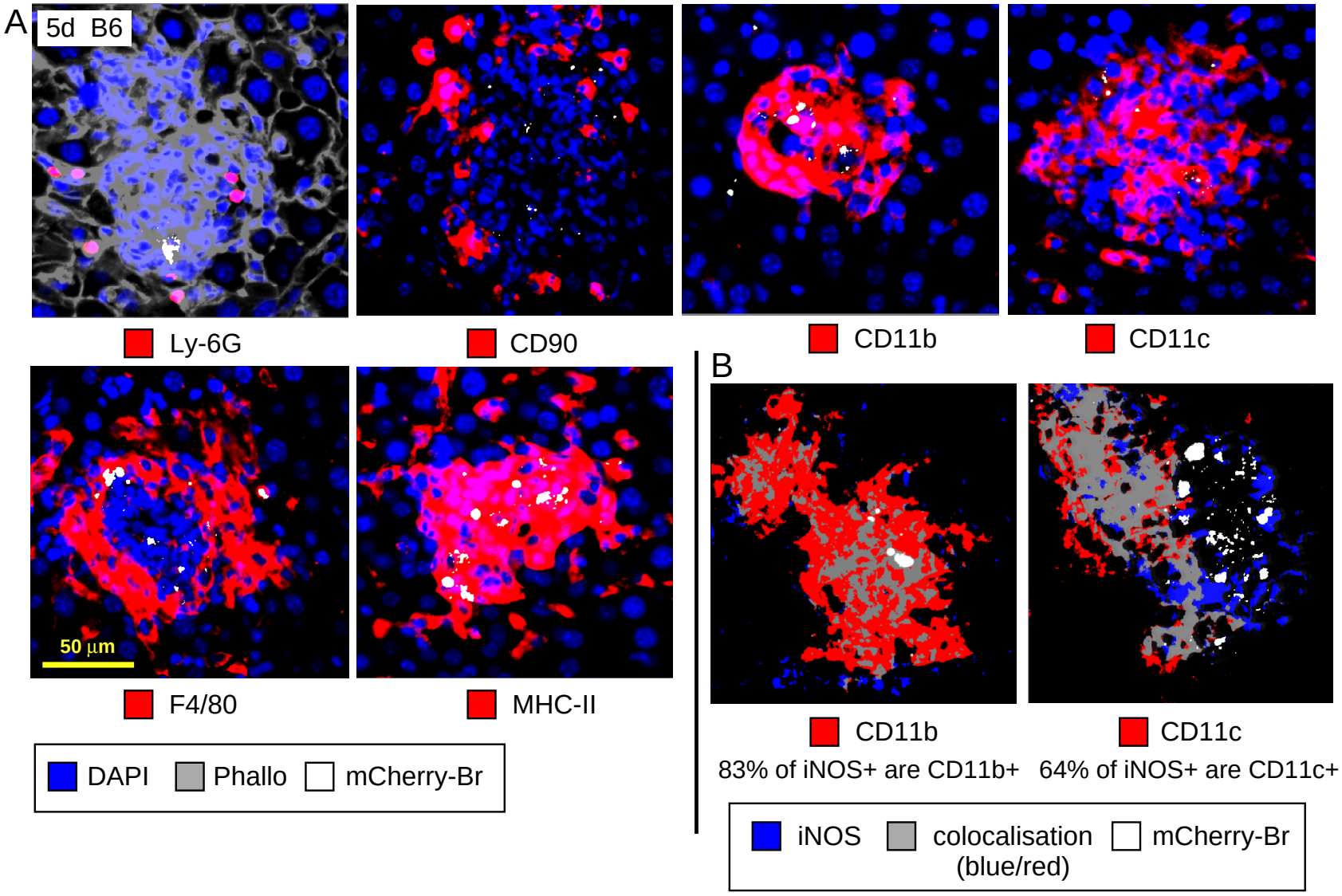

A
5d B6
Ly-6G
CD90
CD11b
CD11c
50 µm
F4/80
MHC-II
DAPI
Phallo
mCherry-Br
B
CD11b
CD11c
83% of iNOS+ are CD11b+
64% of iNOS+ are CD11c+
iNOS
colocalisation (blue/red)
mCherry-Br

Figure S8

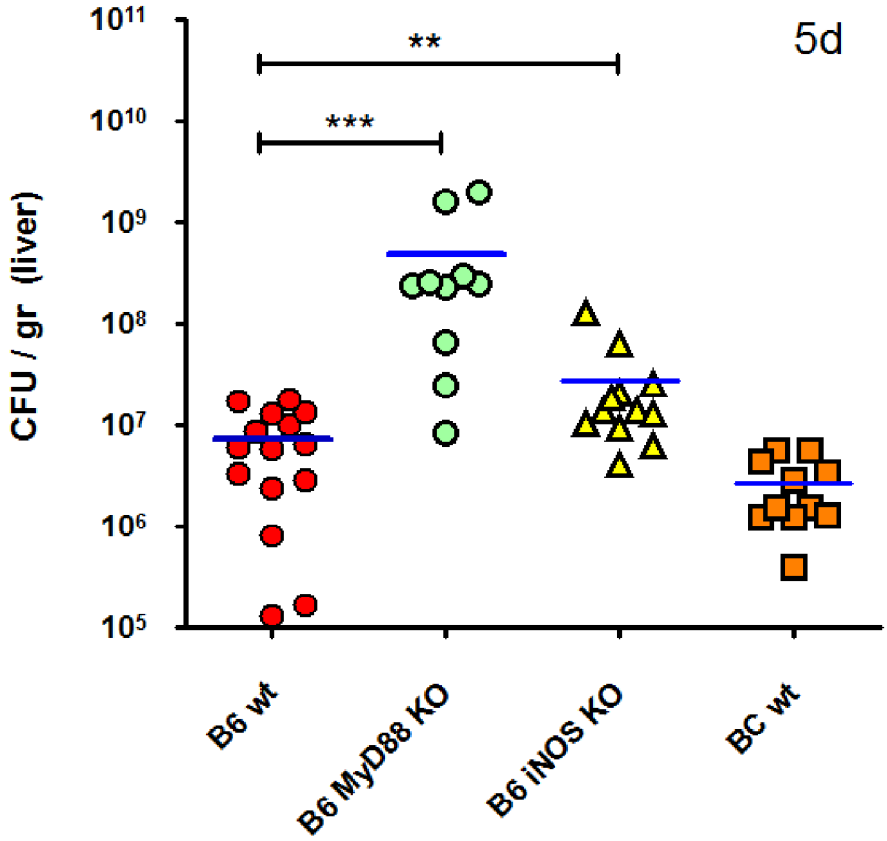

5d
**
***
CFU / gr (liver)
10^11
10^10
10^9
10^8
10^7
10^6
10^5
B6 wt
B6 MyD88 KO
B6 iNOS KO
BC wt

Figure S9

CONCLUSIONS

Brucellosis is one of the world's most widespread zoonotic diseases, causing substantial losses in livestock and debilitating infection in humans. The recent body of work about *Brucella* pathogenesis has significantly added to our understanding of how virulent strains interact with their mammalian hosts, including the establishment inside the host cell and the host immune system manipulation in such a manner as to avoid both killing the host and being cleared from the host. The list of *Brucella* virulence factors is growing fast and is synchronized with advances in the sensitivity and possibilities of new techniques, which enable the identification and analysis of cellular functions that are affected during the infection. However, despite the fact that the mechanisms by which *Brucella* down regulates innate and cell-mediated immune responses have become more and more into focus, we are bound to recognize that few things have improved our knowledge concerning the dynamic behavior of *Brucella* inside its natural host. In this context, the interaction of the bacterium with the immune system during the course of infection, and the ability of *Brucella* to establish long-lasting infection in its host, are far from being understood. On the other hand, the plethora of data gathered in experiments performed on *Brucella* has drawn the fundamental blocks needed to yield a more comprehensive picture of the "battle" between *Brucella* and its host.

This is the perspective from which we realized this work.

Using an experimental host model, the main objective of the current study was to characterize the innate immune system of mammals during a systemic infection with *B. melitensis*. In an attempt to narrow the scope of our research, experiments were mainly realized in the peritoneal cavity and spleen of genetically distinct mouse backgrounds during the early stages of infection. By using a combined histologic and flow-cytometric approach, we intended on one hand to determine the nature of the main effector mechanisms of the immune system activated during the infection and on the other hand to describe the phenotypical characteristics of *B. melitensis* targeted host cells in the spleen during the 5 first days of infection.

Although perhaps engaging in a bit of anthropomorphizing to summarize the findings of this work, we suggest that, following *Brucella* infection, the immune system establishes a hierarchical program for the activation of innate immune cells that is orchestrated by IFN-γ. We propose a model wherein *B. melitensis* is sensed by PRRs, including TLR4 and TLR9, and trapped by F4-80$^+$ resident M∅s, immediately upon its arrival in the spleen. Following the PRRs stimulation, a low inflammatory response is induced, primarily initiated in the peritoneal cavity, that ultimately leads to secretion of IFN-γ by NKs cells, CD4$^+$ and CD8$^+$ T cells. Inflammatory environment activates then the mobilization of CD11b$^+$ myeloid cells from the circulation into the spleen that cluster at foci of bacterial infection and form granulomas structures. Finally, T lymphocytes surround the organized structure and contribute to the maturation of monocytes to inflammatory DCs, which express iNOS and which are required to restrict early infection.

The cellular tropism

Our study revealed dynamism in the cellular environment of the pathogen during the course of infection. Mainly found associated with the M∅s surface marker F4-80$^+$, *B. melitensis* transiently colocalized with MOMA$^+$, CD11c$^+$ and CD11b$^+$ cell surface markers during the early days of infection. Interestingly, *Brucella* was never associated with neutrophils or fibroblastic cells. Initially described as a facultative intracellular pathogen able to infect professional and non-professional phagocytes *in vitro*, our findings demonstrate that, *in vivo*, *B. melitensis* presents a selective cellular infection spectrum. From their introduction within the peritoneal cavity and for the first week of stay in the spleen, this pathogen has always been associated with innate immune cells such as M∅s and DCs. Not consistent with previous studies reporting neutrophils as natural target of *Brucella* [Riley and Robertson, 1984], this observation suggests a privileged link between *Brucella* and myeloid cell lineages, *in vivo*. PMNs are circulating cells implicated in the control of many infection models such as *L. monocytogenes* [Cousens and Wing, 2000] and characterized by a broad spectrum of PRRs and internalizing receptors (see introduction section 3.1). *In vitro* studies highlighted the capacity of neutrophils to engulf *Brucella*, indicating that PMNs are not, *per se*, less able than M∅s to recognize the pathogen [Riley and Robertson, 1984]. Several hypotheses can be proposed to explain why *Brucella* does not infect PMNs *in vivo*: First, as previously demonstrated with *B. abortus* [Barquero-Calvo *et al.*, 2007], *B. melitensis* infection, even when massively

inoculated, does not induce neutrophils influx in the spleen. Absence of PMNs infection could therefore be explained by default in the recruitment of these cells. Second, although innate immune cells share a common panel of PRRs, *Brucella* internalization *in vivo* may be restricted to phagocytes harboring particular cell surface receptors, nonexistent on neutrophil surface. Third, internalization of *Brucella* may be receptor independent and actively regulated by the pathogen. Accordingly, it could necessitate the presence of surface structures only accessible on M∅s and DCs. These hypotheses are in the same direction i.e., -*Brucella* is a furtive pathogen characterized by a cognate restriction of PAMPs limiting its recognition and, thereby crucial for its pathogenesis-.

The DCs relationship

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 cDCs. Flow cytometry and *in situ* staining showed that the infection with *B. melitensis* provoked the migration and subsequent maturation of cDCs in the white pulp during the first 24 hours of infection, followed, latter, by a gradual disappearance. By using heat-killed *B. melitensis* whole extracts, we observed that the maturation process was dose dependent and necessitated very high concentrations of Ags. Comparisons with results obtained in literatures using *Escherichia coli* Ags showed that 100-1000 times more *Brucella* Ags than *E. coli* LPS were needed to trigger cDC maturation [De Trez *et al.*, 2005]. Again, these results are in harmony with the stealthy strategy attributed to virulent *Brucella* spp. Interestingly, histological analysis indicated that none of migrating $CD11c^+$ cells were infected by lived *B. melitensis*, suggesting two distinct groups of DCs in the spleen: the non-infected DCs localized in the white pulp and the infected DCs present in the red pulp. Therefore, an active contribution of the living bacteria to the DCs maturation process has been suggested, as already demonstrated *in vitro* [Salcedo *et al.*, 2008]. However, preliminary results showed that a significant proportion of infected $CD11c^+$ cells were also $CD86^+$, a well-known marker of DCs maturation. In conclusion, *B. melitensis* seems to affect the behavior of DCs in two different ways: First, massive inoculation of bacteria induces a local inflammatory reaction that provokes the migration and maturation of DCs in the white pulp; Second, *Brucella* infected DCs, exclusively localized in the red pulp, can express maturation surface markers but are unable to migrate with their living packages to the lymphoid T cell area. The relationship between *Brucella* and DCs seems complex and necessitates further experiments to be elucidated. However, the preliminary results obtained here suggest the development of *Brucella* determinants that actively hamper the activation of DCs natural behavior, *in vivo*.

The granulomas structures

At the peak of infection, a significant proportion of *B. melitensis* population was gathered in iNOS-producing cellular structures, that we termed granulomas.

The term granuloma is often loosely used to mean "a small nodule" formed during a pathological context. However, the correct use of the term "granuloma" necessitates precaution and requires a careful examination of specially stained tissue under a microscope by a specialist. A right but quite simple definition of granuloma is an organized collection of immune cells where myeloid cells form the bulk of the structure. These cells often, but not invariably, fuse to form multinucleated giant cells and are often referred to as "epithelioid" (because of the vague resemblance to epithelial cells). Epithelioid monocytes have typical characteristics such as elongated and larger nuclei that are thought to be a consequence of their "activation" by the offending Ags. The other key term in the above definition is the word "organized" that refers to a tight, ball-like formation. The monocytes in these formations are typically so tightly clustered that the borders of individual cells are difficult to appreciate.

Granulomas are seen in a wide variety of diseases, both infectious and non-infectious and may be the consequence of infection by pathogens such as bacteria, fungi or worms. Of course, all granulomas, regardless of cause, may contain additional cells and matrix. These include lymphocytes, PMNs, eosinophils, multinucleated giant cells, and fibroblasts. The additional cells are sometimes a clue to the cause of the granuloma. For example, granulomas with numerous eosinophils may be a clue to allergic bronchopulmonary fungal disease, and granulomas with surrounding T lymphocyte suggest a bacterial infection. Another important feature of granulomas is whether or not they contain necrosis. Necrosis refers to dead cells that, under the microscope, appear as a mass of formless debris with no nuclei present. A related term, "caseation" (literally: turning to cheese) refers to a form of necrosis that without a microscope, appears cheese-like ("caseous"), and is typically (but not uniquely) a feature of the granulomas of tuberculosis.

Importantly, in terms of the underlying cause, the difference between granulomas and other types of inflammation is that granulomas form in response to Ags that are resistant to "first-responder" inflammatory cells such as PMNs, M∅s or eosinophils. A granuloma is therefore a second-step immune effector mechanism that can occur in a wide variety of diseases to help the immune response to restrain and eliminate the successful invading pathogen.

It is known for years that the basic characteristic tissue reaction of brucellosis involves mononuclear cells implicated in the formation of granulomic structures even though the nature of these organized aggregates was completely unknown. During our study, we aimed to better understand the dynamic and complexity of these structures and to describe their cellular composition. Present in the spleen and liver of infected animals, *Brucella* granulomas were composed of innate immune cells including inflammatory DCs, and were surrounded by T lymphocytes. Comparative analysis at various time point of infection and using Myd88 knock out mice, suggested a causal link between granulomas development and *Brucella* elimination in the spleen. Moreover, presence of phenotypically similar immune structures in the liver and spleen of infected animals reinforced the hypothesis of granulomas as the "spearhead" of the immune response against *Brucella*. However, the precise role of these immune structures during *Brucella* infection is not clear. During this work, we identified granulomas as massive producer of $NO^{\cdot}$. Interestingly, analysis of iNOS deficiency revealed partial contribution of $NO^{\cdot}$ in the control of *Brucella* proliferation in the spleen and liver of the mouse. Granulomas are complex structures probably characterized by many functions. Notably, it has been proposed that one the role of innate immune cell clustering could be to link the detection of pathogens by the APCs with the innate immune response [Kang *et al.*, 2008]. In other words, granulomas development might be a way to optimize the crosstalk between innate and adaptive immunity. Granulomas development result from the emigration of monocytes from bone marrow into the blood stream and, eventually to infected tissues, where they differentiate to inflammatory DCs and thereby limit bacterial replication. This recruitment process results from the induction of the chemokines MCP-1 and MCP-3, both recognized by CCR2 [Serbina and Pamer, 2006]. Therefore, CRR2 knock out mice cannot mobilize monocytes from the bone marrow and are unable to develop granulomas structures. The utilization of CCR2 knock out mice to study *Brucella* infection should be therefore of great interest to characterize the importance of granulomas in host protection.

The T_H1 importance

As assessed by using immunodeficient mice implicated in the production of IFN-γ, the control of *B. melitensis* infection indubitably results from the capacity of the infected mouse to mount a robust T_H1 response. Although the initial dissemination of the pathogen does not seem to be affected by the lack of T_H1 mediators, the interplay between *B. melitensis* and the innate immune cells is profoundly affected during the latter stages of infection. First, absence of IFN-γ impairs the formation of granuloma structures and thereby avoids the maturation of $CD11b^+$ in inflammatory DCs. Second, defect in T_H1 response

perturb the dissemniation of the bacteria in the spleen with a higher infection of the cells located in the white pulp. Third, the disruption of the immune response allows *Brucella* to massively replicate in phenotypically distinct splenic cells that bear the $CD11c^+$ marker.

The T_H2 contribution

Such important variations in the interaction between *Brucella* and the immune system of the mouse seem to be complex and are far form being understood. However, comparative studies using susceptible Balb/c mice have allowed us to suggest new hypotheses that may contribute to shed light on the complexity of the relationship. One major immune characteristic of Balb/c mice is the typical T_H2-bias of its immune response. T_H2-bias is a complex genetic trait characterizing variation in the propensity of naive T_H cells to differentiate into T_H2 (as opposed to T_H1 or T_H17) cell subsets. T_H2-bias, measured experimentally as the amount of IL-4 produced by effector $CD4^+$ T cells differentiated *in vitro* from naive T_H cells, varies over 50-fold from the high-producer phenotype of Balb/c mice to the low-producer phenotype of C57BL/6 mice and correlates with susceptibility to T_H2-dependent diseases such as leishmaniasis [Bix *et al.*, 1998; Okamoto *et al.*, 2009]. Because T_H responses are exclusive processes, an increase in the production of IL-4 is associated with a proportional diminution of IFN-γ secretion. This observation is consistent with the results obtained in this work showing that the IFN-γ production in Balb/c mice was significantly lower than in C57BL/6 mice during the course of infection. In the spleen, IL-4 is mainly produced by T cell subsets present in the lymphoid sheath of the white pulp, surrounding the central artery [Yoshimoto *et al.*, 1995]. Analysis of the bacterial load and distribution of *B. melitensis* in the spleen revealed a correlation between the presence of the pathogen in the white pulp and the susceptibility of the mouse. Compared to Balb/c mice, the white pulp of the resistant C57BL/6 mice contained very low number of infected cells. In contrast, in the white pulp of Balb/c mice, the number of infected cells, although still weak, was more important and frequently corresponded to $iNOS^-$/F4-80^-/$MHCII^-$ giant cells harboring a dramatic accumulation of bacteria. In the Myd88 deficient C57BL/6 mouse, *Brucella* was significantly found in the white pulp, massively replicated in $iNOS^-$/F4-80^-/$CD11c^+$ cells or $iNOS^-$/ F4-80^-/$MOMA^+$ cells. According to these observations and because the T_H1 response of the mice used in these experiments range from robust (C57BL/6) to reduced (Balb/c) to almost absent (Myd88 KO), it is reasonable to postulate that the Balb/c and T_H1 deficient mice susceptibility results not solely from perturbation in the T_H1 response but also from the upregulation in T_H2 mediators, including IL-4 that may contribute to the development of permissive replication niches for *B. melitensis*.

In other words, absence of T_H1 response and IFN-γ may have two positive impacts on the *Brucella* proliferation: (1) to perturb the formation and activation of essential effector mechanisms implicated in the control of *Brucella*, including granulomas formation and (2) to indirectly increase the production of T_H2 mediators such as IL-4, which in turn are involved in the differentiation of innate immune cells potentially permissive to *Brucella* replication.

The M2 hypothesis

IL-4 is associated with many functions controlling the status of the immune system during an infectious process. One example is the stimulation of M2M∅s program activation. As already discussed in the introduction (section 3.2.2), M2M∅s is a differentiated population of M∅s implicated in the down-regulation of the immune response and in the remodeling of tissues following a inflammation process [Van Ginderachter *et al.*, 2006]. The phenotypical characterization of this subgroup of innate immune cells is not well determined and seems to be related to a particular environment. Until recently, the discrimination between M1M∅s and M2M∅s in mice was mainly demonstrated at the biochemical level, by measuring the metabolism of L-arginine. Within M∅s, L-arginine can be metabolized by three different pathways that result in the production of: (1) L-citrulline and NO· by iNOS; (2) ureum and L-ornithine by arginase; and/or [Cole *et al.*] agmatine by arginine decarboxylase. The cross-regulation of these pathways by T_H1 mediators, such as IFN-γ, and by T_H2 cytokines, such as IL-4, which favor the activity of iNOS and arginase respectively, suggests that the measure of the level of both enzyme activity in distinct M∅s populations reflects their activation state, classical ($iNOS^+$ M1M∅s) or alternative (Arg^+ M2M∅s) [Munder *et al.*, 1998]. In our context, one hypothesis could be that the massively infected cells of the Balb/c and Myd88 knock out mice white pulp are M2M∅s. These alternatively activated M∅s have already been associated with infectious process but principally in the context of parasitic infection that involved T_H2-dependent responses [Dewals *et al.*; Namangala *et al.*, 2001; Gordon, 2003]. By using specific M2M∅s markers including arginase staining, we could shed light on the phenotype of these permissive cells and contribute to better understand the cross regulation of the T_H1 versus T_H2 responses on *Brucella* infection.

Analysis of the contribution of both immune responses during the infection process is an important challenge, crucial to figure out the basis of *Brucella* pathogenesis. Utilization of additional knock out mice involved in T_H1 or T_H2 responses activation will be an important step to confirm or refute the results and hypotheses established during this work. Moreover, it would be of great interest to characterize the *Brucella* infection in the combined IL-12/IL-4 knock out mice in which both T_H1 and T_H2 responses is defective. Assuming that M2M∅s contribute to *Brucella* proliferation in T_H1 deficient context, the double knock out should not be more susceptible than the single T_H1 knock out mice.

The limitations of the model

In many ways, the scientific process can be defined as the generation of increasingly complex models of the natural world. Where the direct study of natural systems is impractical, biological models provide an important tool for progress. However, all models are prey to the criticism that they are not,' the real situation' and that any study using a model is an accurate representation only of the behavior of that model under the circumstances of the test. However, by carefully defining the study parameters, scientists hope that results might prove to be analogous to the situation in nature.

Our model presents certain limitations that may support some points of dispute. To gain insights into the dynamic behavior of the innate immune system following *Brucella* infection in the mouse, we used a traceable strain of the pathogen expressing the bright fluorescent mcherry protein. The limits of detection imposed by the immunohistological techniques used in this work constrained us to use an unusually high dose of bacteria that certainly trigger an inflammation larger than that occurring in conventional models. However, our results revealed that the high infectious dose was not lethal and provoked an infection cleared at the same rate than that occurring when the wild type mouse was inoculated with the conventional dose. Moreover, when both studies of the present thesis are compared regarding the behavior of the immune response induced by both infectious doses, we notice that there is no difference in the nature of the response but rather in the speed of activation of this response. Indeed, the typical recruitment of inflammatory DCs that occurs 5 days post-infection when the mouse is inoculated with the high infectious dose is highlighted 10 days post-infection in the conventional model. Since we found that inflammatory DCs are the main components of granulomas, it is likely that these structures are also formed in the conventional model, but later in the infection process. As a result, we can conclude that the results derived from our study are not artifacts resulting from the experimental conditions and may be used for a

better understanding of the immune response developed against *Brucella* in a general context of systemic infection of animals.

An additional limitation of our experimental model is the inability to analyze the behavior of the bacterium when the population of the pathogen is bellow the one billion individuals. Brucellosis is a chronic disease that is rarely lethal and often characterized by the absence of symptoms. Regarding the stealthy strategies choose by the pathogen to hamper the immune response, it is obvious to say that the strength of the bacterium do not result from its capacity to proliferate within its host but rather to go unnoticed in this hostile environment. Therefore, to understand brucellosis complexity and reveal chronic niche of replication, it is essential to consider the question from the single individual in this context. Accordingly, our experimental procedure can only lead to a dead end if used to consider such questions. However, it is important to remind that the main purpose of the present work was to reveal immune effector mechanisms developed by the mouse to control brucellosis during the first steps of infection. The only contribution that may be done regarding the chronic aspect of the infection is the impact of granulomas on the persistence of the pathogen. Within natural host as well as human, granulomas are also the hallmark of brucellosis especially in the liver. The impact of granulomas on the persistence of other long-lasting pathogens such as *M. tuberculosis* is clearly defined even though the question of the beneficial contribution of these structures on the proliferation of the pathogen is still controversial. The results obtained during our research reveal that the granulomas formed following *Brucella* infection didn't contribute to the persistence of the pathogen but rather proved to be an effective mechanism of the immune response to eradicate the pathogen.

The list of limitations of an experimental procedure is far from being short. In nature, initial infection by the most common routes (aerosol, ingestion or vector-mediated) is usually followed by a period of replication that establishes the infection within the host. The final step is the exit from the infected host and transmission to the next individual. This can occur through several routes, including the shedding of bacteria in contaminated abortion tissue, the creation of aerosols by coughing, or the bite of an insect vector. In almost all models of bacterial infection, animals are deliberately infected with high doses of bacteria grown in rich artificial laboratory media rather than being exposed to the organism in a manner that approximates the natural route of transmission. Consequently, the strain grows in culture require a period in which to adapt to the new environment of the animal host. Furthermore, disease is an incidental consequence of the life cycle of many infectious organisms, and the study of pathogens in isolation from their natural life cycle can therefore be misleading.

Finally, it is important to remind that a model is only a representation of natural processes that will be successful only in so far as it is relevant to that process.

The contribution of the model

In considering the brucellosis in its natural context, many questions arise regarding the interaction of the parasitized host and the invading microorganism. These include: What happens to *Brucella* after it invades the body? What takes place in the host with respect to immune responses and tissue reactions? What basic causes contribute to the illness of patients?

Both animal and human hosts display variable degrees of susceptibility and resistance to *Brucella* organisms. While some species of animals are highly resistant to infection (e.g.: the cat), variations of susceptibility occur within others species, such as in human. Additionally, parasites also differ in their invasive properties and virulence, *B. melitensis* causing the most serious infection in animal and human. Nevertheless, despite such variations, a common theme unified the pathogenesis of brucellosis, i.e. the sequence of events immediately following invasion of the body by the microorganism that ultimately leads to the formation of granulomatous lesions.

Indeed, whatever the nature of the infected host, this sequence can be summarized as follows: surviving at the site of primary invasion, the pathogens enter the lymphatics and become localized in regional lymph nodes where they proliferate and finally reach the blood stream. Dissemination then takes place in secondary localization, particularly in those organs and tissues having an abundance of reticulo-endothelial cells, including the spleen and liver. In these regions, *Brucella* becomes localized within the tissue cells, especially in those cells belonging to the mononuclear family and forming granulomatous structures. Accordingly, the granulomatous lesion is a basic and characteristic tissue reaction in animal and human brucellosis.

In the light of such observations, the characterization of the early steps of infection as well as the elucidation of the nature and roles of the granulomatous structures seem obvious to gain insight into the pathogenesis of brucellosis in its natural context. Therefore, our contribution may be used as the first draft to understand the complexity of the interaction between *Brucella* species and their respective hosts. The comparison of the phenotype of the granuloma structures formed within various species and the study of the impact of the infection by different *Brucella* strains on the establishment of these structures are among interesting perspectives that deserve energy and a particular attention.

To conclude

This work contributed to establish a map describing the itinerary taken by *Brucella* in the mouse after systemic inoculation during the early steps of infection. Although the "hosts" employed to obtain these data are laboratory animals, and differences among the immune systems of mammals occurs, the results nevertheless shed light on the behavior of *Brucella in vivo* that are likely to be useful in further understanding of the disease in the principal target species. Localized at the beginning of our understanding of the host pathogen relationship, the findings of this thesis have revealed the complexity of the mechanisms underlying innate immune cell trafficking and activation during *B. melitensis* infection. Development of new techniques combined to the availability of a broad range of knock out mice has begun to open the doors of the *in vivo* universe. Nevertheless, the perspectives of this work are numerous and much work remains to be done. "Natural route of infection", "tissular tropism" or "chronic cellular niche" are among examples of general themes that are barely addressed and for which many questions remained unresolved. Such knowledge is needed to determine the outcome of the infection and ultimately to design effective vaccines for use in humans, which has been and remains the challenge for the future.

MATERIALS & METHODS

1. Construction of a mcherry-producing *B. melitensis* strain.

1.1. Design and construction of the vector

The mCherry protein, a previously described rapidly maturing variant of the red fluorescent protein DsRed [Shaner *et al.*, 2004], was cloned into the suicide vector pKSoriT-*bla-kan* downstream a strong *Brucella* spp. promoter named P*sojA* [Kohler *et al.*, 2002]. This promoter is described as controlling the expression of the protein translocase SecE.

The vector was constructed as follows: the *mcherry* coding sequence was amplified by PCR from pRSET-B-*mcherry* [Shaner *et al.*, 2004] with the *mcherry*-up and *mcherry*-down primers and ligated into pGemT-Easy (Promega) to generate pGEM-T-*mcherry*. The *mcherry* fragment was then excised from pGEM-T-*mcherry* by *Hin*dIII/*Xba*I double restriction and subsequently cloned into *Hin*dIII/*Xba*I-cut pKSoriT-*bla* (pBluescript II KS vector from Stratagene in which RP4 *oriT* was inserted in order to make this vector mobilizable (Tibor, 2002 #1039) 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 *Not*I/*Xba*I double restriction and subsequently inserted into *Not*I/*Xba*I-cut pKSoriT-*bla-mcherry* to generate pKSoriT-*bla*-P*sojA*-*mcherry*. Finally, the *aphA*4 cassette (a promoterless kanamycin resistance gene (Dozot, 2006 #1045) was excised from pUC4*aphA4* with *Sal*I, and subsequently cloned into the *Xho*I 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 Nal^R 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.

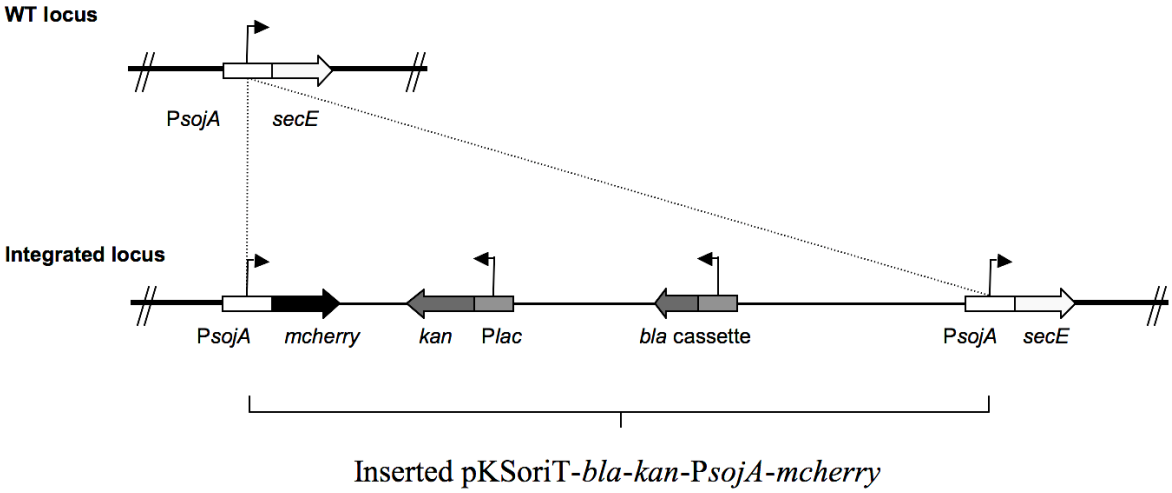


Figure | Schematic representation of the pKSoriT-*bla*-*kan*-P*sojA*-*mcherry* insertion in the *B. melitensis* genome

Primers used in this study	
Primer	Sequence (5'-3')
***mcherry*-up (*Xba*I)**	tctagaatggtgagcaagggcgag
***mcherry*-down (*Hind*III)**	aagcttttacttgtacagctcgtcca
P*sojA*-up (*Not*I)	gcggccgccttgactatggatgcccgtt
P*sojA*-down (*Xba*I)	tctagactctgtctgatcaggcacaa

1.2. Characterization of the fluorescent strain

1.2.1. Infections and bacterial counts in cell line cultures

Syrian hamster kidney fibroblast BHK cells were cultured in DMEM (Gibco) supplemented with10% fetal calf serum in 5% CO2 at 37 °C. Cells were detached from culture flasks with trypsin, resuspended in fresh culture medium and replated at a density of $1x10^5$ in 24-well cell culture plates (Costar). Cells were typically incubated overnight before the addition of bacteria.

Bacterial strains were grown individually with shaking at 37 °C. Sub confluent monolayers of BHK fibroblasts grown in 24-well cell culture plates were inoculated with 500 ml of bacterial strains at a multiplicity of infection [Barrangou *et al.*] of 300 diluted in cell culture medium. The mixture was centrifuged for 10 minutes at 1000 r.p.m. at room temperature and placed under a 5% CO2 atmosphere at 37°C. After 1 hour, the wells were washed three times with phosphate buffered saline (PBS) and were further incubated for 1 hour with cell culture medium supplemented with 50 mg/ml of gentamicin. Then, the medium was replaced by the cell culture medium supplemented with 10 mg/ml of gentamicin until the end of the infection time. The number of intracellular viable brucellae was determined at 48 hours p.i. To this end, the monolayers were washed three times with PBS and treated with 200 ml of 0.1% Triton X-100 (Roche) in PBS for 10 minutes. Lysates were serially diluted and plated onto 2YT plates to count viable bacteria (expressed as colony-forming units, CFU).

1.2.2. Infections and bacterial counts in experimental animals

Balb/c and C57BL/6 mice were injected intraperitoneally with $4x10^4$, $1x10^6$ and $1x10^8$ CFU of *B. melitensis* 16M WT or mCherry-expressing *B. melitensis* 16M in 500µl of PBS. To count residual *Brucella* CFUs in the spleens or livers of mice, at least five mice from each group were examined at each sampling period. Spleens or livers were homogenized in plastic bags with 1 ml sterile PBS containing 0.1% Triton X-100 (Roche). To enumerate viable *Brucella*, homogenized spleen or liver samples were serially diluted 10-fold with PBS and plated onto 2YT medium plates (Luria-Bertani with double quantity of yeast extract). (Difco, Detroit, MI). *Brucella* colonies were counted after a 3 days incubation at 37°C.

2. Materials & Methods relative to the Immunofluorescence microscopy

2.1. Immunofluorescence assay on cell line cultures

Glass coverslips (13 mm diameter) in 24-well plates were seeded with BHK fibroblasts suspended in the culture medium ($1x10^5$ cells/well). Inoculation and washing were performed as described above (1.2.2). The infected cells were fixed with 3% paraformaldehyde for 20 minutes at room temperature, at 48 hours after infection. Then, coverslips were incubated successively in a PBS solution containing 1% blocking reagent (Boeringer) (PBS-BR 1%) and in PBS-BR 1% containing 4'-6-Diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) and bovine purified antibody anti-*brucella*LPS. Primary antibody was detected using goat anti-bovine coupled with FITC (Jackson ImmunoResearch)

2.2. Immunofluorescence assay on mouse tissue

For immunofluorescence detection, spleen and liver samples were fresh frozen in TissueTek OCT (optimal cutting temperature) (Sakura) compound using liquid nitrogen. 5 µm cryosection retrieved on SuperFrost Plus Gold slides (Vwr) were incubated 30 minutes in PBS-BR 1% to prevent non-specific binding of the antibodies, then over night with monoclonal antibodies (mAbs) diluted in PBS-BR 1% containing Alexa Fluor 488 phalloidin (Molecular Probes) and DAPI (Sigma-Aldrich). Double specific staining were incubated in PBS-BR 1% containing either Alexa Fluor 350 phalloidin (Molecular Probes) or DAPI (Sigma-Aldrich) alone. After 3 washes of 5 minutes each in fresh PBS, sections were incubated 2 hours in the dark with a secondary antibody diluted in PBS-BR 1%. Biotin-coupled mAbs were amplified using Alexa Fluor 350 or Alexa Fluor 647 Streptavidin (Molecular Probes) in PBS-BR 1%. When two biotin-coupled mAbs were used, free biotin sites were saturated with an avidin-biotin blocking kit (Vector). Finally, after 3 final washes of 5 minutes each in fresh PBS, still in the dark, cover glasses were attached using fluoro-gel (Electron Microscopy Sciences, Hatfield, PA) and left at -20°C before observation. Labeled tissues sections were visualized under a Zeiss fluorescent inverted microscope (Axiovert 200) equipped with high resolution monochrome camera (AxioCam HR, Zeiss).

Antibodies used for immunofluorescence staining		
Full Name	References	Dilution
Alexa Fluor® 647 anti-mouse F4/80 Antigen	#51-4801 eBioscience	1/100
Alexa Fluor® 647 anti-mouse CD11b	#557686 BD Pharmingen	1/100
Biotin anti-mouse CD11c	#553800 BD Pharmingen	1/100 – 1/50
Allophycocyanine (APC) anti-mouse MHCII (I-A/I-E)	#17-5321 eBioscience	1/100 – 1/50
Biotin anti-mouse CD90.2	#553001 BD Pharmingen	1/100
Biotin anti-mouse CD45R/B220	#553085 BD Pharmingen	1/100
Alexa Fluor® 647 anti-mouse Ly-6G (GR-1)	#51-5931 eBioscience	1/100
Purified anti-mouse iNOS	# 482728 Calbiochem	1/500
Biotin anti-mouse MMØs	#T-2021 BMA Biomedicals	1/100
Secondary staining		
Alexa Fluor® 488 goat anti-rabbit IgG	#A11034 Invitrogen	1/100
Streptavidin Alexa Fluor 350 Conjugate	# S11249 Invitrogen	1/100
Streptavidin Alexa Fluor 647 Conjugate	#S21374 Invitrogen	1/100

3. Materials & Methods relative to the flow cytometric analysis

3.1. Harvest of spleen and peritoneal exudates

At selected time intervals, mice were sacrificed by cervical dislocation. Immediately after being killed, mice were peritoneally washed with 10 ml of ice-cold HBSS and the spleen harvested. Before cytofluorometric analysis, spleens were cut in very small pieces and incubated (37°C, 30 min) into HBSS (Invitrogen Life Technologies) medium containing 4000 U/ml collagenase IV (Roche) and 0.1 mg/ml DNase I fraction IX (Sigma-Aldrich Chimie SARL). This procedure was performed in all experiments of this work.

3.2. Fluorescent activated cell sorter (FACS) analysis

After washing, peritoneal cells and spleen cells were filtered and first incubated in saturating doses of purified 2.4G2 (anti-mouse Fc receptor, ATCC) in 200 ml PBS 0.5% BSA 0.02% NaN3 (FACS buffer) for 10 minutes on ice to prevent antibody binding to Fc receptor. 3-5 $x10^6$ cells were stained on ice with various fluorescent mAbs combinations in FACS buffer and further analyzed on a FACScalibur cytofluorometer (Becton Dickinson, BD). Cells were gated according to size and scatter to eliminate dead cells and debris from analysis.

3.2.1. Intracellular cytokine staining

Peritoneal and spleen cells were incubated for 4 h in RPMI 1640, 5% fetal calf serum with 10 μg/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 minutes using a saponin-based buffer (1x Perm/Wash; BD Pharmingen in FACS buffer) containing the intracellular staining mAbs After final fixation in PBS/1%PFA, the cells were analyzed on a FACSCalibur cytofluorometer. Isotype controls were used as negative controls.

Antibodies used for cytometric analysis		
Full Name	References	Dilution
Extracellular Staining		
Fluorescein isothiocyanate (FITC) anti-mouse CD11b (M1/70)	#557396 BD Pharmingen	1/200
FITC anti-mouse TCR β Chain (H57-597)	# 553170 BD Pharmingen	1/200
Phycoerythrin (PE) anti-mouse I-A/I-E (M5/114.15.2)	# 557000 BD Pharmingen	1/100
PE anti-mouse CD11c (HL3)	# 561044 BD Pharmingen	1/200
PE anti-mouse Ly-6C (AL-21)	# 560592 BD Pharmingen	1/100
PE anti-mouse Ly-6G (1A8)	# 561104 BD Pharmingen	1/100
APC anti-mouse CD4 (RM4-5)	# 561091 BD Pharmingen	1/400
PE anti-mouse CD8α (53-6.7)	# 553033 BD Pharmingen	1/100
Biotin anti-mouse CD90.2 (53-2.1)	# 553002 BD Pharmingen	1/100
APC anti-mouse F4/80 (BM8)	# 17-4801 eBioscience	1/100
Intracellular Staining		
APC anti-mouse IFN-γ (XMG1.2)	# 554413 BD Pharmingen	1/300
APC anti-mouse TNF-α (MP6-XT22)	# 554420 BD Pharmingen	1/100
Purified rabbit anti-mouse iNOS (M-19)	# sc-650 Santa Cruz Biotechonlogy, INC.	1/1500
Secondary Staining		
Alexa Fluor® 647 goat anti-rabbit IgG	# A21244 Invitrogen	1/100

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