Humoral Immunity and CD4+ Th1 Cells Are Both Necessary for a Fully Protective Immune Response upon Secondary Infection with Brucella melitensis

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Humoral immunity and $CD4^+$ Th1 cells are both necessary for a fully protective immune response upon secondary infection with *Brucella melitensis*.

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ABSTRACT

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

INTRODUCTION

Brucella (α-proteobacteria) are small, non-motile, non-spore-forming, facultative intracellular Gram-negative coccobacilli that infect humans as well as domestic (cattle, sheep, swine, camels, etc.) and wild-type (deer, bison, etc.) mammals. Animal infection leads to abortion in pregnant females and orchitis and epididymitis in males, resulting in infertility (1, 2). Human brucellosis is a zoonotic infection transmitted through ingestion, inhalation, or contact with conjunctiva or skin lesions (3). Although it is rarely fatal, it is a severe and debilitating chronic disease without prolonged antibiotic treatment (4, 5). Despite significant progress, the incidence of human brucellosis remains very high in endemic areas, with more than 500,000 new human cases reported annually (6), and this number is considered to be largely underestimated (7). In addition, *Brucella* species are considered as potential biological warfare agents and have been "weaponized" by several governments (8). Since *Brucella* are classed as category B threat agents (8), their use in bioterrorist attacks must be taken seriously and response plans should be designed.

As the complete eradication of *Brucella* would be unpractical due to its presence in a large range of wild mammals (9, 10) and because antibiotic treatment is costly and patients frequently suffer from resurgence of the bacteria (11), vaccination remains the only rational strategy to confer protection to populations living in endemic countries. Unfortunately, there is currently no available vaccine against human brucellosis as all commercially available animal vaccines are based on live attenuated strains of *Brucella* (*B. melitensis* Rev.1, *B. abortus* S19, *B. abortus* RB51) (12, 13) that cause disease in humans. Little real progress in the field of *Brucella* vaccination has been reported in recent decades. One clear cause seems be the empirical nature of research on the *Brucella* vaccine. Indeed, the vast majority of

publications report only the protective ability of candidate vaccines, limiting their analysis to present CFU counts in the spleen after challenge (14, 15). Evaluations of the ability of vaccines to induce IFN-γ producing cells, detected *in vitro* after re-stimulation, and/or a humoral response are also often reported (16-18). Rare studies (19-22) have tried to characterize the nature of the protective immune response induced by vaccination and thus identify potential protective immune markers for the development of a rational strategy to select candidate vaccines. These markers cannot be deduced from studies of the primary immune response against *Brucella*, because, as shown in other infectious models (23-29), primary and secondary immune responses frequently implicate different classes of effectors.

Live vaccines are widely accepted to be superior to inactivated vaccines for protection against brucellosis (19, 30, 31), suggesting that the localization and persistence of *Brucella* antigens are key factors in the development of protective immunity. However, there is no consensual explanation for this fundamental difference. The use of heat-killed (HK) preparations of *Brucella* as adjuvants to induce a Th1 response has been described by some authors (32-35) while others have demonstrated that HK *Brucella* failed to induce the desirable Th1 protective response (19, 31). Transfer experiments suggest that antibodies, $CD4^+$ and $CD8^+$ T cells could be protective (20-22, 36, 37), but these results are subject to multiple interpretations in the context of a chronic infection due to the half-life of the transferred antibodies and cells. The fact that both cell-mediated immunity and antibodies have been reported to independently protect mice against brucellosis may explain why a broad collection of immunogens have been described to elicit a protective response, with sometimes substantial variability in the protocol used (38).

To increase our understanding of the nature of protective mechanisms induced by live vaccines, we developed an original model to compare and analyze in detail the level of protection in the blood and spleen induced by the intraperitoneal (i.p.) injection of heat-killed

(HK) and live virulent strains of *B. melitensis* 16M. The protection levels and elicited immune responses were characterized in several compartments (blood, peritoneal cavity and spleen) and at different times after the i.p. challenge with live *B. melitensis*. In this model, we observed that both HK and live vaccines induce drastic early control of bacteria dissemination in the blood, but that only live vaccines mediate late complete elimination of bacteria in the spleen. Using mice rendered genetically deficient for key elements of the immune response, we tried to identify the lymphocyte populations and signaling pathways associated with these early and late protections. Our results demonstrate that specific antibodies are critical for both protection levels and that their development does not require MyD88/IL-12 signaling pathways, $CD4^+$ T cells or even an activation-induced deaminase (AID)-mediated class switch. However, MyD88/IL-12 signaling pathways and IFN-γ-producing $CD4^+$ T cells are needed to eradicate the bacteria from the spleen. On the whole, these results identify potential preliminary markers of protective immune response against *B. melitensis* and could thus help to develop a rational strategy to identify protective live vaccines against human brucellosis.

MATERIALS AND METHODS

Ethics Statement

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

Mice and reagents

MyD88$^{-/-}$ C57BL/6 mice (39) were obtained from Dr. S. Akira (Osaka University, Japan). IL-12p35$^{-/-}$ C57BL/6 mice (40) from Dr. B. Ryffel (University of Orleans, France). AID$^{-/-}$ C57BL/6 mice (41) from Dr. H Jacobs (The Netherlands Cancer Institute, The Netherlands). MHCII$^{-/-}$ C57BL/6 mice (42) from Jörg Reimann (University of Ulm, Ulm, Germany). RAG1$^{-/-}$ C57BL/6 mice (43) from Dr. S. Goriely (Université Libre de Bruxelles, Belgium). STAT6$^{-/-}$ BALB/c mice (44), MuMT$^{-/-}$ C57BL/6 mice (45) were purchased from The Jackson Laboratory (Bar Harbor, ME). Wild-type C57BL/6 and BALB/c mice were purchased from Harlan (Bicester, UK) and were used as controls. All wild-type and deficient mice used in this study were bred in the animal facility of the Gosselies campus of the Université Libre de Bruxelles (ULB, Belgium).

B. melitensis strain 16M (Biotype1, ATCC 23456) was initially isolated from an infected goat and grown in biosafety level III laboratory facilities. Overnight cultures grown with shaking at 37°C in 2YT media (Luria-Bertani broth with double quantity of yeast extract) and then were washed twice in PBS (3500xg, 10 min.) before use for mice inoculation as previously described (46). When indicated, we used a strain of *B. melitensis*

16M stably expressing the mCherry protein (mCherry-Br), a previously-described rapidly maturing variant of the red fluorescent protein DsRed (47), under the control of the strong *Brucella spp.* Promoter, PsojA (48). Construction of the mCherry-Br strain has been described previously in detail (49).

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

Mice immunization and challenge

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

Antibiotic treatment

Antibiotic treatment was administered to both immunized and control mice for 3 weeks. The oral treatment was a combination of rifampicin (12 mg/kg) and streptomycin (450 mg/kg) (adapted from (50)) prepared fresh daily and given in the drinking water. An additional i.p. treatment was given and consisted of 5 injections of streptomycin (300 mg/kg) throughout the 3 weeks of oral treatment (51). The mice were not in distress. To ensure that

the antibiotic treatment was effective, some mice from each group were sacrificed 1 week prior to the challenge and the CFU counts were evaluated in the spleen.

Bacterial count

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

Cytofluorometric analysis

As previously described (46), spleens were harvested, cut in very small pieces and incubated with a cocktail of DNAse I fraction IX (Sigma-Aldrich Chimie SARL, Lyon, France) (100 µg/ml) and 1.6 mg/ml of collagenase (400 Mandl U/ml) at 37°C for 30 min. After washing, spleen cells were filtered and first incubated in saturating doses of purified 2.4G2 (anti-mouse Fc receptor, ATCC) in 200 µl PBS 0.2% BSA 0.02% NaN3 (FACS buffer) for 20 minutes on ice to prevent antibody binding to Fc receptor. 3-5x10^6 cells were stained on ice with various fluorescent mAb combinations in FACS buffer and further collected on a FACScalibur cytofluorometer (Becton Dickinson, BD). We purchased the following mAbs from BD Biosciences: Fluorescein (FITC)-coupled 145-2C11 (anti-CD3ε), Phycoerythrin (PE)-coupled RM4-5 (anti-CD4), Phycoerythrin (PE)-coupled 53-6.7 (anti-CD8α), Fluorescein (FITC)-coupled 53-2.1 (anti-CD90), Fluorescein (FITC)-coupled 7D4 (anti-CD25), Fluorescein (FITC)-coupled H1.2F3 (anti-CD69), Biotin-coupled AL-21 (anti-LY6C) and Fluorescein (FITC)-coupled avidin. The cells were analyzed on a FACScalibur

cytofluorometer. Cells were gated according to size and scatter to eliminate dead cells and debris from the analysis.

Intracellular cytokine staining

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

Immunofluorescence microscopy

Spleens were fixed for 6 hour at 4°C in 2% paraformaldehyde (pH 7.4), washed in PBS, incubated overnight at 4°C in a 20% PBS-sucrose solution under shaking, and washed again in PBS. Tissues were embedded in the Tissue-Tek OCT compound (Sakura), frozen in liquid nitrogen, and cryostat sections (5µm) were prepared. Tissues sections were rehydrated in PBS, then incubated successively in a PBS solution containing 1% blocking reagent (Boeringer) (PBS-BR 1%) and in PBS-BR 1% containing any of the following mAbs or reagents: DAPI nucleic acid stain, Alexa Fluor 350 or 488 phalloidin (Molecular Probes), Alexa Fluor 647-coupled BM8 (anti-F4/80, Abcam). Slides were mounted in Fluoro-Gel medium (Electron Microscopy Sciences, Hatfield, PA). Labeled tissue sections were visualized with an Axiovert M200 inverted microscope (Zeiss, Iena, Germany) equipped with a high resolution monochrome camera (AxioCam HR, Zeiss). Images (1384x1036 pixels,

0.16µm/pixel) were acquired sequentially for each fluorochrome with A-Plan 10x/0.25 N.A. and LD-Plan-NeoFluar 63x/0.75 N.A. dry objectives and recorded as eight-bit grey-level *.zvi files. At least 3 slides per organ were analyzed from 3 different animals and the results are representative of 2 independent experiments.

In vitro stimulation of peritoneal cells

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

ELISA

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

4 washes with PBS, isotype-specific goat anti-mouse horseradish peroxidase conjugates were added (50 µl/well) at appropriate dilutions (αIgM from Sigma; LO-MG1-13 HRPO, LO-MG2a-9 HRPO, LO-MG3-13 HRPO from LO-IMEX). After 1 hour of incubation at room temperature, plates were washed 4 times in PBS, and 100 µl of substrate solution (BD OptEiA) was added to each well. After 10 minutes of incubation at room temperature in the dark, the enzyme reaction was stopped by adding 25 µl/well of 2N H_2SO_4, and absorbance was measured at 450 nm.

Statistical analysis

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

RESULTS

Administration of both killed and live *Brucella melitensis* induces a protective memory state that limits bacteria dissemination in the blood, but only live-infected mice display a complete bacteria elimination in the spleen.

It is well documented that live vaccines induce better protection against *Brucella* infection compared to killed vaccines (19, 30, 31). However, the efficacy of these protocols is rarely compared in the same study and there is no precise or consensual explanation for this fundamental difference. To increase our understanding of this phenomenon, we performed here a detailed analysis of the protective state, including the humoral and cellular immune response induced by the injection of heat-killed (HK) or live fully virulent *B. melitensis* 16M in mice. In addition, as C57BL/6 and BALB/c mice have been reported to display different levels of resistance to *Brucella* infection (46, 52, 53) and are frequently used in *Brucella* vaccination studies (38), we performed our comparison in both strains of mice.

Mice were injected i.p. with PBS (the control, termed here "naive mice group"), $4x10^4$ CFU of live *B. melitensis,* a classical dose to infect the mice (38) (termed here "live-immunized group") or $5x10^7$ CFU of heat killed (HK) *B. melitensis,* a dose used by other investigators (34, 54) (termed here "HK-immunized group"). In order to avoid the impact of persistent chronic infection in mice injected with live bacteria, all groups were treated 21 days post-injection with antibiotics (rifampicin and streptomycin) for 3 weeks and then left resting for at least 3 weeks before challenge with a high ($5x10^7$ CFU) or low (10^5 CFU) dose of live bacteria. See Figure S1.A for a detailed schematic representation of this protocol. As expected, antibiotic treatment completely eliminated *Brucella* in the spleens of wild-type mice after generally 8 days (Figure S1.B), but 3 weeks of treatment was necessary to eliminate

Brucella from the spleens of several deficient mice displaying high susceptibility to infection, such as MyD88$^{-/-}$ and Il-12p35$^{-/-}$ mice (data not shown).

All naive control mice injected with a high ($5x10^7$ CFU) dose of *B. melitensis* (termed here the "primo infected group") displayed clearly detectable counts of bacteria in the blood after 3 hours (Figure 1.A). We used this blood persistence to quantify the ability of the immunized group to control early systemic dissemination of the bacteria. In striking contrast to naive mice, both the HK and live-immunized groups, when challenged with the same dose, displayed a drastic and highly similar reduction of CFU counts in the blood, with elimination of approximately 99.99% of bacteria from the blood. This demonstrated that both groups possess effector mechanisms able to rapidly limit the blood dissemination of *Brucella*. In agreement, these two groups also presented a significant reduction of CFU counts in the spleen at 1 day post-challenge compared to naive control mice (Figure 1.B). Histological analysis of spleen sections from infected mice challenged with a mCherry-expressing strain of *B. melitensis* showed that the bacteria are located in the same zone and cells in both the primo-infected and live-immunized groups of mice (Figure S2). As described in detail by our group in a previous study (49), these cells are mainly red pulp macrophages (F4/80$^+$, Figure S2) and marginal zone macrophages (MOMA-1$^+$, not shown). At 6 days post-challenge, the live-immunized group displayed highly-significant better control of the bacteria count in the spleen compared with the HK-immunized group. Similar results were obtained in BALB/c and C57BL/6 mice (Figures 1.A and 1.B).

In order to investigate in greater detail the ability of the live and HK-immunized groups to develop complete bacterial clearance in the spleen over the long term, we also challenged these mice with a low and more classical dose (10^5 CFU) of *B. melitensis* (Figures 2.A and 2.B). Kinetic analysis of the bacterial load in the spleen showed that mice handle the infection differently according to the immunization protocol used (Figure 2.A). In

approximately 80% of primo-infected C57BL/6 mice, bacteria escape the immune response and persist in the spleen until 50 days post-infection (Figure 2.B). In contrast, only 30% of C57BL/6 mice from the live-immunized group conserved detectable CFU counts in the spleen at 50 days post-infection. Surprisingly, this reduction was not observed in the live-immunized group of BALB/c mice or in the HK-immunized groups of both strains of mice (Figure 2.B).

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

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

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

In order to determine the importance of these circulating Ig during a challenge with live *Brucella*, we compared the ability of live-immunized groups of wild-type, RAG1$^{-/-}$, MuMT$^{-/-}$ (B cell-deficient) and AID$^{-/-}$ (deficient in isotype-switched Abs, B cells produce only

IgM) C57BL/6 mice to control *Brucella*. When challenged with a high dose ($5x10^7$ CFU) of *B. melitensis*, RAG1$^{-/-}$ and MuMT$^{-/-}$ mice appeared unable to control *Brucella* dissemination as demonstrated by much higher blood CFU counts detected at 3 hours and 3 days compared to wild-type and AID$^{-/-}$ live-immunized groups of mice (Figure 3.A). Following a low-dose challenge (10^5 CFU), we also observed that MuMT$^{-/-}$ mice displayed higher CFU counts in the spleen at 50 days (Figure 3.B) and a reduced frequency of these mice displayed a complete bacterial clearance (Figure 3.C) compared to wild-type and AID$^{-/-}$ mice. These results demonstrate that circulating antibodies are the main effectors limiting early dissemination of *Brucella* in the blood of live-immunized groups and suggest that this early control is also critical to the development of a bacterial clearance in the spleen. In addition, the ability of AID$^{-/-}$ mice to control blood dissemination and perform bacteria eradication in spleen strongly suggests that IgM alone can perform this task and IgG production is not strictly necessary.

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

We and others (46, 53, 55-59) have shown that IFN-γ is a key cytokine-regulating protective cellular immune response against primary *Brucella* infection. IFN-γ is produced by Natural Killer (NK) cells, CD4$^+$ T and CD8$^+$ cells (46, 59) and is crucial for the development of inducing Nitric Oxide Synthase (iNOS) positive granulomas that limit *B. melitensis* infection in the spleen and the liver (49). Here, we analyzed by flow cytometry the phenotype of IFN-γ producing cells at the site of infection, i.e. the peritoneal cavity, of HK- and live-immunized groups of C57BL/6 mice challenged with low (10^5 CFU) doses of live *B. melitensis*. IFN-γ-producing cells were analyzed at 12, 24 and 48 hours post-challenge.

After challenge (10^5 CFU), in the absence of *in vitro* stimulation, an elevated frequency of IFN-γ positive cells was detected at 12 hour in the peritoneal cell population from the live-immunized group (Figure 4.A-B) that progressively decrease at 24 and 48 hours (data not shown). In contrast, in the primo-infected or HK-immunized groups, only a very weak IFN-γ signal was detected in the peritoneal cavity (Figure 4.A-C, and data not shown) during the first 48 hours. The specificity of the IFN-γ signal was confirmed using IFN-$\gamma^{-/-}$ C57BL/6 mice (data not shown). The majority of high IFN-γ producers in the peritoneal cavity in the live-immunized group were $CD4^+$ T cells since a mean of 68% of highly IFN-γ-positive cells were found to co-express CD3ε and CD4 markers (Figure 4.C). These cells also expressed higher levels of CD25, CD69 (activation marker) and Ly-6C (memory T cells marker) (Figure 4D). When stimulated overnight *in vitro* with HK *B. melitensis*, only peritoneal cells from the live-immunized group displayed IFN-γ producing $CD4^+$ T cells. This demonstrates that this group contained *Brucella*-specific memory $CD4^+$ T cells in the peritoneal cavity before challenge (Figure S4). As expected, the live-immunized group of BALB/c mice displayed a ten-fold reduction of the frequency of IFN-γ positive peritoneal cells compared to C57BL/6 mice (Figure 5A-B).

We also investigate the production of IFN-γ in the spleen of C57BL/6 mice during the first 120 hours in all groups, but only the live-immunized group displays a very weak frequency of IFN-γ^+ $CD4^+$ T cells (< 200 cells / 10^6 spleen cells) that progressively peak at 48h, indicating a delayed response in this organ (data not shown).

On the whole, these data suggest that only injection of live *B. melitensis* induces high IFN-γ producers $CD4^+$ peripheral memory T cells able to rapidly react *in vivo* to i.p. inoculation of *Brucella*.

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

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

MyD88, IL-12p35 and MHC-II deficiencies do not impair the ability of live-immunized groups to display lower *Brucella* CFU counts in the blood following a high-dose challenge compared to naive infected mice (Figure 6.A). A comparative analysis of the humoral immune response in these deficient mice was performed two days before challenge and showed that all groups displayed high levels of *Brucella*-specific IgM but extremely variable levels of different *Brucella*-specific IgG isotypes (Figure S3.B). In particular, $MHC\text{-}II^{-/-}$ mice presented very low levels of *Brucella*-specific IgG1, IgG2a and IgG3. These results demonstrate that MyD88/IL-12p35 signaling pathways are not implicated in the early control of *Brucella* dissemination. They also suggest that, as previously observed with $AID^{-/-}$ mice (figure 3.A), specific IgM alone could suffice to perform this task.

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

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

DISCUSSION

Brucellae seem perfectly well adapted to their mammalian hosts. They furtively infect mammals, causing only minor inflammation, modify the vesicular environment of phagocytic cells to safely grow intracellularly and disseminate in all tissues (60). However, though *Brucella* infection remains largely silent, brucellosis induces potentially serious complications over the long term (3, 4, 61). As antibiotic-treated patients frequently display bacteria resurgence (11, 62), the development of a safe protective vaccine remains the only realistic strategy to protect exposed populations. Empirical research has failed to develop a safe protective vaccine for humans (13, 63) and, despite a plethora of publications on the murine model of brucellosis, our understanding of the secondary immune response against *Brucella* is currently very poor. Immune markers used to determine the efficacy of vaccination are commonly based on the primary immune response against *Brucella*. However, it has been often observed in several other infectious models (23-29) that the primary and secondary responses do not necessarily use same classes of effector mechanisms. In a recent study (59) using a large panel of genetically-deficient mice, we attempted to clearly identify the effector cells and signaling pathways implicated in the primary immune response against *B. melitensis* infection. We showed that IFN-γ-producing $CD4^+$ Th1 cells play a crucial role in the control of bacteria, but that a deficiency in $CD8^+$ T cell, B cell, Th2 and Th17 responses does not qualitatively affect the course of the infection. We also demonstrated that Th1 induction requires functional TLR9/MyD88/IL-12p35 signaling pathways (46, 49, 59). In the present study, we have developed an original model to characterize the effector mechanisms involved in the control of a secondary infection by *B. melitensis*. Mice were injected with HK or live virulent *Brucella melitensis* 16M and were treated with antibiotics after 21 days. After a resting phase, the mice were challenged with the same living bacteria. Protection was

analyzed at two distinct levels. Early protection was measured by the ability of the immune response to reduce dissemination of the bacteria by the blood stream. The late immune protection was scored by the frequency of animals that were not able to completely eradicate bacteria from their spleen at 50 days post-challenge. The spleen was chosen as the control organ because *Brucella* has been shown to persist for long periods of time (up to 100 days) in this organ (64). In our model, the absence of bacteria in the spleen has been always correlated with complete elimination of bacteria in the liver (data not shown). However, a reservoir in other tissues cannot be formally excluded.

Control of intracellular pathogens such as bacteria and protozoa usually requires $CD4^+$ T cell-, IFN-γ- and/or tumor necrosis factor (TNF)-dependent activation of macrophages. This leads to an upregulation of antimicrobial effector mechanisms, including the acidification of phagolysosomes and the expression of inducible nitric oxide synthase (iNOS, NOS2 (65)). Although antibodies are frequently regarded as irrelevant for the control of intracellular bacteria and protozoa, more recent studies demonstrate that they may contribute both to development of the disease as well as to its control (66). Antibody-mediated aggravation of infections with intracellular pathogens might be due to Fc-receptor-mediated facilitation of entry of the pathogen into the host cell or to macrophage deactivation conveyed by inhibitory Fc receptors (67-69). Conversely, antibody-dependent control of intracellular microbes may result from antibody binding to the pathogen during intermittent extracellular phases, leading to opsonization and classical complement activation (70).

In our model, we observed that humoral immunity is necessary for full protection upon secondary infection (see Table 1). Circulating specific antibodies are crucial to control the early dissemination of *Brucella* by the blood stream following challenge by intraperitoneal injection. They are also critical for the development of sterilizing immunity in the spleen at 50 days post-challenge. Thus, though B cells appear to be dispensable (59) or even detrimental

(71, 72) during primary infection, they play an important positive role in the control of secondary infection. Surprisingly, our results demonstrate that $CD4^+$ T cells, MyD88/IL-12p35 signaling pathways and even the AID-mediated class switch are dispensable to obtain protective circulating antibodies. No other isotype seems to play a crucial role as deficiency in $CD4^+$ T cells, MyD88 and IL-12p35 affects various IgG isotypes, but does not reduce the early control of infection. Interestingly, *Brucella*-specific IgM are maintained in the absence of chronic infection, as antibiotic-treated mice remained protected for 3 months against a challenge infection (data not shown). IgM-mediated immunity is usually considered to be short-lived and only effective during the early stages of infection. Our findings indicate that IgM may be of greater utility during chronic bacterial infections than previously thought. Other researchers have also provided evidence for long-term IgM responses, although such reports are relatively rare (73). Similar results have been reported in experimental models of infection by intracellular bacteria such as *Borrelia hermsii* (74) and *Ehrlichia muris* (75). As T cell-independent activation of B cells is generally dependent on pattern recognition receptors (PRRs) (76), we can hypothesize that *Brucella* pathogen-associated molecular patterns (PAMPs) are implicated in the activation of *Brucella*-specific B cells and that PRRs recognizing these PAMPs may act by a MyD88-independent signaling pathway. Our observations that long-lived protective IgM responses can be generated *in vivo* by *Brucella* infection suggest that it may be feasible to target IgM production as part of vaccination strategies.

Early protective immunity mediated by *Brucella*-specific circulating antibodies developed following inoculation of both HK and live bacteria. In striking contrast, development of late sterilizing immunity in the spleen required previous injection of live bacteria. This ultimate protection level is closely correlated with the presence of both circulating *Brucella*-specific antibodies and peritoneal Th1 $CD4^+$ T cells able to quickly

produce high IFN-γ counts after *Brucella* challenge (see Table 1). The absence of antibodies (MuMT$^{-/-}$ mice) or CD4+ T cells (MHC-II$^{-/-}$ mice) leads to persistence of the bacteria in the spleen, demonstrating that both effector mechanisms must act together to eradicate *Brucella* from peripheral tissues. HK *Brucella* administration fails to induce peritoneal CD4^{+} T cells able to produce high IFN-γ counts after *Brucella* challenge, suggesting that the dynamics of intracellular infection are critical to induce this effector mechanism. Analysis of genetically-deficient mice showed that the development of IFN-γ producing CD4^{+} T cells is strictly dependent on MyD88/IL-12p35 signaling pathways. This result is not expected or predictable on the basis of previous studies. IFN-γ and IRF1 deficient mice, but not RAG, IL-12 or MyD88 deficient mice, succumb to primary infection by *Brucella* (46, 55, 58, 59, 77, 78), suggesting that IFN-γ can be induced at low level by MyD88/IL-12-independent pathways. Our results confirm the importance to use IL-12 inducing adjuvant in *Brucella* vaccination. Failure of HK *Brucella* immunization to induce IFN-γ producing CD4^{+} T cells could explain the absence of IgG2a in the serum of the HK-immunized group, as the development of this isotype is well known to be dependent on IFN-γ (79).

Several past (21) and more recent studies (18, 80, 81) have proposed that CD4^{+} and CD8^{+} T cells can both play important role in the control of *Brucella* infection whereas other studies favors the implication of CD8^{+} (82-84) or CD4^{+} (85, 86) T cells. Interestingly, we observed that IFN-γ-producing CD4^{+} T cells are not replaced by IFN-γ-producing CD8^{+} T cells in the absence of MHC-II-dependent antigen-presenting pathways, as was previously observed during the *Brucella* primary response (59). This demonstrates that primary *Brucella* infection induces low-quality responding CD8^{+} T cells unable to participate in the secondary immune response. Several recent reports suggest that the failure of the immune system to maintain a CD8^{+} T cell response during chronic brucellosis results from bacterial evasion dependent on the virulence factor, TcpB (64), and show *in vitro* that *Brucella* induces

intracellular retention of the MHC-I down-modulating cytotoxic $CD8^+$ T cell response (87). The identification of $CD4^+$ T cells as key lymphocyte subsets is critical to determine which antigen presenting pathways (MHC-I or MHC-II) must be targeted by vaccination protocol.

Several reports in the *Mycobacterium tuberculosis* model suggest that the ability of memory Th1 $CD4^+$ T cells to fight intracellular bacteria could be dissociated from IFN-γ production (88-91). As IFN-$\gamma^{-/-}$ mice succumb rapidly to *Brucella* infection (55, 92), we have not been able to test this hypothesis in our *Brucella* model. However, we have shown previously that IFN-γ-producing $CD8^+$ T cells fail to protect mice during primary *Brucella* infection (59), suggesting that IFN-γ production is not the only property of $CD4^+$ T cells implicated in the control of *Brucella*. The nature of any such additional factors in our model has not yet been determined. Recent studies (23, 93) on the *L. monocytogenes* model suggest that the ability of T cells to regulate the local recruitment of innate effector cells can be crucial to the protective secondary response. Comparison of chemokine production by $CD4^+$ and $CD8^+$ T cells during brucellosis could provide interesting new areas of investigation.

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

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

frequency of IFN-γ-producing cells in the live-immunized group of BALB/C mice compared to C57BL/6 mice after challenge in the peritoneal cavity. These results are important in vaccination as results and conclusions could be affected by the choice of one mouse strain.

It has been hypothesized (52) that IL-4 production in BALB/c mice reduces IFN-γ production and adversely affects the protective immune response to *Brucella*. We have demonstrated previously that IL-4 receptor deficiency (59) or neutralization of IL-4/IL-13 receptor signaling pathways with STAT-6 deficiency (data not shown) does not improve the ability of C57BL/6 and BALB/c mice to control primary *Brucella* infection. In this study, we observed that STAT-6 deficiency in the live-immunized group of BALB/c mice significantly increases the rate of *Brucella* elimination after secondary infection. Indeed, STAT-$6^{-/-}$ BALB/c mice display a level of control similar to wild-type C57BL/6 mice. This surprising result suggests that sterilizing immunity in the spleen is affected by IL-4/IL-13 in BALB/c mice. As IFN-γ-producing cell frequency in the peritoneal cavity after challenge of STAT-$6^{-/-}$ BALB/c does not seem to be higher, we hypothesize that IL-4 and/or IL-13 could act on other unidentified crucial effector mechanisms. This interesting phenomenon suggests that neutralization of IL-4 could improve the efficacy of *Brucella* vaccination and requires further study.

Though previous studies on *Brucella* vaccination have reported the importance of the induction of specific antibodies (21, 36, 37) and $CD4^+$ T cells (19, 21, 37) in protection, our study is the first, to our knowledge, to (i) formally demonstrate by using genetically-deficient mice and without manipulation such as transfer experiments the complementary role played by both humoral immunity and Th1 $CD4^+$ T cells in the clearance of *Brucella* during secondary infection and (ii) identify the signaling pathways implicated in the development of these effector mechanisms. These results could improve our ability to develop protective vaccines or therapeutic treatments against brucellosis. Our observations suggest that the

development of protective vaccines requires the selection of a vaccination protocol favoring humoral immunity, antigen presentation to $CD4^+$ T cells, IL-12 production and absence of IL-4.

The great majority of vaccination studies analyzed the isotype induced by their vaccine candidate and discussed the interest of IFN-γ/IL-12 dependent isotype in the control of *Brucella* infection. It is usually assumed that the induction by $CD4^+$ T cells of the production of IgG2 antibodies from B cells is critical to control the course of murine and ovine *B. melitensis* infection (85, 94). In contrast, our results strongly suggest that the nature of isotype is not a critical parameter in vaccination.

As functional Th1 $CD4^+$ T cells only developed following the administration of live bacteria in our model, live vaccines seem to remain the easiest and most potent tools for the production of candidate protective vaccines. However, live-attenuated strains retain generally unacceptable levels of virulence for human vaccination. Gamma-irradiated *Brucella* do not divide but conserve metabolic activity and protect mice against virulent bacterial challenge without signs of residual virulence (95). Thus, inactivated, yet metabolically active, microbes could represent a promising strategy for safe vaccination against *B. melitensis*.

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FIGURE LEGENDS

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

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

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

Figure 4. Comparison of IFN-γ^+ cell frequency after challenge in C57BL/6 mice immunized previously with live or HK *B. melitensis*. C57BL/6 mice were immunized i.p. either with live ($4x10^4$ CFU, Live-immunized group) or HK bacteria (10^8 CFU, HK-immunized group). All mice were treated with antibiotics as described in the Materials and Methods and then were challenged with either a low dose (10^5 CFU) of live bacteria, as indicated, and sacrificed at the selected time. Peritoneal cells were collected and analyzed by flow cytometry. **A,** Cells were analyzed for Forward Size Scatter (FSC) versus IFN-γ production. The figure shows representative dot plots from individual peritoneal cavities in each group. Numbers under the line of plots indicate the number of cells in gate R1 (blue) or gate R2 (red) out of 10^5 peritoneal cavity cells acquired. **B,** The graph represents the number of IFN-γ positive cells per 10^5 peritoneal cells acquired in gate R1. Each data point represents the value obtained from an individual peritoneal cavity and the data are representative of two

independent experiments. Grey bars represent the median. Significant differences are denoted by an asterisk (*). **, *** denote $p<0.01$, $p<0.001$, respectively. **C,** Total (gate R1) and highly (gate R2) IFN-γ-positive cells in Live-immun group were analyzed for CD3, CD4 and CD8α expression. Numbers next to the circles indicate the percentage of $CD3^+CD4^+IFN\text{-}\gamma^+$ T cells in gate R1 and R2. **D,** $CD4^+$ (gate R3) or $CD4^+IFN\text{-}\gamma^+$ peritoneal T cells (gate R4) were selected and analyzed for the expression of a panel of activation markers: CD90, CD25, CD69 and LY6C. The data are represented for each group by the total Mean Fluorescence Intensity (mfi). The percentage of positive gated events is also shown for each marker.

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

Figure 6. Comparison of protection in wild-type and deficient C57BL/6 mice immunized previously with live *B. melitensis*. Wild-type (WT), $MyD88^{-/-}$, $IL\text{-}12p35^{-/-}$ and $MHCII^{-/-}$ C57BL/6 mice were immunized i.p. with live *B. melitensis* ($4x10^4$ CFU) and were treated

with antibiotics as described in the Materials and Methods. **A**, Mice were challenged with a high dose of *B. melitensis* ($5x10^7$ CFU) and bled at the selected time. The data represent the CFU per ml of blood. **B, C**, Mice were challenged with a low dose of *B. melitensis* (10^5 CFU) and sacrificed at 50 days post challenge. **B**, The data represent the CFU per gram of spleen and are representative of two independent experiments. Grey bars represent the median. The percentage of mice that are still positive for *Brucella* in the spleen at 50 days post challenge is represented in (**C**). These results are pooled from at least two independent experiments. Significant differences are denoted by an asterisk (*). *, **, *** denote $p<0.05$, $p<0.01$, $p<0.001$, respectively. "Pri" means primo group.

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

Figure 8. Comparison of protection and IFN-γ^+ cell frequency after challenge in wild-type or STAT6$^{-/-}$ BALB/c mice immunized previously with live *B. melitensis*. Wild-type

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

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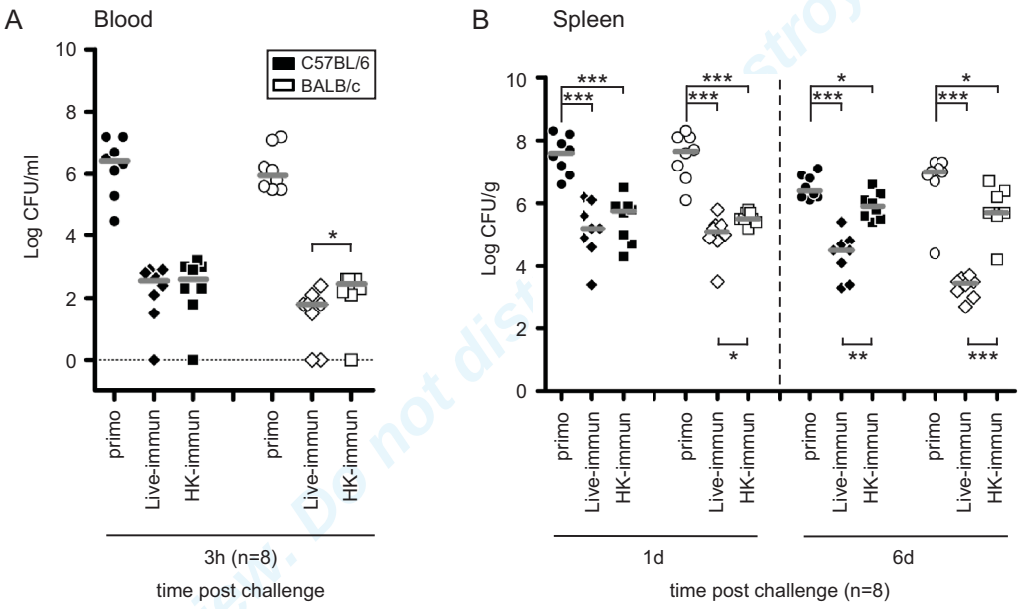

Figure 1

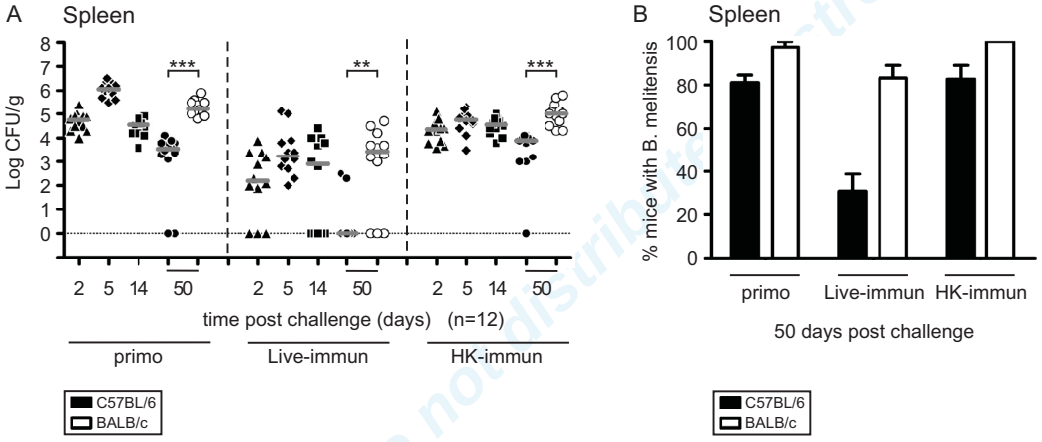

Figure 2

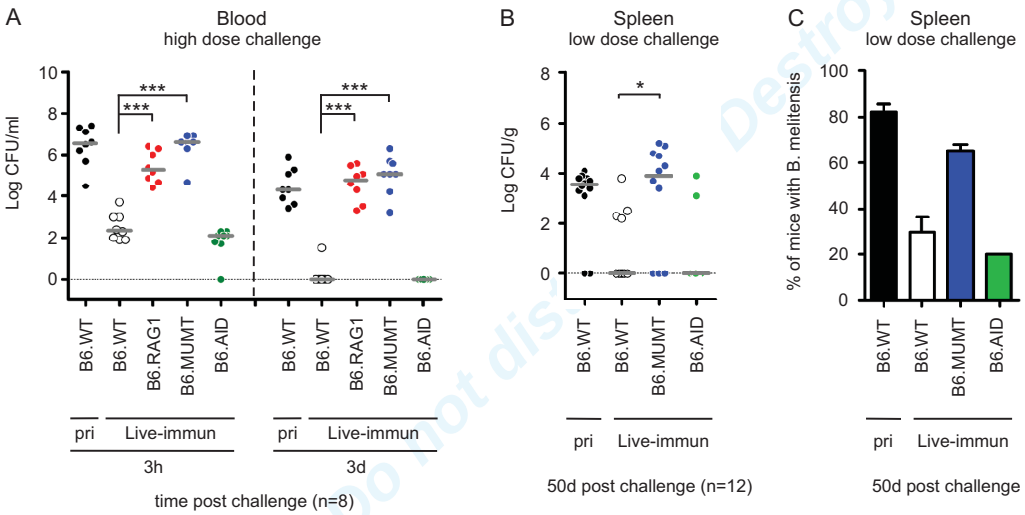

Figure 3

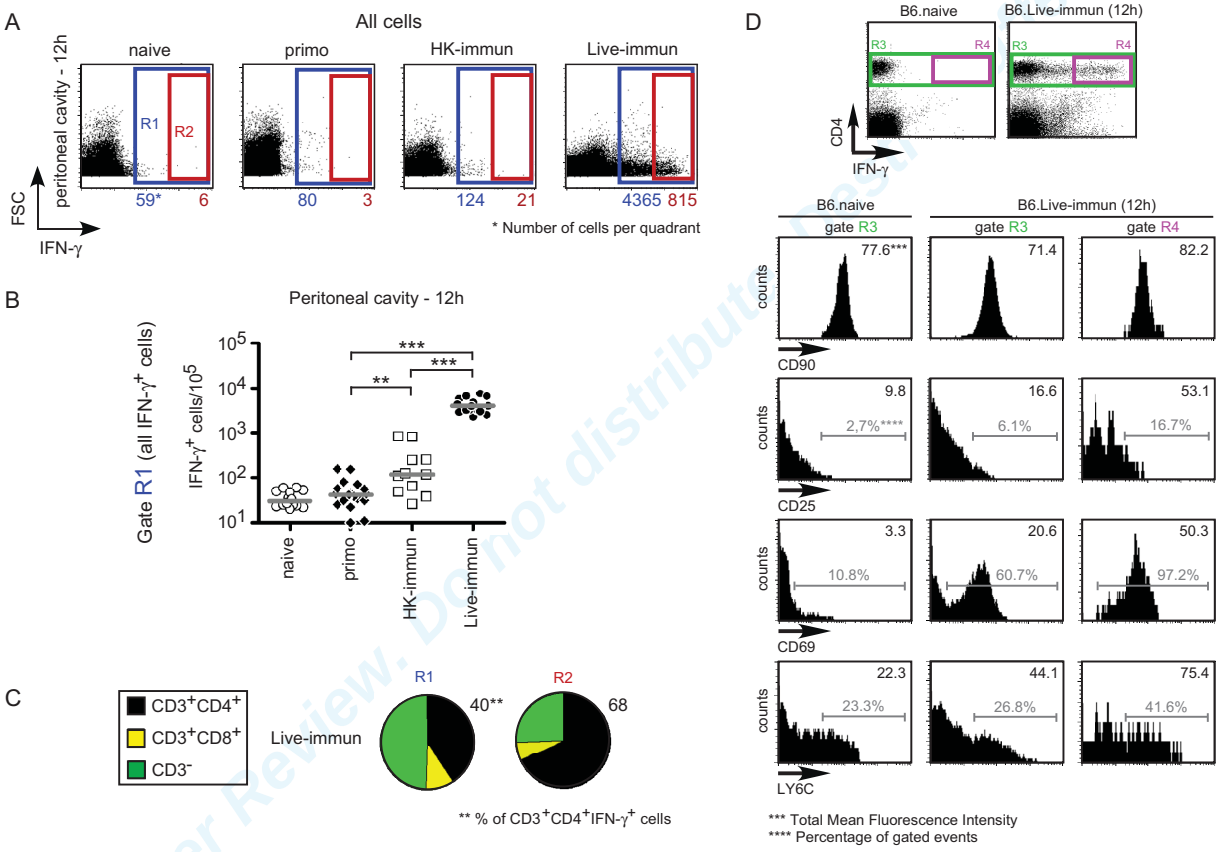

Figure 4

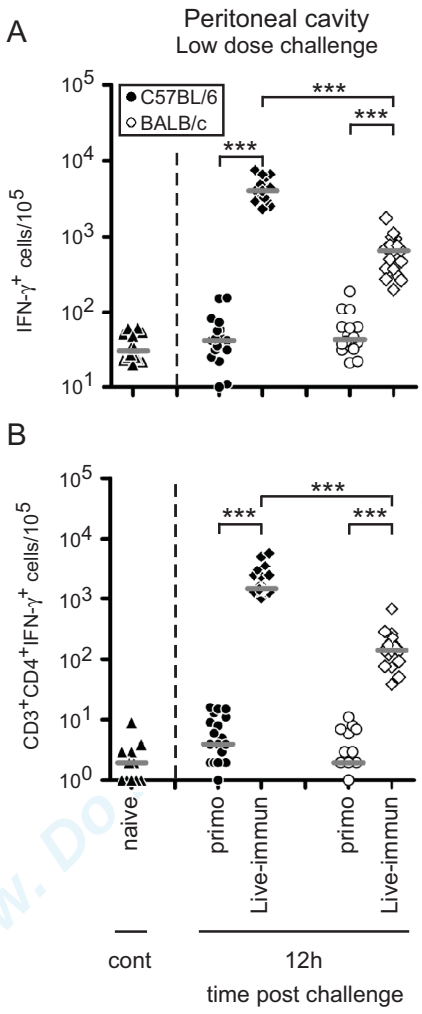

Figure 5

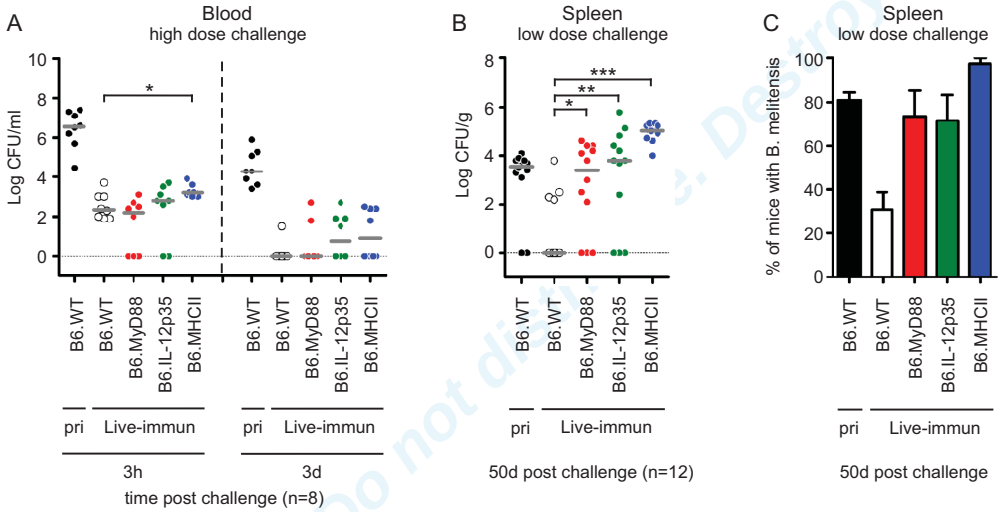

Figure 6

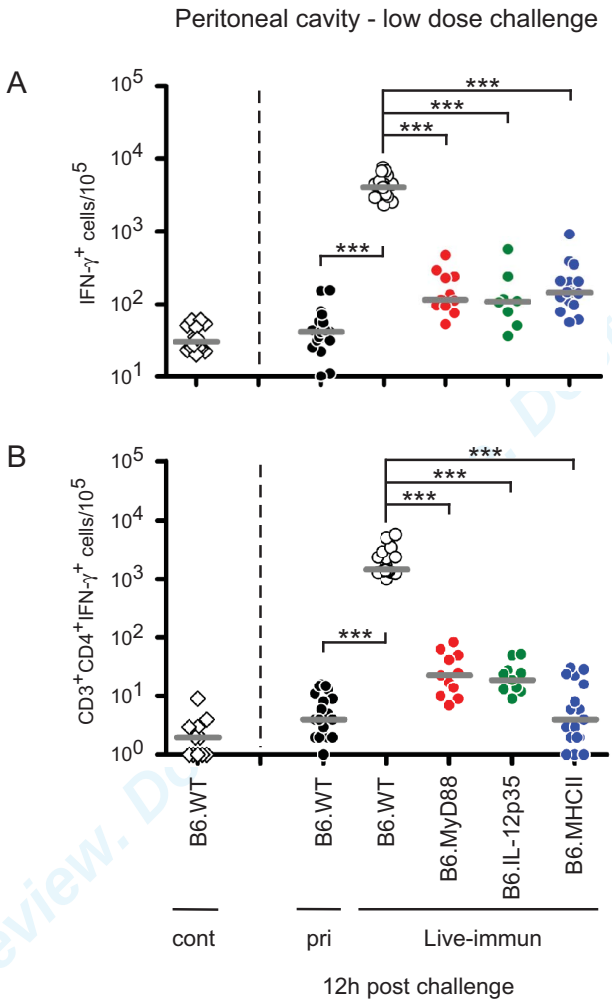

Figure 7

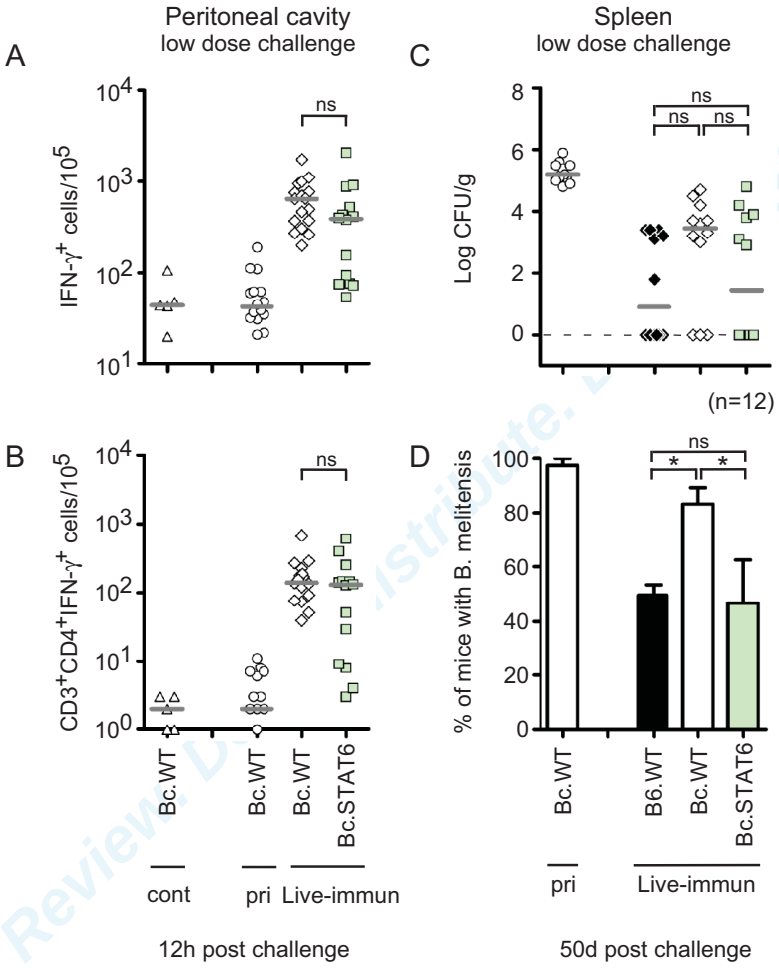

Figure 8

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

C57BL/6 mice	**WT primo**	**WT HK-immun**	**WT Live-immun**	**MuMT$^{-/-}$ Live-immun**	**AID$^{-/-}$ Live-immun**	**MyD88$^{-/-}$ Live-immun**	**IL-12p35$^{-/-}$ Live-immun**	**MHCII$^{-/-}$ Live-immun**
Circulating IgM	-	+++	+++	-	+++	++	++++	++
Circulating IgG	-	++	+++	-	-	++	+++	-
Control of bacteria dissemination in the blood	-	+++	+++	-	+++	+++	+++	++
IFN-γ^+ cells (peritoneal cavity) *	-	+	+++	+++	+++	+	+	+
IFN-γ^+CD4$^+$ T cells (peritoneal cavity) *	-	+	+++	+++	+++	+	+	-
% of mice displaying a complete bacteria elimination in the spleen **	**19**	**17**	**69**	**33**	**80**	**26**	**28**	**2**

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