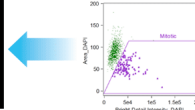
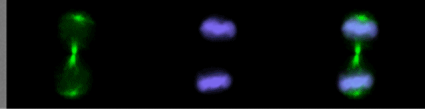
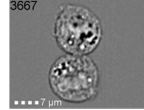




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Humoral Immunity and CD4⁺ Th1 Cells Are Both Necessary for a Fully Protective Immune Response upon Secondary Infection with *Brucella melitensis*

Marie-Alice Vitry,* Delphine Hanot Mambres,* Carl De Trez,[†] Shizuo Akira,[‡] Bernhard Ryffel,^{§,¶} Jean-Jacques Letesson,^{*1} and Eric Muraille^{||,1}

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*. In this study, we characterize the cells and signaling pathways implicated in the generation of a protective immune memory response following priming by the injection of heat-killed or live *Brucella melitensis* 16M. Using a panel of gene-deficient mice, we demonstrated that during a secondary recall response, both the *Brucella*-specific humoral response and CD4⁺ Th1 cells must act together to confer protective immunity in the spleen to *B. melitensis* infection. Humoral protective immunity is induced by the inoculation of both heat-killed 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. *The Journal of Immunology*, 2014, 192: 3740–3752.

Brucella (α -proteobacteria) are small, nonmotile, 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 >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). Because *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, and *B. abortus* RB51) (12, 13) that cause disease in humans. Little real progress in the field of *Brucella* vaccination has been recently reported. One clear cause seems to be the empirical nature of research on the *Brucella* vaccine. Indeed, the vast majority of publications reports only the protective ability of candidate vaccines, limiting their analysis to present CFU counts in the spleen after challenge (14,

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The online version of this article contains supplemental material.

Abbreviations used in this article: AID, activation-induced deaminase; HK, heat-killed; MHC-II, MHC class II; MuMT^{-/-}, B cell-deficient; PFA, paraformaldehyde; RT, room temperature.

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15). Evaluations of the ability of vaccines to induce IFN- γ -producing cells, detected *in vitro* after restimulation, 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* Ags 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), whereas others have demonstrated that HK *Brucella* failed to induce the desirable Th1-protective response (19, 31). Transfer experiments suggest that Abs, 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 Abs and cells. The fact that both cell-mediated immunity and Abs 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 *i.p.* injection of 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 Abs 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 University of Namur (permit number 05-558).

Mice and reagents

MyD88^{-/-} C57BL/6 mice (39) were obtained from Dr. S. Akira (Osaka University, Osaka, Japan), IL-12p35^{-/-} C57BL/6 mice (40) from Dr. B. Ryffel (University of Orleans), AID^{-/-} C57BL/6 mice (41) from Dr. H. Jacobs (The

Netherlands Cancer Institute), MHC class II (MHC-II)^{-/-} C57BL/6 mice (42) from Jörg Reimann (University of Ulm, Ulm, Germany), and RAG1^{-/-} C57BL/6 mice (43) from Dr. S. Goriely (Université Libre de Bruxelles). STAT-6^{-/-} BALB/c mice (44) and B cell-deficient (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 Laboratories (Bicester, U.K.) and 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.

B. melitensis strain 16M (Biotype1; ATCC 23456; American Type Culture Collection) was initially isolated from an infected goat and grown in biosafety level 3 laboratory facilities. Overnight cultures were grown with shaking at 37°C in 2YT media (Luria-Bertani broth with double quantity of yeast extract) and then washed twice in PBS (3500 × g, 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 HK *B. melitensis*, bacteria from an overnight liquid culture in 2YT media were washed twice in PBS (3500 × g, 10 min) before heating at 80°C for 1 h. To confirm the killing, an aliquot was plated onto 2YT medium.

Mice immunization and challenge

Mice were injected *i.p.* with 4 × 10⁴ CFUs live or 10⁸ CFUs HK *B. melitensis* in 500 μ l PBS. Control animals were injected with the same volume of PBS. Infectious doses were validated by plating serial dilutions of inoculums. Three weeks after immunization, mice were given antibiotics for 3 wk to clear the infection. After resting for an additional 3 wk, they were challenged *i.p.* with either a low dose (10⁵ CFUs) or high dose of *B. melitensis* (5 × 10⁷ CFUs). 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 wk. The oral treatment was a combination of rifampicin (12 mg/kg) and streptomycin (450 mg/kg) (adapted from Ref. 50) prepared fresh daily and given in the drinking water. An additional *i.p.* treatment was given and consisted of five injections of streptomycin (300 mg/kg) throughout the 3 wk 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 wk prior to the challenge, and the CFU counts were evaluated in the spleen.

Bacterial count

Spleens 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 plated them onto 2YT medium. The CFUs were counted after 4 d of culture at 37°C. For bacterial counts in the blood, 70 μ l blood was collected from the tail with heparinated capillaries at selected time points and diluted in PBS/0.1% Triton X-100 (Sigma-Aldrich). Serial dilutions in PBS were performed and plated onto 2YT medium. The CFUs were counted after 4 d of culture at 37°C.

Cytofluorometric analysis

As previously described (46), spleens were harvested, cut in very small pieces, and incubated with a mixture of DNase I fraction IX (Sigma-Aldrich Chimie SARL, Lyon, France) (100 μ g/ml) and 1.6 mg/ml 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; American Type Culture Collection) in 200 μ l PBS/0.2% BSA/0.02% Na₃N (FACS buffer) for 20 min on ice to prevent Ab binding to FcR. A total of 3–5 × 10⁶ cells was stained on ice with various fluorescent mAb combinations in FACS buffer and further collected on an FACSCalibur cytofluorometer (BD Biosciences). We purchased the following mAbs from BD Biosciences: FITC-coupled 145-2C11 (anti-CD3 ϵ), PE-coupled RM4-5 (anti-CD4), PE-coupled 53-6.7 (anti-CD8 α), FITC-coupled 53-2.1 (anti-CD90), FITC-coupled 7D4 (anti-CD25), FITC-coupled H1.2F3 (anti-CD69), biotin-coupled AL-21 (anti-LY6C), and FITC-coupled avidin. The cells were analyzed on an FACSCalibur cytofluorometer (BD Biosciences). 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 (Life Technologies Laboratories), 10% FCS with 1 μ M GolgiStop (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% paraformaldehyde (PFA) for 15–20 min on ice. These cells were then permeabilized for 30 min using a saponin-based buffer (10 \times Perm/Wash in FACS buffer; BD Pharmingen) and stained with allophycocyanin-coupled XMG1.2 (anti-IFN- γ ; BD Biosciences). After final fixation in PBS/1% PFA, cells were analyzed on an FACSCalibur cytofluorometer (BD Biosciences). No signal was detectable with control isotypes.

Immunofluorescence microscopy

Spleens were fixed for 6 h at 4°C in 2% PFA (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 (5 μ m) were prepared. Tissue sections were rehydrated in PBS and then incubated successively in a PBS solution containing 1% blocking reagent (PBS-BR 1%; Boehringer Ingelheim) 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), and 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, Jena, Germany) equipped with a high-resolution monochrome camera (Axio-Cam HR; Zeiss). Images (1384 \times 1036 pixels, 0.16 μ m/pixel) were acquired sequentially for each fluorochrome with A-Plan 10 \times /0.25 numerical aperture and LD-Plan-NeoFluar 63 \times /0.75 numerical aperture dry objectives and recorded as eight-bit gray-level .zvi files. At least three slides per organ were analyzed from three different animals, and the results are representative of two independent experiments.

In vitro stimulation of peritoneal cells

Mice were injected i.p. with 4 \times 10⁴ CFUs live or 10⁸ CFUs HK *B. melitensis* in 500 μ l 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 d later by washing the peritoneal cavity with 10 ml cold RPMI 1640. Cells were centrifuged and then cultured in RPMI 1640 supplemented with 10% FCS, 1% L-glutamine, 1% nonessential amino acids, 1% pyruvate sodium, and 0.1% gentamicin in six-well plates with 10⁷ cells/well in a volume of 2 ml. For stimulation, a concentration of 2 \times 10⁷ bacteria/ml HK *B. melitensis* was used. Cells were then incubated for 7 h at 37°C, 5% CO₂. After adding 1 μ M GolgiStop (BD Pharmingen), the incubation was continued for an additional 13 h 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 ELISA. Polystyrene plates (269620; Nunc) were coated with HK

B. melitensis (10⁷ CFUs/ml). After incubation overnight at 4°C, plates were blocked for 2 h at room temperature (RT) with 200 μ l PBS-3.65% casein. Then plates were incubated for 1 h at RT with 50 μ l serial dilutions of the serum in PBS-3.5% casein. The sera from unimmunized mice were used as the negative control. After four washes with PBS, isotype-specific goat anti-mouse HRP conjugates were added (50 μ l/well) at appropriate dilutions (anti-IgM from Sigma-Aldrich; LO-MG1-13 HRPO, LO-MG2a-9 HRPO, and LO-MG3-13 HRPO from LOIMEX). After 1 h of incubation at RT, plates were washed four times in PBS, and 100 μ l substrate solution (BD OptEiA; BD Biosciences) was added to each well. After 10 min of incubation at RT in the dark, the enzyme reaction was stopped by adding 25 μ l/well 2 N H₂SO₄, and absorbance was measured at 450 nm.

Statistical analysis

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

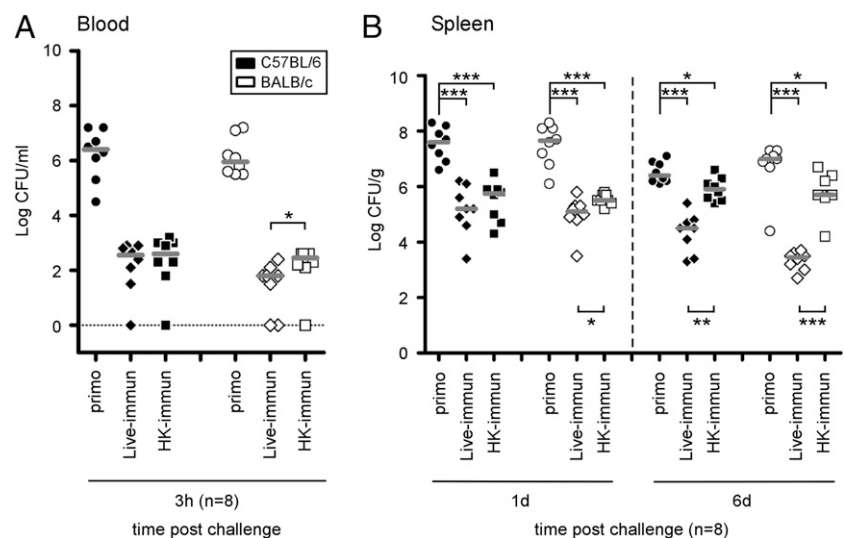
Results

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

It is well documented that live vaccines induce better protection against *Brucella* infection compared with 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 in this study a detailed analysis of the protective state, including the humoral and cellular immune response induced by the injection of 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, referred to in this study as the naive group), 4 \times 10⁴ CFUs of live *B. melitensis*, a classical dose to infect the mice (38) (referred to in this study as the live-immunized group) or 5 \times 10⁷ CFUs of HK *B. melitensis*, a dose used by other investigators (34, 54) (referred to in this study as the HK-immunized group). To avoid the impact of persistent chronic infection in mice injected with live bacteria, all groups

FIGURE 1. Comparison of protective immunity induced by a high-dose challenge in C57BL/6 or BALB/c mice immunized previously with live or HK *B. melitensis*. C57BL/6 and BALB/c wild-type (WT) mice were immunized i.p. either with live (Live-immun group; 4 \times 10⁴ CFUs) or HK bacteria (HK-immun group; 10⁸ CFUs), as indicated. All mice were treated with antibiotics as described in the *Materials and Methods*, then challenged with a high dose of live bacteria (5 \times 10⁷ CFUs), and bled or sacrificed for spleen harvesting at the selected time. The data represent the CFUs per milliliter of blood (**A**) or CFUs per gram of spleen (**B**). Gray bars represent the median. These results are representative of two independent experiments. Significant differences are denoted by asterisks. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

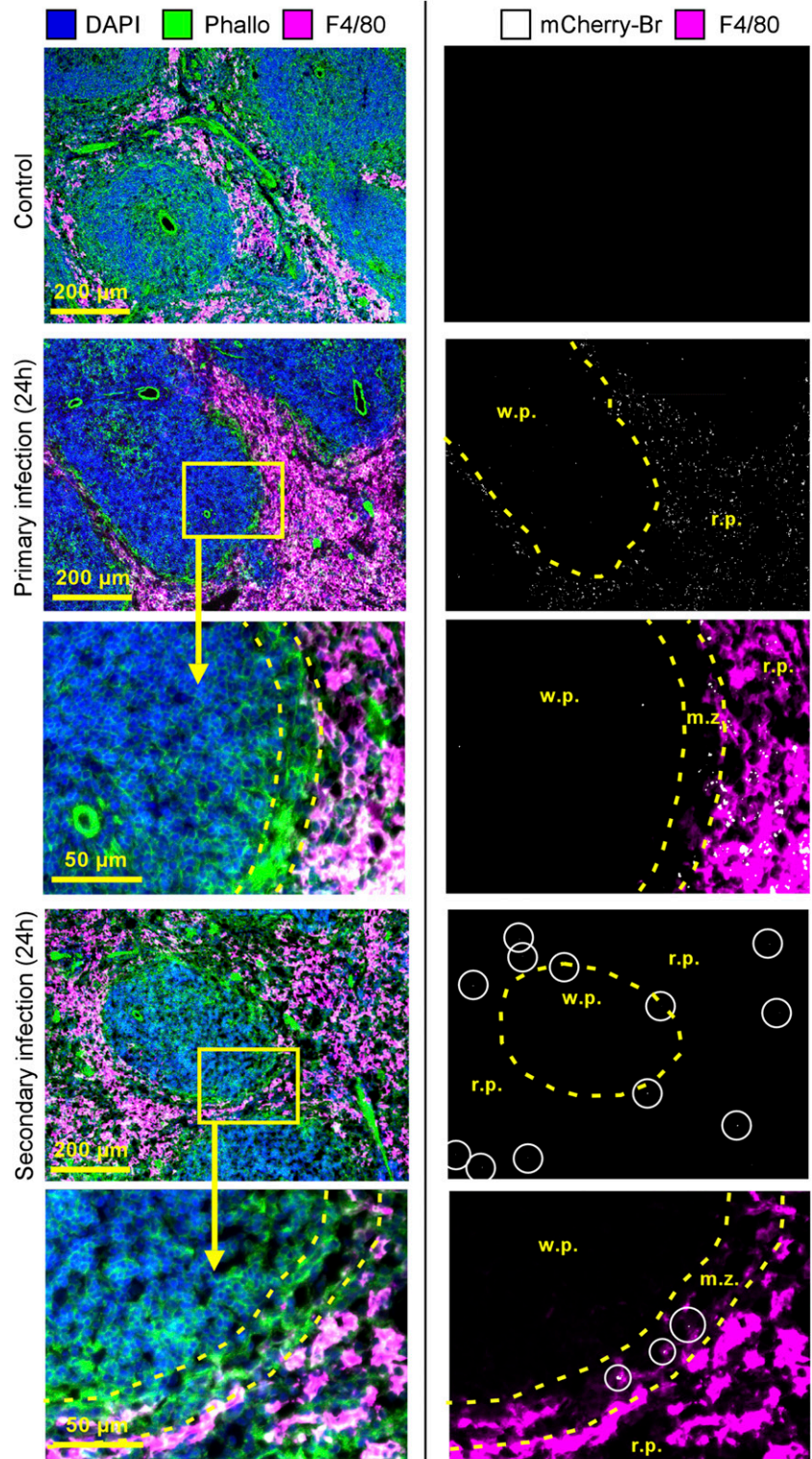


were treated 21 d postinjection with antibiotics (rifampicin and streptomycin) for 3 wk and then left resting for at least 3 wk before challenge with a high (5×10^7 CFUs) or low (10^5 CFUs) dose of live bacteria. See Supplemental Fig. 1A for a detailed schematic representation of this protocol. As expected, antibiotic treatment completely eliminated *Brucella* in the spleens of wild-type mice after generally 8 d (Supplemental Fig. 1B), but 3 wk of treatment was necessary to eliminate *Brucella* from the spleens of several gene-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 (5×10^7 CFUs) dose of *B. melitensis* (referred to in this study as the primo-infected group) displayed clearly detectable counts of bacteria in the blood after 3 h (Fig. 1A). 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

FIGURE 2. Phenotype of infected spleen cells following secondary infection. Wild-type C57BL/6 mice were immunized i.p. with live *B. melitensis* (4×10^4 CFUs). Mice were treated with antibiotics as described in the *Materials and Methods*, then challenged with a high dose of bacteria (5×10^7 CFUs), and sacrificed at the selected time. Naive mice were infected with the same dose to compare the cell phenotypes between secondary and primary infection. The spleens were harvested and fixed. Frozen sections were examined by immunohistochemistry for bacteria (mCherry signal) and F4/80-expressing cells at 24 h postinfection. The insets in the *second* and *fourth* panels are shown in the panels directly following each at high magnification. The panels are color-coded by Ag as indicated. The data are representative of two independent experiments. Scale bars, 200 or 50 μ m. m.z., marginal zone; r.p., red pulp; w.p., white pulp.



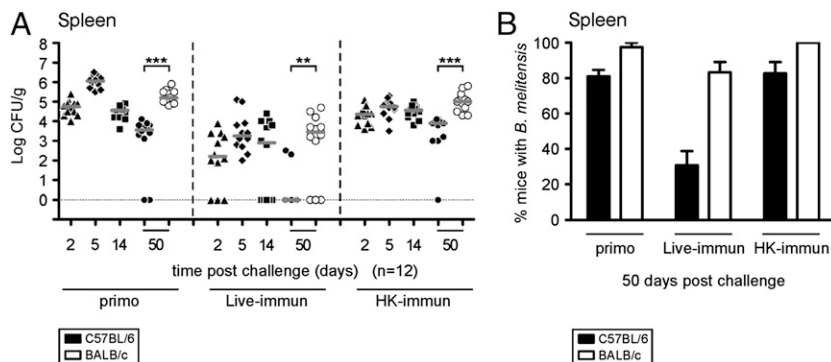


FIGURE 3. Comparison of protective immunity induced by a low-dose challenge in C57BL/6 or BALB/c mice immunized previously with live or HK *B. melitensis*. C57BL/6 and BALB/c wild-type mice were immunized i.p. either with live (Live-immun group; 4×10^4 CFUs) or HK bacteria (HK-immun; 10^8 CFUs). All mice were treated with antibiotics as described in the *Materials and Methods*, then challenged with a low dose of bacteria (10^5 CFUs), and sacrificed at the selected time. **(A)** The data represent the CFUs per gram of spleen from one representative experiment. Gray bars represent the median. The mean \pm SEM of the percentage of mice that are still positive for *Brucella* in the spleen 50 d postchallenge is represented in **(B)**. These data are pooled from at least two independent experiments. Significant differences are denoted by asterisks. $**p < 0.01$, $***p < 0.001$.

same dose, displayed a drastic and highly similar reduction of CFU counts in the blood, with elimination of $\sim 99.99\%$ 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 d postchallenge compared with naive control mice (Fig. 1B). Histological analysis of spleen sections from infected mice challenged with an 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 (Fig. 2). As described in detail by our group in a previous study (49), these cells are mainly red pulp macrophages (F4/80⁺, Fig. 2) and marginal zone macrophages (metalophilic macrophage Ab-1⁺, not shown). At 6 d postchallenge, 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 (Fig. 1).

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 CFUs) of *B. melitensis* (Fig. 3). Kinetic analysis of the bacterial load in the spleen showed that mice handle the infection differently according to the immunization protocol used (Fig. 3A). In $\sim 80\%$ of primo-infected C57BL/6 mice, bacteria escape the immune response and persist in the spleen until 50 d postinfection (Fig. 3B). In contrast, only 30% of C57BL/6 mice from the live-immunized group conserved detectable CFU counts in the spleen at 50 d postinfection. 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 (Fig. 3B).

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. To identify immune parameters associated with resistance to infection, we

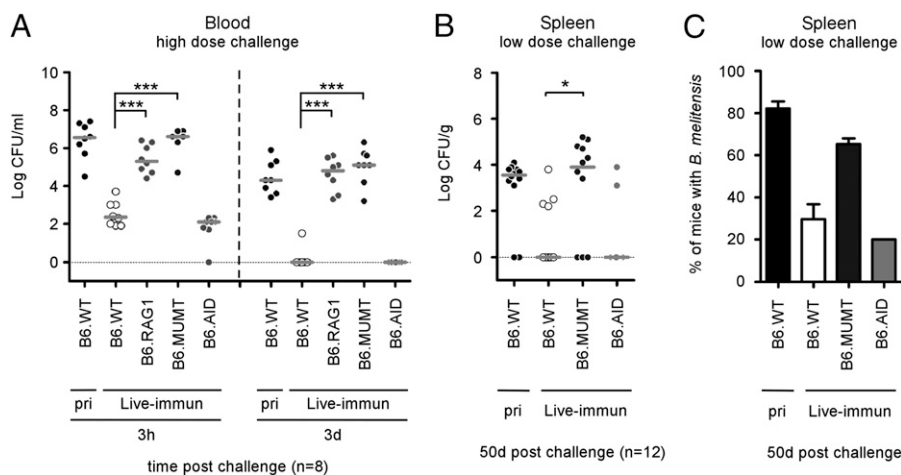


FIGURE 4. Comparison of protection among wild-type (WT), RAG1^{-/-}, MuMT^{-/-} (MUMT), and AID^{-/-} C57BL/6 mice immunized previously with live *B. melitensis*. WT, RAG1^{-/-}, MuMT^{-/-}, and AID^{-/-} C57BL/6 mice were immunized i.p. with live *B. melitensis* (Live-immun; 4×10^4 CFUs) and were treated with antibiotics as described in the *Materials and Methods*. **(A)** Mice were challenged with a high dose of *B. melitensis* (5×10^7 CFUs) and bled at the selected time. The data represent the CFUs per milliliter of blood. **(B and C)** Mice were challenged with a low dose of *B. melitensis* (10^5 CFUs) and sacrificed 50 d postchallenge. **(B)** represents the CFUs per gram of spleen. These data are representative of three independent experiments. Gray bars represent the median. **(C)** displays the mean \pm 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 asterisks. $*p < 0.05$, $***p < 0.001$. pri, primo group.

compared the development of humoral and cellular immune responses in both the HK- and live-immunized groups.

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

The presence of specific Igs against *Brucella* Ags in the serum of the HK- and live-immunized groups of wild-type C57BL/6 mice was investigated by ELISA at 61 d postimmunization (Supplemental Fig. 2A). The results showed that both groups displayed high levels of specific IgM, IgG1, and IgG3 Abs against *Brucella* Ags. It is interesting to note that *Brucella*-specific IgG2a were observed only in the live-immunized group.

To determine the importance of these circulating Ig during a challenge with live *Brucella*, we compared the ability of control or live-immunized groups of wild-type, RAG1^{-/-}, MuMT^{-/-}, and AID^{-/-} (deficient in isotype-switched Abs, B cells produce only IgM) C57BL/6 mice to control *Brucella*.

As expected, when challenged with a high dose (5×10^7 CFUs) of *B. melitensis*, all naive control groups appeared unable to

control *Brucella* dissemination and display a similar high level of bacteria in blood (Fig. 4A for wild-type and data not shown for gene-deficient mice). Among the live-immunized group, wild-type and AID^{-/-} mice appear able to control *Brucella* dissemination and display strongly reduced blood CFU counts at 3 h and 3 d. In contrast, RAG1^{-/-} and MuMT^{-/-} mice present high blood CFU counts similar to naive wild-type mice (Fig. 4A).

We (55) and others (56) have previously observed that B cell-mediated humoral immunity does not contribute positively to the control of a primary *Brucella* infection. Indeed, in contrast to RAG1^{-/-} mice that present high CFU counts in the spleen following infection by 4×10^4 CFUs of *B. melitensis*, wild-type, AID^{-/-}, and MuMT^{-/-} mice display a close similar or even better (in the case of MuMT^{-/-} mice) ability to control primary *Brucella* infection in the spleen (55 and data not shown for AID^{-/-} mice). In a secondary recall response, we observed that wild-type and AID^{-/-} mice displayed a similar ability to control bacteria persistence in the spleen at 50 d following a low-dose challenge (10^5 CFUs) (Fig. 4B, 4C). In striking contrast, MuMT^{-/-} mice displayed higher CFU counts in the spleen at this time (Fig. 4B),

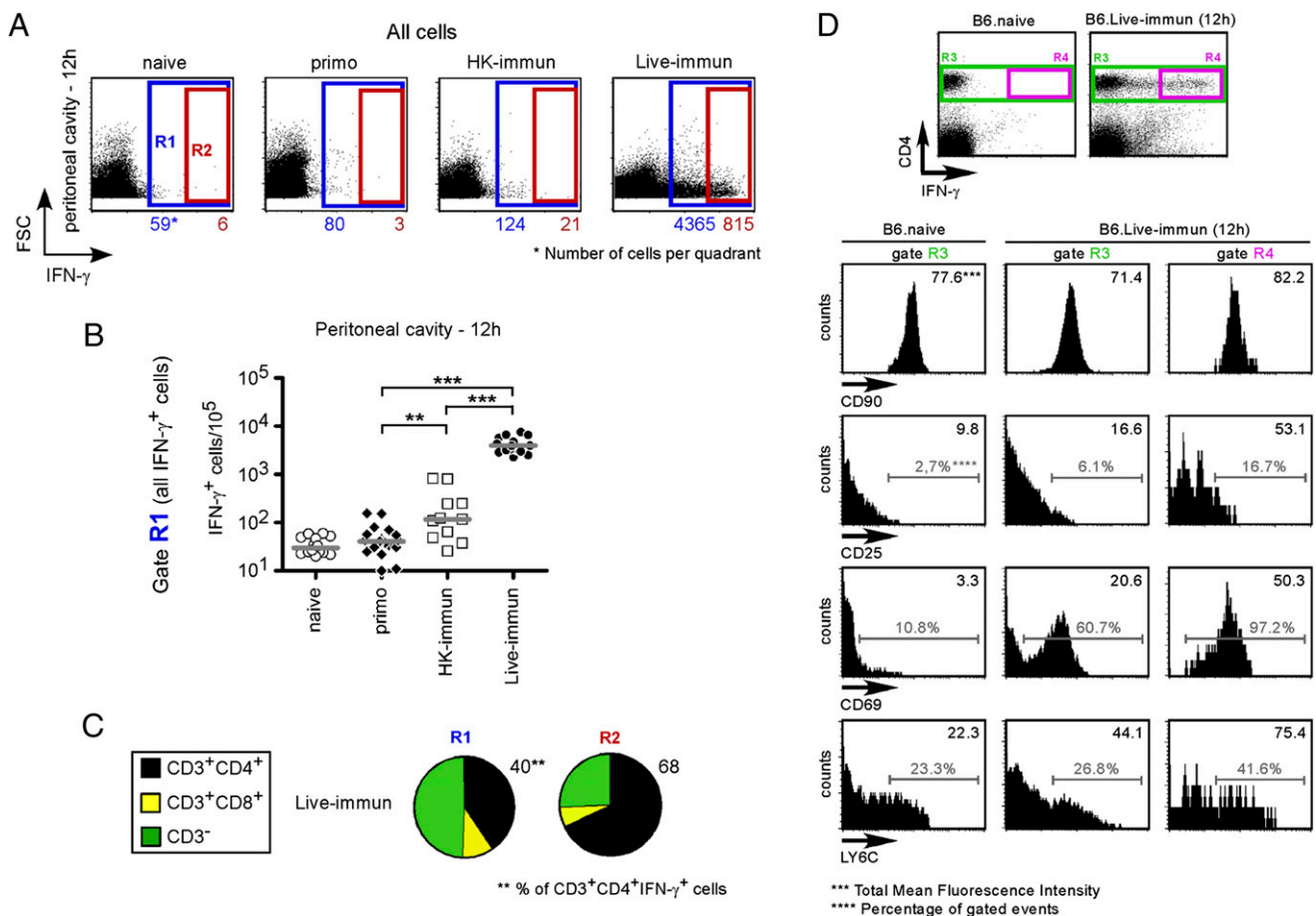


FIGURE 5. Comparison of IFN- γ ⁺ cell frequency after challenge in C57BL/6 mice immunized previously with live or HK *B. melitensis*. C57BL/6 mice were immunized i.p. with either live (Live-immun; 4×10^4 CFUs) or HK bacteria (HK-immun; 10^8 CFUs). All mice were treated with antibiotics as described in the *Materials and Methods*, challenged with a low dose (10^5 CFUs) of live bacteria, 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. Gray bars represent the median. Significant differences are denoted by an asterisk. **(C)** Total (gate R1) and highly (gate R2) IFN- γ -positive cells in the Live-immun group were analyzed for CD3, CD4, and CD8 α expression. Numbers next to the circles indicate the percentage of CD3⁺CD4⁺IFN- γ ⁺ T cells in gates R1 and R2. **(D)** CD4⁺ (gate R3) or CD4⁺IFN- γ ⁺ 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. The percentage of positive gated events is also shown for each marker. ** $p < 0.01$, *** $p < 0.001$.

and a reduced frequency of these mice displayed a complete bacterial clearance (Fig. 4C) compared with wild-type and $AID^{-/-}$ mice.

Taken together, these results demonstrate that circulating Abs 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 B. 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, 57–60) have shown that IFN- γ is a key cytokine-regulating protective cellular immune response against primary *Brucella* infection. IFN- γ is produced by NK cells, CD4⁺ T, and CD8⁺ cells (46, 55) and is crucial for the development of inducible NO synthase-positive granulomas that limit *B. melitensis* infection in the spleen and the liver (49). In this study, 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 CFUs) doses of live *B. melitensis*. IFN- γ -producing cells were analyzed at 12, 24, and 48 h postchallenge.

After challenge (10^5 CFUs), in the absence of in vitro restimulation, an elevated frequency of IFN- γ -positive cells was detected at 12 h in the peritoneal cell population from the live-immunized group (Fig. 5A, 5B) that progressively decreased at 24 and 48 h (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 (Fig. 5A–C and data not shown) during the first 48 h. 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 because a mean of 68% of highly IFN- γ -positive cells were found to coexpress CD3e and CD4 markers (Fig. 5C). These cells also expressed higher levels of CD25, CD69 (activation marker), and Ly-6C (memory T cells marker) (Fig. 5D). 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 (Supplemental Fig. 3). As expected, the live-immunized group of BALB/c mice displayed a 10-fold reduction of the frequency of IFN- γ -positive peritoneal cells compared with C57BL/6 mice (Fig. 6).

We also investigated the production of IFN- γ in the spleen of C57BL/6 mice during the first 120 h in all groups, but only the live-immunized group displayed a very weak frequency of IFN- γ ⁺ CD4⁺ T cells (<200 cells/ 10^6 spleen cells) that progressively peaked at 48 h, 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 producer 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 bacterial eradication in the spleen of mice immunized with live B. melitensis

Our previous studies (49, 55) showed that $MyD88^{-/-}$, $MHC-II^{-/-}$, and $IL-12p35^{-/-}$ C57BL/6 mice display high susceptibility to primary *B. melitensis* infection. All of these gene-deficient mice display higher CFU counts in the spleen, suggesting a key role for

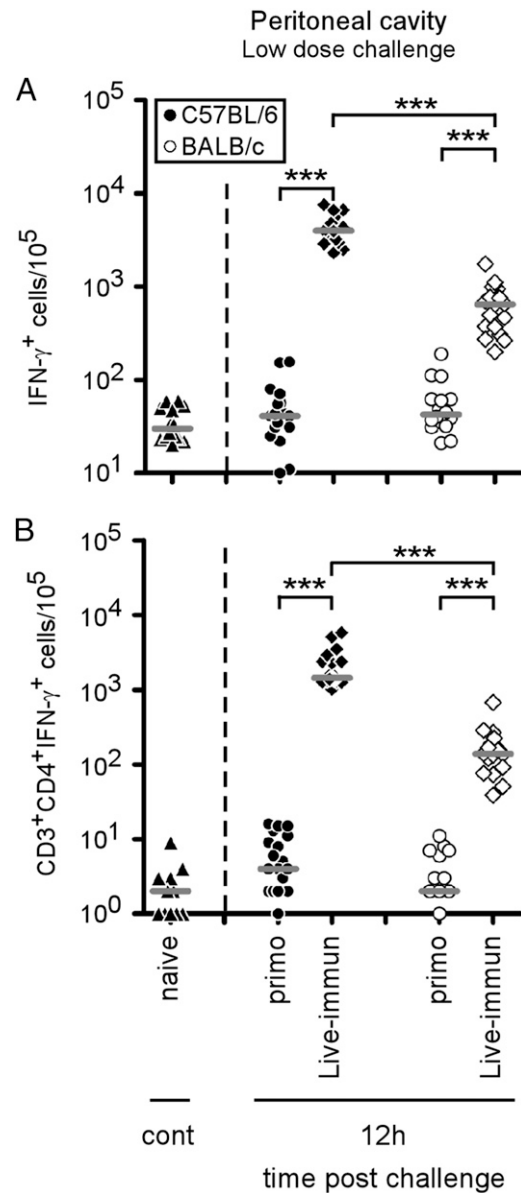


FIGURE 6. IFN- γ ⁺ peritoneal cell frequency after challenge in C57BL/6 or BALB/c mice immunized previously with live *B. melitensis*. C57BL/6 and BALB/c mice were immunized i.p. with live *B. melitensis* (Live-immun; 4×10^4 CFUs) and treated with antibiotics as described in the *Materials and Methods*. (A and B) Mice were then challenged with 10^5 CFUs of *B. melitensis* and sacrificed at 12 h postchallenge. Peritoneal cells were collected and analyzed by flow cytometry. Cells were first analyzed for forward size scatter versus IFN- γ production and then for cell-surface markers. The data represent the number of IFN- γ -positive cells (A) or CD3⁺CD4⁺IFN- γ ⁺ T cells (B) per 10^5 peritoneal cells acquired. Each data point represents the value obtained from an individual peritoneal cavity, and the data are representative of two independent experiments. Gray bars represent the median. Significant differences are denoted by asterisks. *** p < 0.001. cont, control.

IFN- γ -producing CD4⁺ T cells in the control of primary *Brucella* infection.

To confirm the link between complete bacteria clearing in the spleen occurring during a secondary recall response 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 gene-deficient mouse strains. Live-immunized groups of wild-type,

MyD88^{-/-}, IL-12p35^{-/-}, and MHC-II^{-/-} C57BL/6 mice were challenged with high (5×10^7 CFUs) or low (10^5 CFUs) doses of live *B. melitensis*, and their ability to control *Brucella* dissemination in the blood and confer protective immunity in the spleen was assessed (Fig. 7).

MyD88, IL-12p35, and MHC-II deficiencies do not impair the ability of both naive control group (data not shown for gene-deficient mice) and live-immunized groups to display lower *Brucella* CFU counts in the blood following a high-dose challenge compared with naive infected mice (Fig. 7A). A comparative analysis of the humoral immune response in these deficient mice was performed 2 d before challenge and showed that all groups displayed high levels of *Brucella*-specific IgM but extremely variable levels of different *Brucella*-specific IgG isotypes (Supplemental Fig. 2B). In particular, MHC-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 (Fig. 4A), 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 (Fig. 7B, 7C). 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 h postchallenge in the peritoneal cavity (Fig. 8).

Finally, as BALB/c mice displayed reduced IFN- γ production in both the peritoneal cavity and the spleen (Fig. 6) and impaired protective immunity in the spleen (Fig. 3), we analyzed the impact of IL-4/IL-13R 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 (Fig. 9A, 9B), the live-immunized group of STAT-6^{-/-} mice displayed lower CFU counts (Fig. 9C) and significantly better elimination of *B. melitensis* in the spleen (Fig. 9D) compared with 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 (61). However, though *Brucella* infection remains largely silent, brucellosis induces potentially serious complications over the long term (3, 4, 62). As antibiotic-treated patients frequently display bacteria resurgence (11, 63), 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, 64), 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 (55) using a large panel of gene-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, 55). In the current study, we have developed an experimental model (based on Ref. 50) to characterize the effector mechanisms involved in the control of a secondary infection by *B. melitensis*. Mice were injected with HK or live virulent *B. melitensis* 16M and treated with antibiotics after 21 d. 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 bloodstream. The late immune protection was scored by the frequency of animals that were not able to completely eradicate

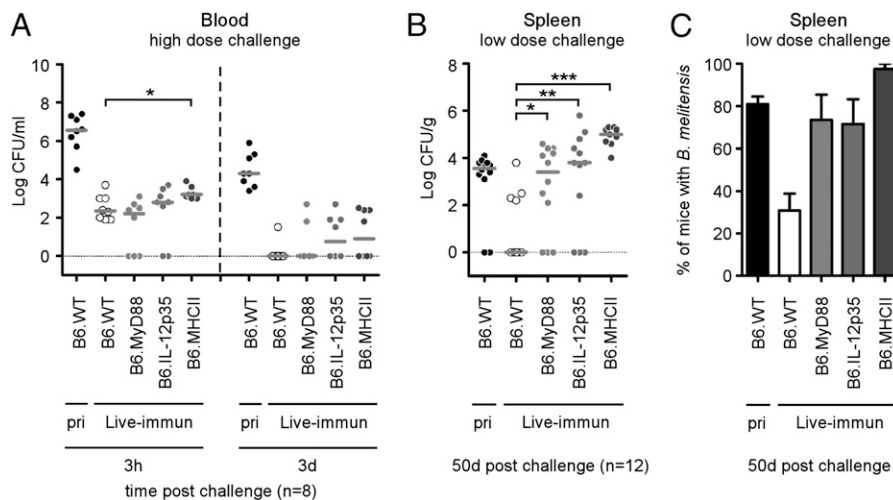


FIGURE 7. Comparison of protection in wild-type (WT) and deficient C57BL/6 mice immunized previously with live *B. melitensis*. WT, MyD88^{-/-}, IL-12p35^{-/-}, and MHC-II^{-/-} C57BL/6 mice were immunized i.p. with live *B. melitensis* (4×10^4 CFUs) and treated with antibiotics as described in the *Materials and Methods*. (A) Mice were challenged with a high dose of *B. melitensis* (5×10^7 CFUs) and bled at the selected time. The data represent the CFUs per milliliter of blood. (B and C) Mice were challenged with a low dose of *B. melitensis* (10^5 CFUs) and sacrificed at 50 d postchallenge. (B) The data represent the CFUs per gram of spleen and are representative of two independent experiments. Gray bars represent the median. The mean \pm SEM of the percentage of mice that are still positive for *Brucella* in the spleen at 50 d postchallenge is represented in (C). These results are pooled from at least two independent experiments. Significant differences are denoted by asterisks. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. pri, primo group.

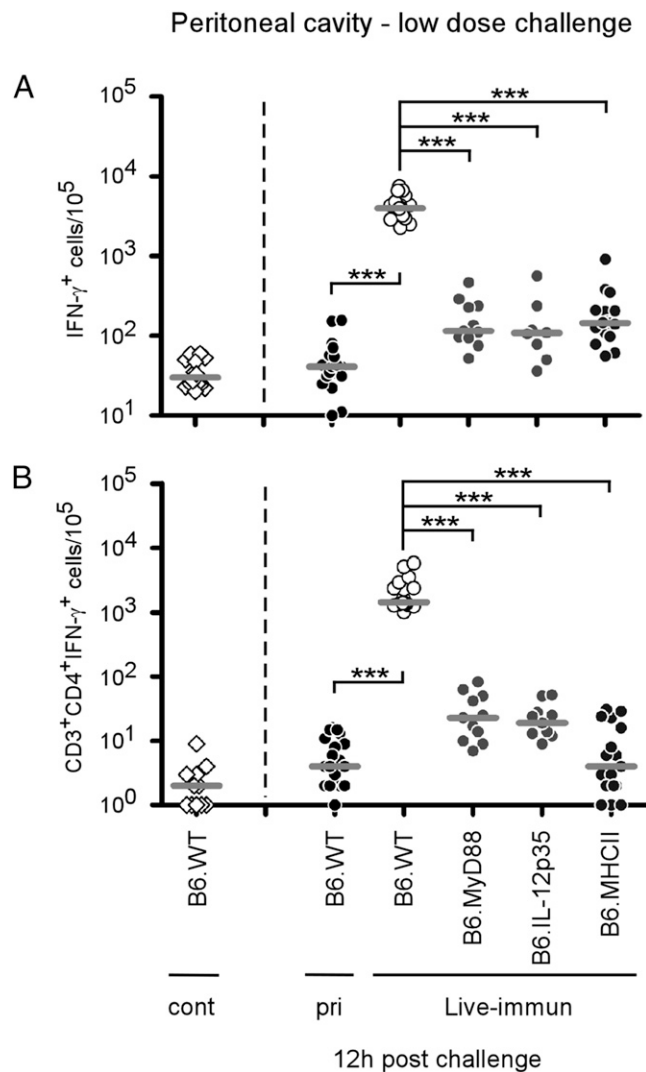


FIGURE 8. Comparison of IFN- γ ⁺ cell frequency after challenge in wild-type (WT) and deficient C57BL/6 mice immunized previously with live *B. melitensis*. WT, MyD88^{-/-}, IL-12p35^{-/-}, and MHC-II^{-/-} C57BL/6 mice were immunized i.p. with live *B. melitensis* (Live-immun; 4×10^4 CFUs) and treated with antibiotics as described in the *Materials and Methods*. Mice were then challenged with a low dose of *B. melitensis* (10^5 CFUs) and sacrificed at the selected time. Peritoneal cells were collected and analyzed by flow cytometry. Cells were first analyzed for forward size scatter versus IFN- γ production and then for cell-surface markers. The data represent the number of IFN- γ -positive cells (**A**) and the number of CD3⁺CD4⁺IFN- γ ⁺ T cells (**B**) per 10^5 peritoneal cells acquired. Each data point represents the value obtained from an individual peritoneal cavity, and the data are representative of two independent experiments. Gray bars represent the median. Significant differences are denoted by asterisks. *** $p < 0.001$. cont, control; pri, primo group.

bacteria from their spleen at 50 d postchallenge. The spleen was chosen as the control organ because *Brucella* has been shown to persist for long periods of time (up to 100 d) in this organ (65). 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 TNF-dependent activation of macrophages. This leads to an upregulation of antimicrobial effector mechanisms, including the acidification of phagolysosomes and the expression of inducible NO synthase [NO

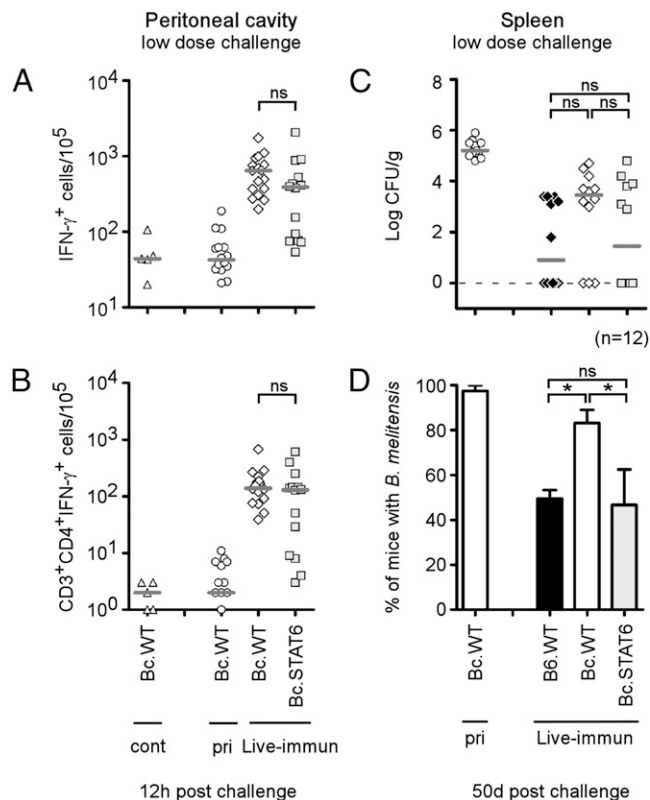


FIGURE 9. Comparison of protection and IFN- γ ⁺ cell frequency after challenge in wild-type (WT) or STAT6^{-/-} BALB/c mice immunized previously with live *B. melitensis*. WT and STAT6^{-/-} BALB/c mice were immunized i.p. with live *B. melitensis* (Live-immun; 4×10^4 CFUs) and treated with antibiotics 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 CFUs) and sacrificed at the selected time. (**A** and **B**) To characterize the elicited immune response, mice were sacrificed at 12 h postchallenge, and peritoneal cells were collected and analyzed by flow cytometry. Cells were first analyzed for forward size scatter versus IFN- γ production and then for cell-surface markers. The data represent the number of IFN- γ -positive cells (**A**) and number of CD3⁺CD4⁺IFN- γ ⁺ cells (**B**) per 10^5 peritoneal cells acquired. (**C** and **D**) To estimate the elicited protection, mice were sacrificed at 50 d postchallenge, and the spleens were harvested. (**C**) The data represent the CFUs per gram of spleen and are representative of two independent experiments. Gray bars represent the median. The mean \pm 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. * $p < 0.05$. pri, primo group.

synthase 2 (66)]. Although Abs 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 (67). Ab-mediated aggravation of infections with intracellular pathogens might be due to FcR-mediated facilitation of entry of the pathogen into the host cell or to macrophage deactivation conveyed by inhibitory FcRs (68–70). Conversely, Ab-dependent control of intracellular microbes may result from Ab binding to the pathogen during intermittent extracellular phases, leading to opsonization and classical complement activation or to opsonophagocytosis (71).

In our model, we observed that humoral immunity is necessary for full protection upon secondary infection (Table I). Circulating specific Abs are crucial to control the early dissemination of *Brucella* by the bloodstream following challenge by i.p. injection. They are also critical for the development of sterilizing immunity

in the spleen at 50 d postchallenge. Thus, although B cells appear to be dispensable (55) or even detrimental (56, 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 Abs. 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 mo 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 use 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 (76), we can hypothesize that *Brucella* pathogen-associated molecular patterns are implicated in the activation of *Brucella*-specific B cells and that pattern recognition receptors recognizing these pathogen-associated molecular patterns 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.

Brucella-specific circulating Abs mediated the early protective immunity 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 Abs and peritoneal Th1 CD4⁺ T cells able to quickly produce high IFN- γ counts after *Brucella* challenge (Table I). The absence of Abs (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 gene-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 IFN regulatory factor 1-deficient mice, but not RAG-, IL-12-, or MyD88-deficient mice, succumb to primary infection by *Brucella* (46, 55, 57, 60, 77, 78), suggesting that IFN- γ can be induced at low level by MyD88/IL-12-independent pathways. Our results confirm the importance of using 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⁺ memory T cells are not replaced by IFN- γ -producing CD8⁺ memory T cells in the absence of MHC-II-dependent Ag-presenting pathways. We previously observed during the *Brucella* primary response that in absence of MHC-II, IFN- γ -producing CD8⁺ T cells develop but are not able to control *Brucella* infection (55). Taken together, these results demonstrate that primary *Brucella* infection induces low-quality responding CD8⁺ T cells playing a minor role in primary control of infection and unable to participate in the secondary immune response. A recent report suggests 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 (65). The identification of CD4⁺ T cells as key lymphocyte subsets is critical to determine which

Table I. 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	HK- Immunized	Live- Immunized	MuMT ^{-/-} Live- Immunized	AID ^{-/-} Live- Immunized	MyD88 ^{-/-} Live- Immunized	IL-12p35 ^{-/-} Live- Immunized	MHC-II ^{-/-} Live- Immunized
Circulating IgM	–	+++	+++	–	+++	++	++++	++
Circulating IgG	–	++	+++	–	–	++	+++	–
Control of bacteria dissemination in the blood	–	+++	+++	–	+++	+++	+++	++
IFN- γ ⁺ cells (peritoneal cavity) ^a	–	+	+++	+++	+++	+	+	+
IFN- γ ⁺ CD4 ⁺ T cells (peritoneal cavity) ^a	–	+	+++	+++	+++	+	+	–
Percentage of mice displaying a sterilizing immunity in the spleen ^b	19	17	69 (protected)	33	80 (protected)	26	28	2

^aData considered for IFN- γ production concern the analysis of peritoneal cells 12 h after a low-dose challenge (10⁵ CFU of *Brucella*), without restimulation. Data are not shown for IFN- γ production of MuMT^{-/-} and AID^{-/-} mice.

^bNumbers indicate the mean of the percentage of mice that are still positive for *Brucella* in the spleen 50 d postchallenge. Mean was calculated with data from at least two independent experiments, each including a minimum of 10 mice.

WT, wild-type.

Ag-presenting pathways (MHC class 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 (87–90). As IFN- γ ^{-/-} mice succumb rapidly to *Brucella* infection (57, 91), 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 (55), 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, 92) on the *Listeria 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 an immune response eradicating *Brucella* in the spleen. Unlike in C57BL/6 mice, the injection of live bacteria in BALB/c mice does not improve their capacity to clear 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 with 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-4R deficiency (55) or neutralization of IL-4/IL-13R 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.

Although previous studies on *Brucella* vaccination have reported on the importance of the induction of specific Abs (21, 36, 37) and CD4⁺ T cells (19, 21, 37) in protection, our study is the first, to our knowledge, to: 1) formally demonstrate by using gene-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 2) 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, Ag 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 Abs from B cells is critical to control the course of murine and ovine *B. melitensis* infection (85, 93). 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. The γ -irradiated *Brucella* do not divide but conserve metabolic activity and protect mice against virulent bacterial challenge without signs of residual virulence (94). Thus, inactivated, yet metabolically active, microbes could represent a promising strategy for safe vaccination against *B. melitensis*.

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Disclosures

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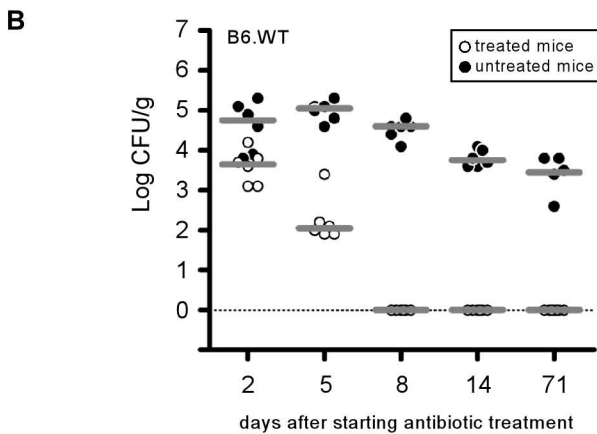
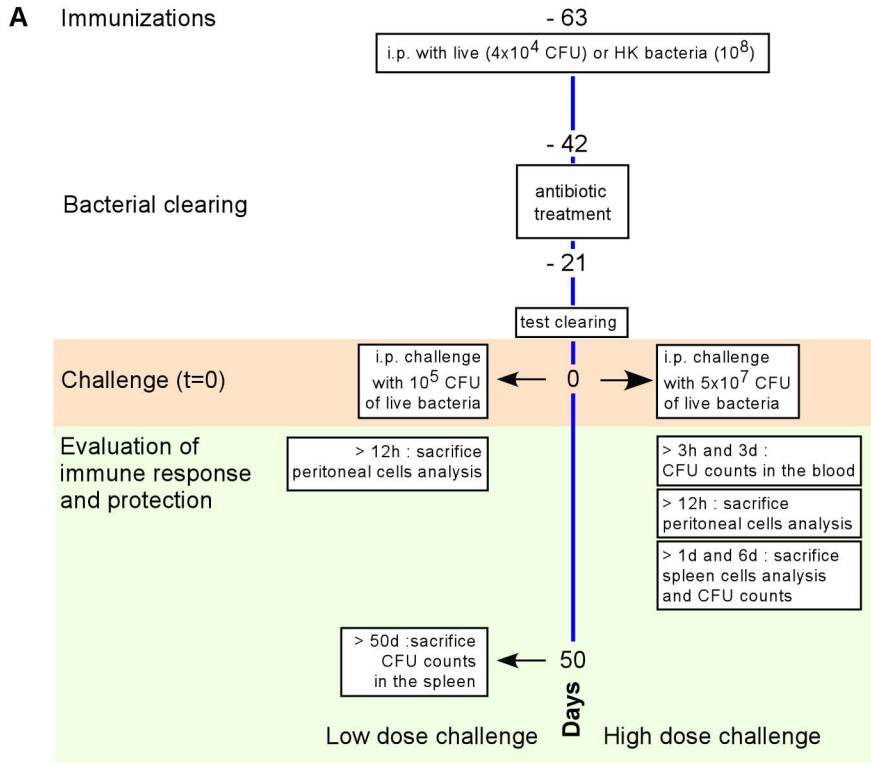


Figure S1. Experimental design and antibiotic treatment efficacy. **A**, Mice were immunized i.p. on day -63 with live (4×10^4 CFU) or heat-killed (HK) (10^8 CFU) *B. melitensis*. Three weeks after immunization, antibiotic treatment was administered daily for 3 weeks, starting on day -42. To ensure that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

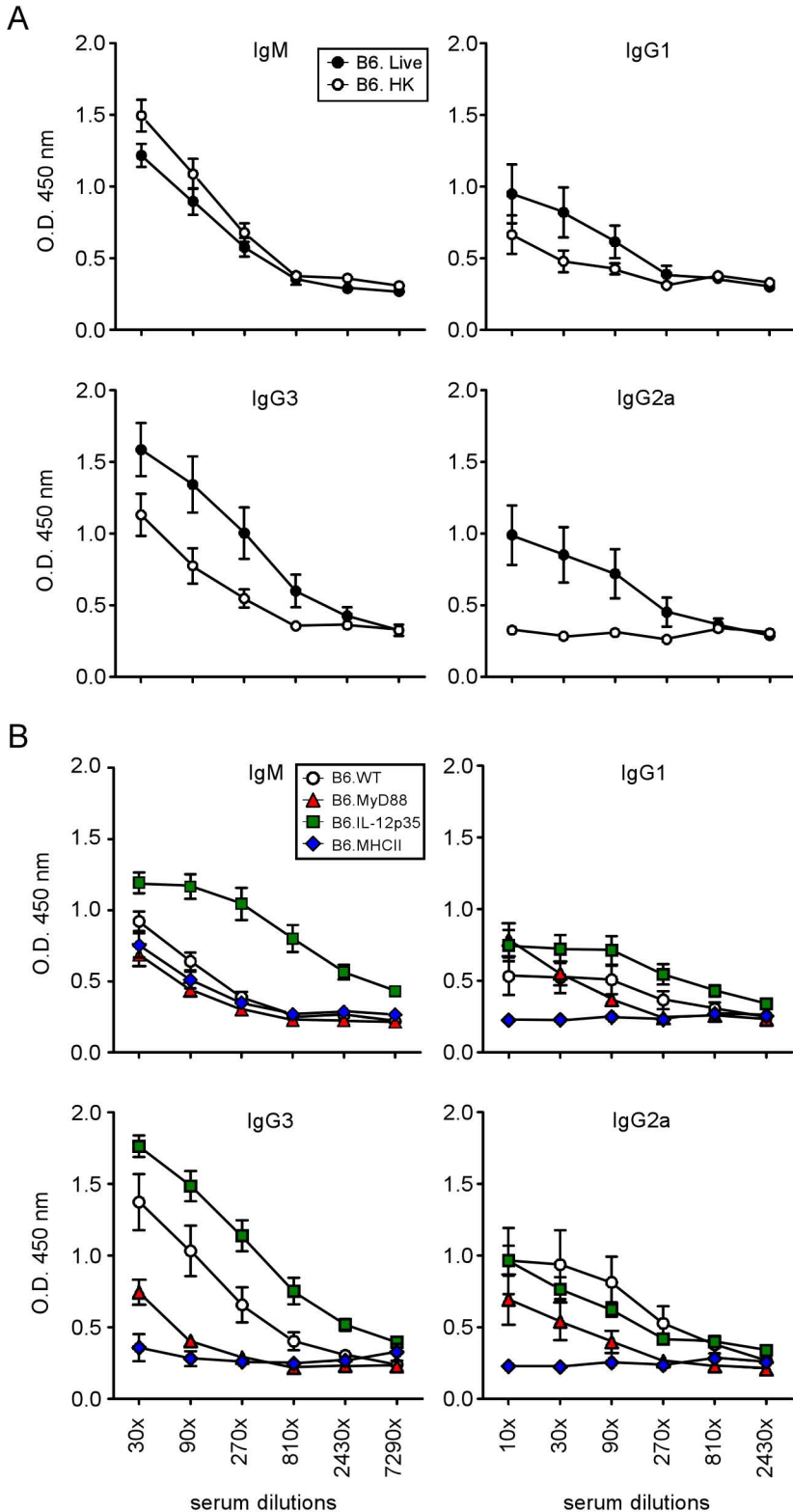


Figure S2. Analysis of *Brucella*-specific antibodies in the serum of wild-type, MyD88^{-/-}, IL-12p35^{-/-} and MHCII^{-/-} C57BL/6 mice immunized previously with live or heat-killed *B. melitensis*. **A. Wild-type mice were immunized i.p. with live *B. melitensis* (4×10^4 CFU) or heat-killed (HK) (10^8 CFU) *B. melitensis*. **B.** Wild-type and deficient mice were immunized i.p. with live *B. melitensis* (4×10^4 CFU). All mice were treated with antibiotics as described in the Materials and Methods. Sera were collected 2 days before challenge and ELISA was performed to detect the isotype distribution of *Brucella*-specific antibodies. The data represent the mean \pm SEM of 8 mice.**

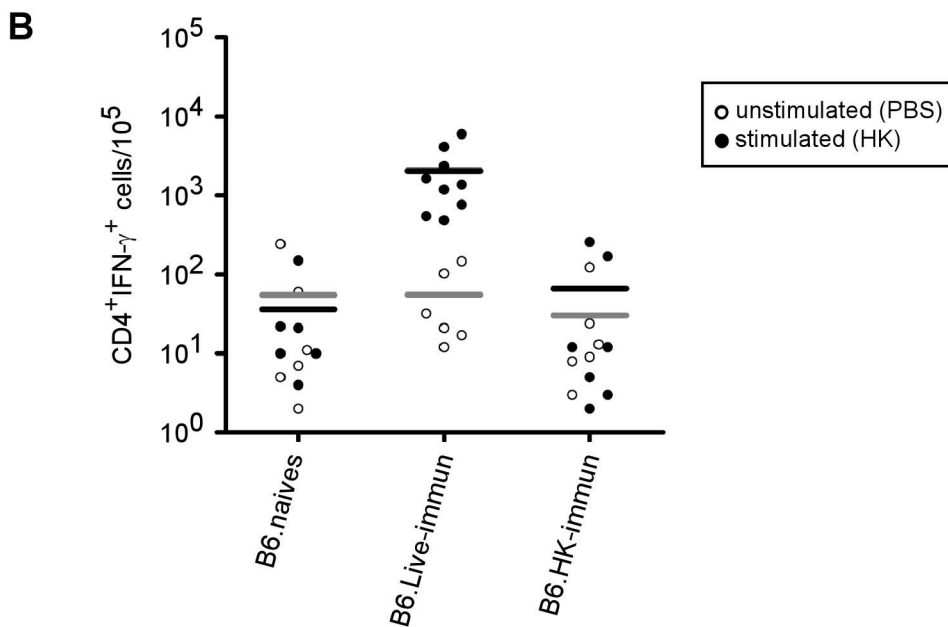
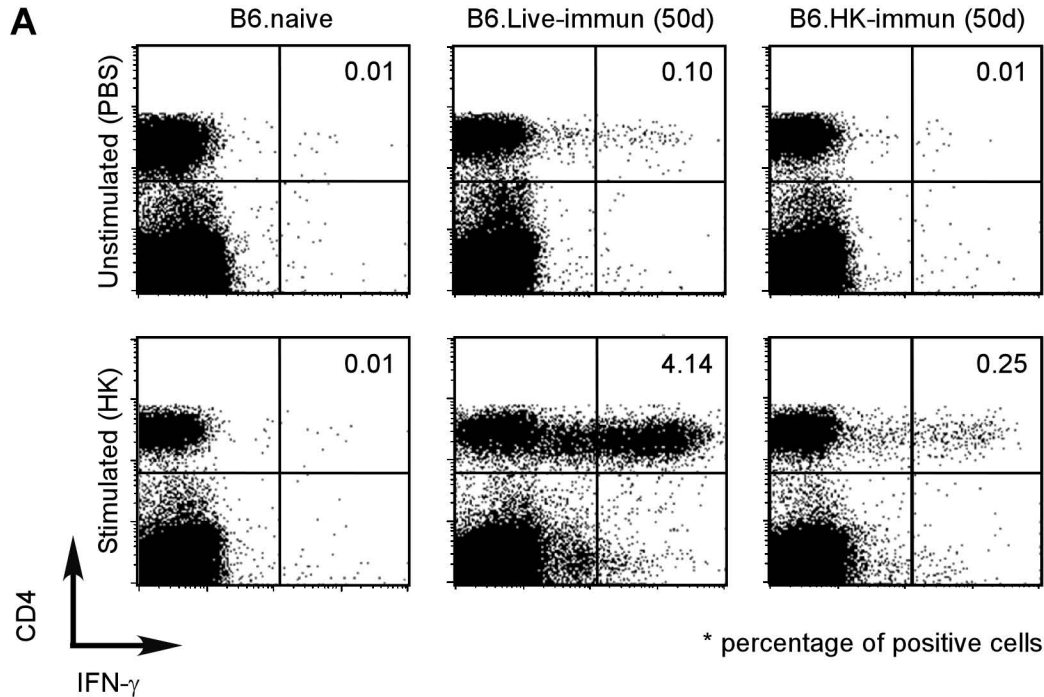


Figure S3. *In vitro* stimulation of peritoneal cells from wild-type C57BL/6 mice immunized previously with live or HK *B. melitensis*. Mice were immunized i.p. with live (4×10^4 CFU) or heat-killed (HK) (10^8 CFU) *B. melitensis* and were treated with antibiotics as described in the Materials and Methods. Instead of an *in vivo* challenge, mice were sacrificed 60 days after immunization and peritoneal cells were recovered for *in vitro* stimulation with PBS (unstimulated) or HK *B. melitensis* (10^7 CFU) for 20 hours. Cells were then analyzed by flow cytometry. **A**, Cells were analyzed for CD4 expression versus IFN-gamma production. The figure shows representative dot plots from a pool of two peritoneal cavities in each group. Numbers indicate the percentage of IFN-gamma producing CD4⁺ T cells. **B**, The graph represents the number of CD4⁺IFN-gamma⁺ cells per 10^5 peritoneal cells acquired. Each data point represents the value obtained from an individual peritoneal cavity and the data are representative of two independent experiments. Grey bars represent the median of unstimulated groups whereas black bars represent the median of stimulated groups.