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Mice Lacking Components of Adaptive Immunity Show Increased *Brucella abortus virB* Mutant Colonization▽

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The *Brucella abortus* type IV secretion system (T4SS), encoded by the *virB* genes, is essential for survival in mononuclear phagocytes in vitro. In the mouse model, a *B. abortus virB* mutant was initially able to colonize the spleen at the level of the wild type for approximately 3 to 5 days, which coincided with the development of adaptive immunity. To investigate the relationship between survival in macrophages cultivated in vitro and persistence in tissues in vivo, we tested the ability of mutant mice lacking components of adaptive immunity to eliminate the *virB* mutant from the spleen during a mixed infection with the *B. abortus* wild type. *Ifng*$^{-/-}$ or β_2*m*$^{-/-}$ mice were able to clear the *virB* mutant to the same degree as control mice. However, spleens of *Rag1*$^{-/-}$ mice and *Igh6*$^{-/-}$ mice were more highly colonized by the *virB* mutant than control mice after 14 to 21 days, suggesting that, in these mice, there is not an absolute requirement for the T4SS to mediate persistence of *B. abortus* in the spleen. Macrophages isolated from *Igh6*$^{-/-}$ mice killed the *virB* mutant to the same extent as macrophages from control mice, showing that the reduced ability of these mice to clear the *virB* mutant from the spleen does not correlate with diminished macrophage function in vitro. These results show that in the murine model host, the T4SS is required for persistence beyond 3 to 5 days after infection and suggest that the T4SS may contribute to evasion of adaptive immune mechanisms by *B. abortus*.

Brucella abortus is one of the causative agents of brucellosis, a chronic zoonotic infection characterized by bacterial persistence in the reticuloendothelial system (RES) of the liver, spleen, and lymph nodes. The chronic infection of the RES observed during human brucellosis can be studied in the murine model host. A signature-tagged transposon screen identified a type IV secretion system (T4SS) as an essential virulence factor for persistence in the murine RES (11). The T4SS is encoded by the *virB* locus on chromosome II of the *B. abortus* genome. Mutational inactivation of the T4SS reduces the ability of *B. abortus* to survive and/or replicate in human epithelial cell lines (HeLa cells), murine bone marrow-derived macrophages, and macrophage-like cell lines (3, 5, 8, 14, 29, 30), and similar phenotypes have been described for *Brucella melitensis* and *Brucella suis virB* mutants (7, 10, 16, 24). Within murine bone marrow-derived macrophages, *B. abortus* traffics to an endoplasmic reticulum (ER)-like compartment, where it localizes to ER exit sites. In contrast, *virB* mutants fail to acquire ER markers (3, 4). These differences in cellular trafficking are reflected in the kinetics of bacterial growth and/or killing: whereas wild-type (wt) *B. abortus* is able to replicate in macrophages in vitro, *virB* mutant bacteria are eliminated within the first 24 h after infection (3, 29, 30).

These observations raise the question whether the T4SS is required for evasion of macrophage killing mechanisms in vivo. The role of the T4SS in evading two major macrophage killing mechanisms was recently addressed by comparing the ability of mice defective for production of reactive nitrogen intermediates (*Nos2*$^{-/-}$ mice) and reactive oxygen intermediates (gp91*phox*$^{-/-}$mice) with their congenic wild-type mice to check growth of *B. abortus* and an isogenic *virB* mutant in the RES. This study showed that neither the inducible nitric oxide synthase nor NADPH oxidase was responsible for the severe in vivo growth defect of the *virB* mutant (30).

Since *B. abortus virB* mutants are 100-fold attenuated for intracellular growth within only 5 to 8 h of in vitro infection of macrophages, it seems reasonable to expect that, if bacteria entered macrophages at the onset of infection, then attenuation in vivo should manifest early during infection of mice as well. However, a recent screen of 672 *Brucella melitensis* signature-tagged transposon mutants for genes required for colonization of murine spleens 5 days after infection did not identify any transposon insertions in the *virB* locus (18). This result was somewhat puzzling, since screening of a smaller *B. abortus* signature-tagged mutant bank (178 mutants) in mice identified two insertions in the *virB* locus (11). One major difference in the design of the latter screen was the recovery of mutants from the spleen at 2 weeks and 8 weeks after infection. A possible interpretation of these in vivo data is that, unlike during in vitro growth in host cells, the T4SS is only required for bacterial growth in the RES at late times (>5 days) after infection. In order to address this question experimentally, we studied the kinetics of infection of a *virB* mutant in both wild-type and mutant mouse strains.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. Bacterial strains used were *Brucella abortus* 2308 and its isogenic mutant, BA41 (11), which has an insertion of mTn*5*Km2 at nucleotide 1232 of the *B. abortus virB* locus (GenBank accession number AF226278). This insertion is located 59 bp downstream of the *virB1* gene

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and is polar on the expression of downstream genes in the *virB* operon (31). Strains were cultured on tryptic soy agar (TSA; Difco/Becton-Dickinson, Sparks, MD) or in tryptic soy broth at 37°C on a rotary shaker. Bacterial inocula for infection of mice were cultured on TSA plus 5% blood (1). For cultures of strain BA41, kanamycin (Km) was added to the culture medium at 100 mg/liter. All work with live *B. abortus* was performed at biosafety level 3.

Infection of mice. C57BL/6, B6.129S7-Rag1^{tm1Mom} (21) mice carrying a targeted knockout of the gene encoding recombination activating gene 1, B6.129S2-*Cd4*^{tm1Cgn} (27) mice carrying a targeted knockout of the gene encoding CD4, B6.129S2, *Igh*-6^{tm1Mak} (15) mice carrying a targeted knockout of the gene encoding immunoglobulin Mu chain, and B6.129S7-Ifng^{tm1Ts}/J (6) mice carrying a targeted knockout of the gene encoding interferon gamma (IFN-γ) were obtained from the Jackson Laboratory (Bar Harbor, ME). B6 and B6.129-*B2m*^{tm1Jae}N12 (33) mice carrying a targeted knockout of the gene encoding β_2-microglobulin were obtained from Taconic Farms (Germantown, NY). Mice were held in microisolator cages with sterile bedding and water and irradiated feed in a biosafety level 3 facility. For infection experiments, groups of five knockout mice and five age-matched controls per time point were inoculated intraperitoneally (i.p.) with 0.1 ml of phosphate-buffered saline (PBS) containing 5×10^5 to 10×10^5 CFU of *B. abortus*. At the appropriate time points, mice were euthanized by CO_2 asphyxiation, and the spleens were collected aseptically at necropsy. The spleens were homogenized in 3 ml of PBS, and serial dilutions of the homogenate were plated on TSA and TSA plus Km for enumeration of CFU. For mixed infection experiments, the log ratio was calculated as the geometric mean of CFU mutant/CFU wild type recovered from spleens. Experimental groups each contained five knockout mice and five age-matched controls from the same supplier, aged 6 to 10 weeks. All animal experiments were approved by the Texas A&M University Laboratory Animal Care and Use Committee or the UC Davis Institutional Animal Care and Use Committee and were conducted in accordance with institutional guidelines.

Confirmation of mutant mouse phenotypes. Flow cytometry analysis was used to confirm mutant phenotypes of $\beta_2m^{-/-}$, *Cd4*$^{-/-}$, and *Igh6*$^{-/-}$ mice. Spleens from infected mice were homogenized in 3 ml PBS, and serial dilutions were plated on TSA or TSA plus Km to enumerate CFU. The remaining cells were labeled for analysis by flow cytometry. Briefly, after passing the cells through a 100-μm cell strainer and treating the samples with ACK buffer (0.15 M NH_4Cl, 1.0 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.2) to lyse red blood cells, the cells were washed with PBS (Gibco) containing 1% bovine serum albumin (PBS-BSA). Portions of the splenocytes were stained at 4°C with the appropriate monoclonal antibodies (MAb): either fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD8a (Ly-2) MAb, FITC-conjugated rat anti-mouse CD45R/B220 MAb (Pharmingen, San Diego, CA), or FITC-conjugated rat anti-mouse CD4 (L3T4) MAb. The cells were washed with PBS-BSA and fixed with 4% formaldehyde for 1 h. Finally, samples were washed as before and resuspended in PBS-BSA. Flow cytometry analysis was performed using a FACSCalibur (Becton Dickinson, San Diego, CA), and data were collected for 10,000 cells/sample (data not shown). Mice that did not exhibit the expected phenotypes were eliminated from the data analysis.

Isolation of bone marrow-derived macrophages. Bone marrow-derived macrophages were isolated from C57BL/6, *Igh6*$^{-/-}$, and *Cd4*$^{-/-}$ mice following standard protocols. Briefly, after aseptically obtaining femurs from the mice, the bone marrow cells were flushed out with 6 ml of cold RPMI medium 1690 (RPMI; Gibco, Rockville, MD). Cells were pelleted at 1,000 rpm for 10 min at 4°C. The cells were resuspended in BMM medium (RPMI supplemented with 20% heat-inactivated fetal bovine serum, L-cell conditioned medium, 1 mM glutamine, 1% nonessential amino acids, and 1% antibiotic-antimycotic [Gibco, Rockville, MD]) and placed in petri dishes at 37°C in 5% CO_2. At day 3 cells were fed by adding 10 ml of BMM medium and incubated for an additional 5 days in the presence of CO_2. At day 7, the bone marrow-derived macrophages were harvested by removing the medium and adding cold PBS. Macrophages were centrifuged at 1,000 rpm for 10 min at 4°C, resuspended in RPMIsup (RPMI supplemented with 20% heat-inactivated fetal bovine serum, 1 mM glutamine, and 1% nonessential amino acids), and counted for the infection assays.

Isolation of resident peritoneal macrophages. Resident peritoneal macrophages were isolated from C57BL/6, *Igh6*$^{-/-}$, and *Cd4*$^{-/-}$ post mortem, following standard protocols. Briefly, peritoneal fluid was harvested after injecting the animal's abdomens with cold RPMI supplemented with heparin. After injection, the abdomen of the animal was massaged and the liquid was extracted from the peritoneal cavity. Fluids were centrifuged at 1,200 rpm for 10 min at 4°C, and the pellets were resuspended in RPMI supplemented with 20% heat-inactivated fetal bovine serum, 1 mM glutamine, 1% nonessential amino acids, and 1% antibiotic-antimycotic (Gibco, Rockville, MD) and placed in petri dishes at 37°C in 5% CO_2. After incubation for 3 h at 37°C under 5% CO_2, nonadherent cells were removed from the petri dishes by aspiration, and the adherent macrophages were rinsed twice with PBS, counted, and plated in 24-well plates with fresh medium without antibiotic-antimycotic for subsequent infection.

Splenic adherent cells. Splenic cells were isolated from C57BL/6 and *Igh6*$^{-/-}$ mice by homogenizing spleens in 3 ml of RPMI supplemented with 20% heat-inactivated fetal bovine serum, 1 mM glutamine, 1% nonessential amino acids, and 1% antibiotic-antimycotic (Gibco, Rockville, MD). Cells were centrifuged at 1,000 rpm for 10 min at 4°C. The cells were resuspended in 10 ml of the above medium and placed in petri dishes at 37°C in 5% CO_2 overnight. The nonadherent cells were removed from the petri dishes by aspiration, and the adherent cells were rinsed twice with PBS, counted, and plated in 24-well plates with fresh medium without antibiotic-antimycotic for subsequent infection.

Macrophage infection. For macrophage killing assays, 24-well microtiter plates were seeded with macrophages at a concentration of 2×10^5 cells/well in 0.5 ml of RPMIsup and incubated overnight at 37°C in 5% CO_2. The inocula were prepared by growing with shaking in tryptic soy broth for 24 h and then subsequent dilution in RPMIsup to a concentration of 4×10^7 CFU/ml. Approximately 2×10^7 bacteria in 0.5 ml of RPMIsup, containing *B. abortus* 2308 (wild type) or its isogenic *virB* mutant, were added to each well of macrophages. Three independent assays were performed with triplicate samples, and each experiment included control (C57BL/6) macrophages together with either *Igh6*$^{-/-}$ or *Cd4*$^{-/-}$ macrophages. Microtiter plates were centrifuged at $250 \times g$ for 5 min at room temperature in order to synchronize infection. Cells were incubated for 20 min at 37°C in 5% CO_2, and free bacteria were removed by three washes with PBS. RPMIsup plus 50 mg gentamicin per ml was added to the wells, and the cells were incubated at 37°C in 5% CO_2. After 1 h, the RPMIsup plus 50 μg/ml gentamicin was replaced with medium containing 25 μg/ml gentamicin. Wells were sampled at time points between 1 and 48 h after infection by aspirating the medium, lysing the macrophages with 0.5 ml of 0.5% Tween 20, and rinsing each well with 0.5 ml of PBS. Viable bacteria were quantified by dilution in sterile PBS and plating on TSA and TSA plus Km. For macrophage infection with opsonized bacteria, *Brucella abortus* 2308 or the *virB* mutant was treated with a 1:4,000 dilution of naïve rabbit serum, anti-*Brucella* rabbit serum (Difco), or PBS (non-opsonized) for 1 h at 37°C, as described by Bellaire et al. (2). This dilution of both naïve and immune sera was confirmed by microscopy to be nonagglutinating for *Brucella abortus*. After opsonization, inocula were prepared and bone marrow-derived macrophages infected as described above.

Statistical analysis. For determination of statistical significance between experimental groups at an individual time point, either a Student's *t* test or analysis of variance (ANOVA) was performed on the data after logarithmic conversion. A P value of <0.05 was considered significant.

RESULTS

Infection kinetics of the *B. abortus* wild type and *virB* mutant in mice. Determining the time point at which the *virB* mutant first begins to be eliminated from the spleen would give some insight into how the T4SS contributes to persistent infection by *B. abortus*. To this end, a mixed infection experiment was used to assess the level of colonization of both the wild type and the *virB* mutant in the same animals. In a previous study, the rate of clearance of BA41 (*virB*) from mouse spleens during mixed infection was found to be comparable to that observed when mice were inoculated individually with *B. abortus* BA41 (11). The results of this study showed that by 14 days postinoculation, approximately 10-fold more wild-type than mutant bacteria were recovered from splenic tissue in either mixed or individual infections, thereby validating the use of mixed infection for our current study.

To determine when the *virB* mutant begins to be cleared from splenic tissue, we performed a kinetic study of infection of BA41 compared to its isogenic wild-type strain 2308 (Fig. 1). To reproduce the experimental design of the original STM screens (11, 18), BALB/c mice were used. An inoculum consisting of 5×10^5 CFU containing a 1:1 mixture of *B. abortus* 2308, and BA41 (*virB*) was administered i.p. to the mice.

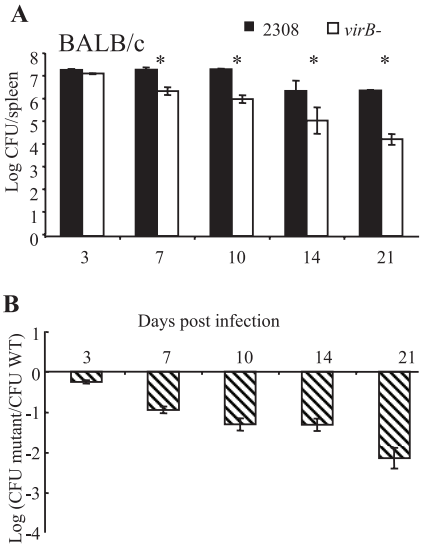

FIG. 1. Kinetics of bacterial load in the spleens of BALB/c ByJ mice following mixed infection with *B. abortus* 2308 (wt) and the *virB* mutant. Mice were inoculated i.p. with 5×10^5 CFU of a 1:1 mixture of wt and the *virB* mutant. Data points represent the geometric means of CFU recovered from groups of five mice per time point ± the standard error. (A) Log CFU of wt and *virB* mutant. Asterisks denote significant differences ($P < 0.05$) between geometric means of *virB* mutant and wt CFU as determined with Student's *t* test. (B) Competitive index, calculated as the ratio of log CFU *virB* mutant/log CFU wild type.

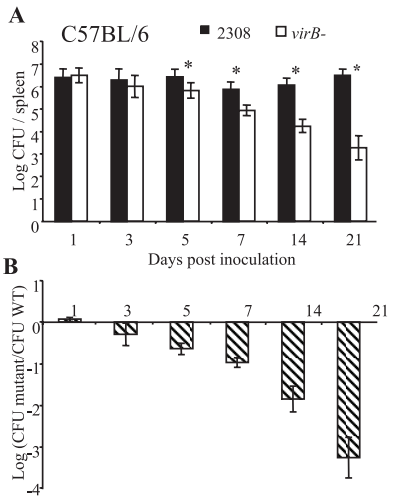

FIG. 2. Kinetics of bacterial load in the spleen following mixed infection of C57BL/6 mice with *B. abortus* 2308 (wt) and the *virB* mutant. Mice were inoculated i.p. with 5×10^5 CFU of a 1:1 mixture of wt and *virB* mutant. Each data point represents the geometric mean of CFU recovered from groups of 10 mice per time point ± the standard error. For each time point, data are combined from two independent experiments, each containing five mice per time point. (A) Log CFU of wt and the *virB* mutant. Asterisks denote significant differences ($P < 0.05$) between geometric means of *virB* mutant and wt CFU as determined with Student's *t* test. (B) Competitive index, calculated as the ratio of log CFU *virB* mutant/log CFU wild type.

Groups of five mice were sacrificed at the time points indicated for determination of splenic CFU. The results of this experiment showed that the CFU of the *virB* mutant recovered from the spleens was nearly identical to that of the wild type at 3 days postinfection (Fig. 1A). The mean log ratio of wild type to *virB* mutant in each mouse was also not significantly different from 1.0 until day 7 postinfection (Fig. 1B), suggesting that the *virB* mutant is initially able to colonize the spleen at levels similar to the wild type.

Based on these results, we hypothesized that one possible explanation for the different observations obtained using in vivo and in vitro models could be that, in addition to its described effect on intracellular trafficking in macrophages, the T4SS may allow *B. abortus* to evade other components of immunity in vivo. To test this hypothesis, we screened a group of mutant mice to identify strains that allowed prolonged persistence of the *virB* mutant.

Since the clearance of the *virB* mutant from the spleens of the mice correlated with the development of adaptive immunity, we examined the ability of mice defective in components of adaptive immunity to eliminate the *virB* mutant. Many of these mutant mice are available on a C57BL/6 strain background but not on a BALB/c background. C57BL/6 mice have been described as more resistant to *B. abortus* infection than BALB/c mice (22); therefore, we repeated the initial experiment using C57BL/6 mice to determine whether the clearance of the *virB* mutant proceeded with similar kinetics in this mouse strain. In this experiment, we included additional time points to determine more precisely when mice start to clear the *virB* mutant. The results (Fig. 2A and B) showed that both the wild type and the *virB* mutant colonized C57BL/6 mice at similar levels during the first 24 h and that a significant difference ($P < 0.05$) between numbers of *B. abortus* 2308 and the *virB* mutant was first observed at 5 days postinfection. At days 14 and 21 postinfection, the recovery of the *virB* mutant was reduced by 2 (day 14) and 3 (day 21) orders of magnitude compared to the wild type. Thus, although the C57BL/6 mice cleared the *virB* mutant more rapidly between 7 and 21 days, the capacity of the *virB* mutant to colonize the spleen during the first few days of infection was similar in both mouse strains.

An IFN-γ defect does not rescue the *virB* mutant. To learn how the T4SS mediates evasion of host immune mechanisms to initiate persistent infection, we first identified immune mechanisms required for clearance of the *virB* mutant from murine spleens. Toward this goal, knockout mice with specific defects in components of the immune response were used to identify mouse strains with a reduced ability to eliminate the *virB* mutant from the spleen. We hypothesized that identification of mouse mutations that can rescue the *virB* mutant, but do not permit increased replication of wild-type *B. abortus*, may pinpoint immune mechanisms that are circumvented by *B. abortus* using its T4SS.

One host response mechanism that has been shown to be elicited in mice by *B. abortus* infection during the time when numbers of the *virB* mutant start to decline is the production of IFN-γ (9, 12). Since IFN-γ production has been shown to be crucial for controlling *B. abortus* infection in mice (22, 32), we postulated that the *Ifng*$^{-/-}$ mice, which are unable to produce IFN-γ (6), might be deficient in controlling replication of the *virB* mutant. To test this idea, we performed a mixed infection study in *Ifng*$^{-/-}$ mice (Fig. 3C) in parallel with C57BL/6 mice

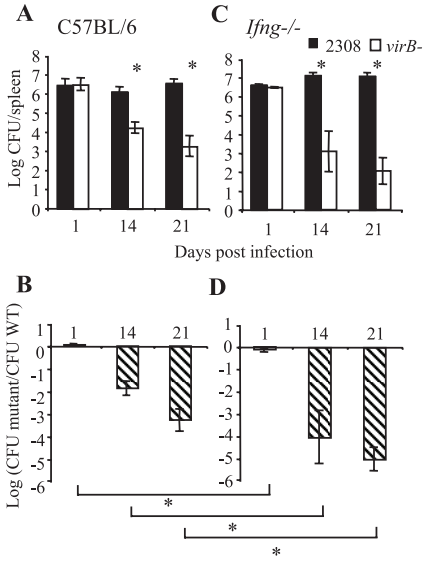

FIG. 3. Recovery of the *virB* mutant and wild type from spleens of C57BL/6 mice (A and B) and *Ifng*$^{-/-}$ mice (C and D) after mixed infection. (A and C) Mean CFU of wild-type and *virB* mutant *B. abortus* recovered from mice ($n = 5$). (B and D) Data points are expressed as competitive index (ratio log CFU mutant/log CFU wt) and represent the geometric mean of data from five mice ± the standard error. Each mouse was infected i.p. with 5×10^5 CFU of a 1:1 mixture of *B. abortus* wild type and *virB* mutant. Graphs are representative of two independent experiments. Asterisks denote a significant difference ($P < 0.05$) between geometric means of *virB* mutant and wt CFU as determined with Student's *t* test. Asterisks in the lower panels (B and D) indicate a significant difference between geometric means of CFU recovered from congenic control mice and mutant mice at each time point. (See text for details.)

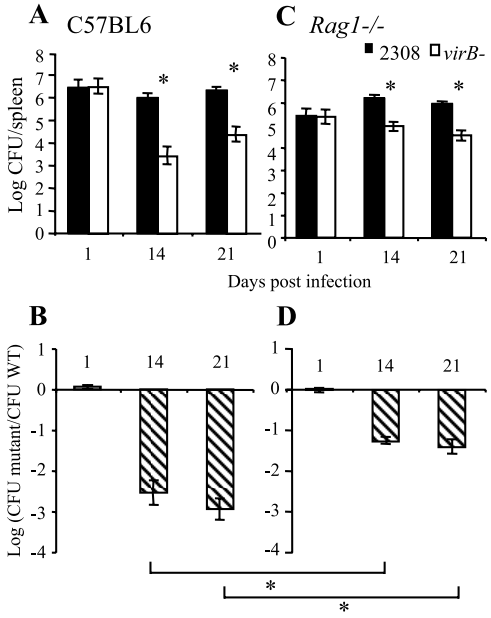

FIG. 4. Recovery of *virB* mutant and wild type from spleens of C57BL/6 mice (A and B) and *Rag1*$^{-/-}$ mice (C and D) after mixed infection. (A and C) Mean CFU of wild-type and *virB* mutant *B. abortus* recovered from mice ($n = 15$). (B and D) Data points are expressed as competitive index (ratio log CFU mutant/log CFU wt) and represent the geometric mean of data from 15 mice ± the standard error. Each mouse was infected i.p. with 5×10^5 CFU of a 1:1 mixture of *B. abortus* wild type and *virB* mutant. Data are combined from three independent experiments, and each group contained five mice per time point. Asterisks in the upper panels (A and C) denote a significant difference ($P < 0.05$) between geometric means of *virB* mutant and wt CFU, as determined with Student's *t* test. Asterisks in the lower panels (B and D) indicate significant differences between geometric means of CFU recovered from congenic control mice and mutant mice at each time point. (See text for details.)

(Fig. 3A). Based on the infection kinetics determined in C57BL/6 mice, we chose 14- and 21-day time points for comparison of C57BL/6 and knockout mice, as the 2- and 3-log difference between CFU of the *virB* mutant and of the wild type recovered at these time points (Fig. 2A and B) would enable us to detect significant differences between control and knockout mice without the use of large groups of experimental animals.

If IFN-γ-activated macrophages controlled replication of the *virB* mutant in vivo, then we expected that the *virB* mutant would exhibit increased survival in the *Ifng*$^{-/-}$ mice. However, the *virB* mutant was recovered in lower numbers from the *Ifng*$^{-/-}$ mice than from C57BL/6 controls (Fig. 3A and C), suggesting that this is not the case. As has been observed previously (22), wild-type *B. abortus* was recovered in significantly higher numbers from the spleens of *Ifng*$^{-/-}$ mice than C57BL/6 mice at both 14 ($P = 0.03$) and 21 ($P = 0.01$) days postinfection. At days 1 and 21 postinfection, we observed no significant difference between the ratios of wild-type *B. abortus* to *virB* mutant in *Ifng*$^{-/-}$ mice and the C57BL/6 controls (Fig. 3B and D). On day 14, the *Ifng*$^{-/-}$ mice actually showed increased clearance of the *virB* mutant compared to the C57BL/6 mice ($P < 0.05$). Based on these results, we concluded that the *Ifng*$^{-/-}$ mice do not have a specific defect in elimination of the *virB* mutant.

***Rag1*$^{-/-}$ mice permit increased splenic persistence of a *B. abortus virB* mutant.** To determine whether complete inactivation of adaptive immunity rescues the *virB* mutant, we compared the ability of *Rag1*$^{-/-}$ mice and the congenic C57BL/6 controls to clear the *virB* mutant after mixed infection. *Rag1*$^{-/-}$ mice are unable to generate functional B or T cells because of a defect in the recombinase-activating gene required for generating immunoglobulin and T-cell receptor molecules (21). The results (Fig. 4A and C) of this experiment showed that both C57BL/6 mice and *Rag1*$^{-/-}$ mice had lower bacterial loads of the *virB* mutant than wild-type *B. abortus* in the spleen at days 14 and 21. However, the ratio of BA41 (*virB*) to *B. abortus* 2308 (wild type) was higher in the *Rag1*$^{-/-}$ mice than in the C57BL/6 controls (Fig. 4B and D). The spleens of *Rag1*$^{-/-}$ mice contained a *virB* mutant/wt ratio of 1:22 in their spleens at day 21, whereas the C57BL/6 mice had a splenic *virB* mutant/wt ratio of 1:1,200, showing that *Rag1*$^{-/-}$ mice were deficient at clearing the *virB* mutant (Fig. 4B and D). Thus, the *Rag1*$^{-/-}$ mutation caused a partial rescue of the *virB* mutant in the mouse spleen without affecting recovery of wt *B. abortus*.

CD4$^+$ T cells and B cells, but not CD8$^+$ T cells, contribute to controlling persistence of the *virB* mutant. Since *Rag1*$^{-/-}$ mice lack functional CD4$^+$ T cells as well as CD8$^+$ T cells and mature B cells, we tested whether mutant mice, in which individual genes required for CD4$^+$ T-cell, CD8$^+$ T-cell, or B-cell function are knocked out, are also more permissive for persis-

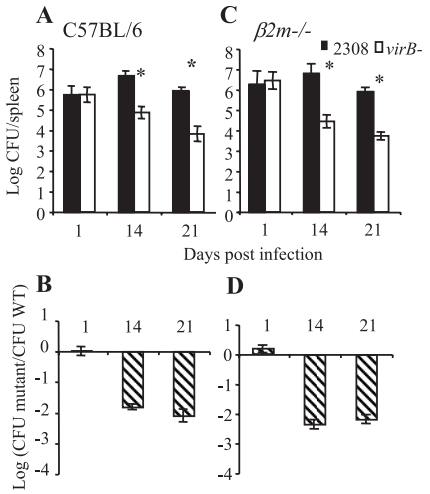

FIG. 5. Recovery of *virB* mutant and wild type from spleens of C57BL/6 mice (A and B) and $\beta_2m^{-/-}$ mice (C and D) after mixed infection. (A and C) Mean CFU of wild-type and *virB* mutant *B. abortus* recovered from mice ($n = 5$). (B and D) Data points are expressed as competitive index (ratio log CFU mutant/log CFU wt) and represent the geometric means of data from five mice ± the standard error. Each mouse was infected i.p. with 5×10^5 CFU of a 1:1 mixture of *B. abortus* wild type and *virB* mutant. Asterisks in the upper panels denote a significant difference ($P < 0.05$) between geometric means of *virB* mutant and wt CFU, as determined with Student's *t* test. Geometric means of CFU recovered from congenic control mice and mutant mice did not differ significantly ($P > 0.05$).

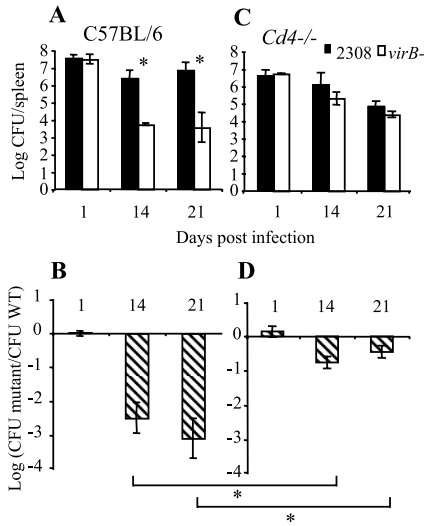

FIG. 6. Recovery of *virB* mutant and wild type from spleens of C57BL/6 mice (A and B) and $Cd4^{-/-}$ mice (C and D) after mixed infection. (A and C) Mean CFU of wild-type and *virB* mutant *B. abortus* recovered from mice ($n = 5$). (B and D) Data points are expressed as competitive index (ratio log CFU mutant/log CFU wt) and represent the geometric means of data from five mice ± the standard error. Each mouse was infected i.p. with 5×10^5 CFU of a 1:1 mixture of *B. abortus* wild type and *virB* mutant. Asterisks in the upper panels (A and C) indicate a significant difference ($P < 0.05$) between geometric means of *virB* mutant and wt CFU as determined with Student's *t* test. Asterisks in the lower panels (B and D) denote significant differences between geometric means of CFU recovered from congenic control mice and mutant mice at each time point. (See text for details.)

tence of the *B. abortus virB* mutant. As with IFN-γ, $CD8^+$ T cells have been shown to be important for controlling *B. abortus* infection. Oliveira and Splitter reported that $CD8^+$ T cells isolated from *B. abortus*-infected mice are able to kill macrophages infected in vitro with *B. abortus* vaccine strain 19 (26). We reasoned that in vivo, a defect in $CD8^+$ T-cell function might rescue the *virB* mutant. To test this idea, we performed mixed infections of C57BL/6 mice and mice deficient in β_2-microglobulin (β_2m; a subunit of major histocompatibility complex [MHC] class I), which are unable to generate $CD8^+$ cytotoxic T cells due to an inability to synthesize functional MHC class I protein (33). Figure 5A to D show that both the absolute numbers of the wild type and *virB* mutant and the ratio of *virB* mutant to wild-type *B. abortus* recovered from $\beta_2m^{-/-}$ mice were similar at days 1, 14, and 21 postinfection. These results show that abrogation of $CD8^+$ T-cell function in the $\beta_2m^{-/-}$ mice does not increase the persistence in the spleen of the *virB* mutant. These results suggested that the T4SS does not mediate evasion of $CD8^+$ T-cell-dependent immune functions.

To determine whether mice lacking $CD4^+$ T cells can rescue the *B. abortus virB* mutant, we performed a mixed infection study using $Cd4^{-/-}$ mice (Fig. 6A to D). At day 1 postinfection, *B. abortus* 2308 and the *virB* mutant were recovered in equivalent numbers from both C57BL/6 mice and the $Cd4^{-/-}$ mice, showing that the T4SS defect in the *virB* mutant does not affect initial colonization of either mouse strain. At day 14 postinfection, the $Cd4^{-/-}$ mice had a higher ratio of *virB* mutant to wild type (1:5.7) in the spleens than did C57BL/6 mice (1:300). Further, at day 14 postinoculation, there were significantly more CFU of the *virB* mutant in the spleens of $Cd4^{-/-}$ mice than in the spleens of C57BL/6 mice ($P < 0.001$). Remarkably, at day 21, the CFU of the *virB* mutant in spleens of $Cd4^{-/-}$ mice were not significantly different from control mice, but CFU of the wild type were significantly lower ($P = 0.01$) in spleens of the $Cd4^{-/-}$ mice than in the spleens of C57BL/6 mice. Thus, the $Cd4^{-/-}$ mice reduced CFU of the *virB* mutant by 1.5 orders of magnitude during the first 21 days postinfection, while control mice reduced CFU of the mutant by 3.5 orders of magnitude (Fig. 6A to D).

The finding that the *virB* mutant was eliminated more slowly by the $Cd4^{-/-}$ mice than by C57BL/6 mice during competitive infection prompted us to determine whether this was also the case if mice were inoculated individually with the wild type or *virB* mutant (Fig. 7). As observed during mixed infections, the $Cd4^{-/-}$ mice harbored fewer CFU of *B. abortus* 2308 in the spleen than C57BL/6 mice at 14 and 21 days after infection (Fig. 7A). The *virB* mutant was recovered in similar numbers from C57BL/6 and $Cd4^{-/-}$ mice at day 14, and at day 21 recovery of the mutant from $Cd4^{-/-}$ mice was slightly higher than C57BL/6 mice; however, this difference was not significant (Fig. 7B).

Both $Rag1^{-/-}$ mice and $Cd4^{-/-}$ mice are defective in B-cell function (21, 27). The lack of functional B cells in these mice may therefore contribute to the reduced ability of these mice to clear the *virB* mutant. To determine whether mice lacking B cells can clear the *B. abortus virB* mutant from mice, we performed a mixed infection study using $Igh6^{-/-}$ mice and control mice (Fig. 8A to D). $Igh6^{-/-}$ mice are deficient in immuno-

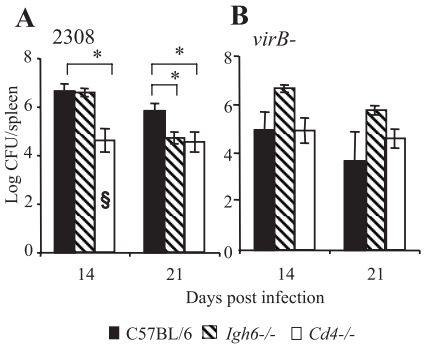

FIG. 7. Recovery of *B. abortus* 2308 (A) and the *virB* mutant (B) from spleens of control, *Igh6* $^{-/-}$, and *Cd4* $^{-/-}$ mice following inoculation with individual strains. Bars represent the geometric means ± standard errors of data from groups of five mice, except where indicated by §, for which $n = 3$. Statistical analysis between geometric means of the CFU of mutant and wild type recovered was performed using ANOVA. Asterisks denote significant differences ($\alpha = 0.05$) between CFU recovered from mutant mice compared to C57BL/6 mice.

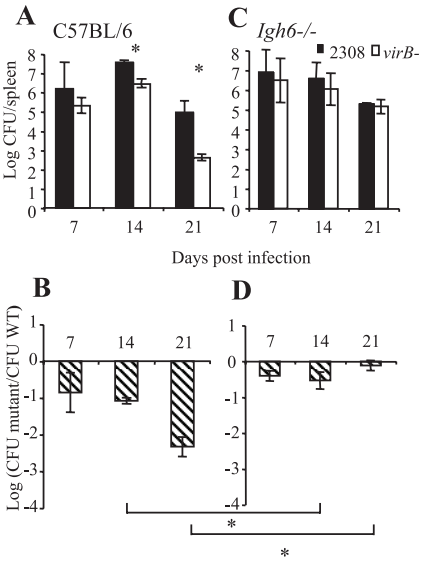

FIG. 8. Recovery of *virB* mutant and wild type from spleens of C57BL/6 mice (A and B) and *Igh6*$^{-/-}$ mice (C and D) after mixed infection. (A and C) Mean CFU of wild-type and *virB* mutant *B. abortus* recovered from mice ($n = 5$). (B and D) Data points are expressed as competitive index (ratio log CFU mutant/log CFU wt) and represent the geometric mean of data from five mice ± the standard error. Each mouse was infected i.p. with 5×10^5 CFU of a 1:1 mixture of *B. abortus* wild type and *virB* mutant. Asterisks in the upper panels (A and C) denote a significant difference ($P < 0.05$) between geometric means of *virB* mutant and wt CFU as determined with Student's *t* test. Asterisks in the lower panels (B and D) indicate a significant difference between geometric means of CFU recovered from congenic control mice and mutant mice at each time point. (See text for details.)

globulin M heavy chain, which is required for normal B-cell development (15). At days 14 and 21 postinfection, the *Igh6*$^{-/-}$ mice had a *virB* mutant to wild type ratio of 1:1.3 in the spleen, while the ratio in C57BL/6 mice was 1:213. Further, the recovery of the *virB* mutant from spleens of *Igh6*$^{-/-}$ mice at day 21 was significantly higher than the recovery from C57BL/6 mice ($P < 0.01$). A second experiment was performed, in which the wild type and *virB* mutant were administered individually to *Igh6*$^{-/-}$ mice (Fig. 7). The results of this experiment showed that the wild type was recovered from both *Igh6*$^{-/-}$ and control mice in similar numbers at 14 days postinoculation; however, at day 21, the *Igh6*$^{-/-}$ mice harbored lower levels of wild-type *B. abortus* in the spleen. The *virB* mutant was recovered in higher numbers than wild type at day 14 and day 21, but these differences were not significant. Taken together, the mixed and individual infection experiments revealed that the *Igh6*$^{-/-}$ mice were slightly better at controlling replication of wild-type *B. abortus* but were more permissive for persistence of the *virB* mutant. Since the *Igh6*$^{-/-}$ mice exhibited the most marked defect in clearing the *virB* mutant and no defect in clearing wt *B. abortus*, we chose this mouse strain for further characterization.

Macrophages isolated from *Igh6*$^{-/-}$ mice do not differ from control macrophages in their ability to control replication of wild-type and *virB* mutant *B. abortus*. Since *Igh6*$^{-/-}$ mice permitted persistence of the *virB* mutant for a longer duration than control mice, we next asked the question whether the *Igh6* mutation could have pleiotropic effects that reduce the ability of macrophages from these mice to control intracellular replication. Although *Igh6*$^{-/-}$ mice have been widely used to study B-cell-dependent responses to infection, no information is available on whether the *Igh6*$^{-/-}$ mutation affects macrophage function. However, given the central role of the macrophage as a site for replication and as an effector cell controlling *Brucella* replication, we considered this possibility. To this end, we compared the ability of macrophages derived from *Igh6*$^{-/-}$ knockout mice to limit intracellular replication of *B. abortus* 2308 or the *virB* mutant. For these experiments, a gentamicin protection assay was used to quantify replication of *B. abortus* in bone marrow-derived macrophages isolated from C57BL/6 mice and *Igh6*$^{-/-}$ mice (Fig. 9A and B). Each experiment was performed in parallel with macrophages from control and knockout mice. These results showed that wild-type *B. abortus* decreased in numbers until 24 h after inoculation and then exhibited a net increase in CFU between 24 and 48 h. In contrast, the *virB* mutant declined below the limit of detection by 24 h and remained undetectable thereafter. Bone marrow-derived macrophages isolated from C57BL/6 or *Igh6*$^{-/-}$ mice did not differ in their ability to control replication of either wild-type *B. abortus* or the *virB* mutant.

Based on these results, we examined whether other macrophage populations, namely, adherent splenocytes and resident peritoneal macrophages isolated from C57BL/6 mice and *Igh6*$^{-/-}$ mice, differed in their ability to limit replication of the *virB* mutant during the first 24 h after inoculation (Fig. 9C to F). In resident peritoneal macrophages from *Igh6*$^{-/-}$ or control mice, wild-type *B. abortus* was present in similar numbers at 1 h and 24 h postinoculation (Fig. 9C). In contrast, the *virB* mutant declined in numbers by 1 order of magnitude between 1 h and 24 h (Fig. 9D). In adherent splenocytes, wild-type *B. abortus* exhibited a net increase in numbers between 1 and 24 h (Fig. 9E), while CFU of the *virB* mutant decreased by approximately 1 order of magnitude (Fig. 9F). No differences were observed between cells isolated from C57BL/6 or *Igh6*$^{-/-}$ mice. Taken together, the results of these experiments showed

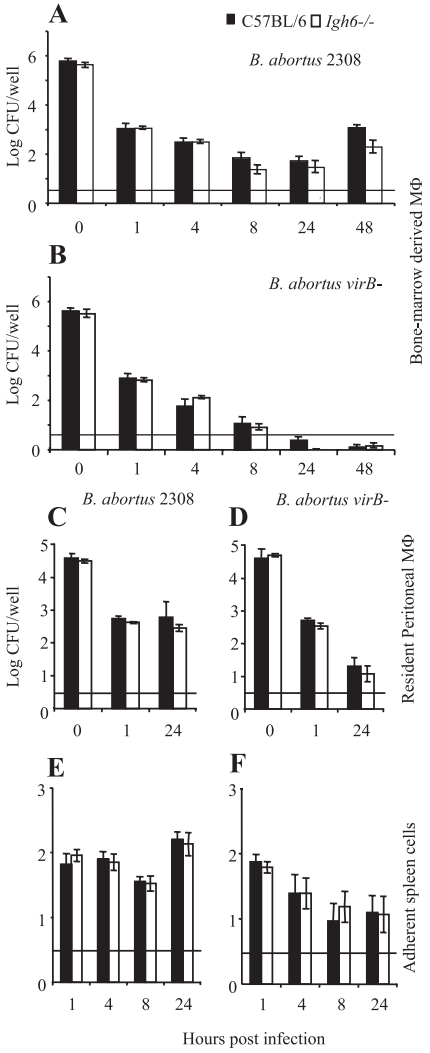

FIG. 9. Recovery of *B. abortus* 2308 or *B. abortus virB* mutant after infection of bone marrow-derived macrophages (MΦ) (A and B), resident peritoneal MΦ (C and D), and adherent splenocytes (E and F) isolated from C57BL/6 or *Igh6*$^{-/-}$ mice. Each graph represents the combined data of two or three independent experiments containing triplicate samples. Data points represent the means ± standard errors. No significant differences were observed in the ability of macrophages from C57BL/6 or *Igh6*$^{-/-}$ mice to control replication of *B. abortus* 2308 (A, C, and E) or the *virB* mutant (B, D, and F).

that the *Igh6*$^{-/-}$ mutation did not affect the ability of macrophages to take up or kill either wild-type *B. abortus* or the *virB* mutant after in vitro infection (Fig. 9A to D). Similar results were obtained using bone marrow-derived or resident peritoneal macrophages isolated from *Cd4*$^{-/-}$ mice (data not shown). These results show that the increased in vivo persistence of the *virB* mutant in *Igh6*$^{-/-}$ mice does not correlate with an inherent defect in the ability of their macrophages to control intracellular replication of the *virB* mutant in vitro.

Killing of the *B. abortus virB* mutant by macrophages is not affected by the presence of *Brucella*-specific antibodies. One possible explanation for the increased persistence of the *virB* mutant in B-cell-deficient *Igh6*$^{-/-}$ mice could be that uptake of opsonized *B. abortus* via Fc receptors may lead to increased

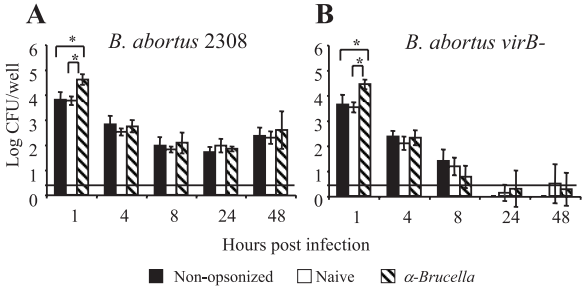

FIG. 10. Recovery of *B. abortus* 2308 (A) or the *B. abortus virB* mutant (B) after opsonization of bacteria and infection of bone marrow-derived macrophages. Bacteria were either nonopsonized, opsonized with naïve rabbit serum, or opsonized with *Brucella*-specific rabbit serum. Each graph represents the combined data of two independent experiments containing triplicate samples. Data points represent the means ± standard deviations. Significant differences ($\alpha = 0.05$) between samples were determined by ANOVA and are indicated with asterisks.

killing of the *virB* mutant by macrophages, compared to wild-type *B. abortus*, which has been shown to survive within macrophages after opsonophagocytosis (2). To test this possibility, we quantified the ability of bone marrow-derived macrophages to kill *B. abortus* 2308 or the *virB* mutant after opsonization with naïve or *B. abortus*-immune rabbit serum (Fig. 10). While opsonization with *B. abortus*-specific serum increased uptake of both wild-type and *virB* mutant *B. abortus*, resulting in 10-fold-greater bacterial numbers at 1 h postinfection, this difference was no longer evident at later time points: at 4, 8, 24, and 48 h, there was no significant difference between intracellular numbers of opsonized and nonopsonized bacteria.

DISCUSSION

The results of this study demonstrate that the *B. abortus* T4SS is not required for initial colonization of mice after i.p. inoculation but is required for persistence beyond 3 days postinfection. In light of results from previous studies, in which *Brucella* localized to phagocytic cells in the spleens of mice (13, 20, 23), this result was surprising, because data obtained using infection of macrophages or macrophage cell lines cultivated in vitro showed a role for the T4SS in intracellular survival that was manifested as early as 4 to 8 h postinfection (Fig. 9). Our findings are in accordance with those from Lestrate et al., who did not identify any *virB* mutants in two different screens for *B. melitensis* signature-tagged mutants attenuated at 5 days postinfection (18, 19), as well as those of Rajashekara et al. (28), who showed that a *B. melitensis virB* mutant was able to disseminate from the site of injection, suggesting that in *B. melitensis* the T4SS is also not essential for initial colonization of mice. The infection kinetics of the *virB* mutant in C57BL/6 mice (a decrease in numbers 3 to 5 days after mixed infection [Fig. 2]) correlate with the timing of adaptive immune responses, as *Brucella*-specific IgM antibodies first appear at 3 to 5 days after infection (data not shown). We therefore hypothesized that the T4SS allows *B. abortus* to evade an immune mechanism that is activated in the mouse after the first 3 days of infection.

To test this hypothesis, we screened a group of mutant

TABLE 1. Effects of mouse mutations on persistence of wild-type and *virB* mutant *B. abortus* relative to responses in C57BL/6 mice

Mouse strain	Principal defect	Effect on persistence of[a]:	
		Wild type	*virB* mutant
Ifng$^{-/-}$	IFN-γ	+	0
$\beta_2m^{-/-}$	MHC class I, CD8$^+$ T cells	0	0
Rag-1$^{-/-}$	B and T cells	0	+
Cd4$^{-/-}$	CD4$^+$ T cells	−	+/0[b]
Igh6$^{-/-}$	B cells	0/−[c]	+

[a] +, the *B. abortus* strain persists at a higher level in mutant mice than in C57BL/6 mice; −, the *B. abortus* strain persists at a lower level in the mutant mouse strain than in C57BL/6; 0, no effect on the level of persistence.

[b] The *virB* mutant persisted at a higher level in mutant mice after mixed infection, but no significant difference was observed after individual infection.

[c] No effect on persistence of wild-type *B. abortus* was observed after mixed infection, but after individual infection of *Igh6*$^{-/-}$ mice, spleens were colonized at a lower level 21 days postinfection.

mouse strains deficient in different immune components to identify mutations that rescue the survival defect of the *virB* mutant. Since the *virB* mutant BA41 was originally identified based on its inability to persist during mixed infection with wild-type *B. abortus*, we had evidence that coinfection with wild-type *B. abortus* does not rescue the *virB* mutant, for example, by type IV secretion of bacterial proteins that may globally disarm the immune response. Further, in this study as in a previous study (11), we recovered 100- to 1,000-fold-fewer CFU of the *virB* mutant from mice between 14 and 21 days compared to wild-type *B. abortus*, irrespective of whether a mixed inoculum was administered or whether the *virB* mutant and wild type were administered separately to mice. Based on this phenotype, we reasoned that the immune mechanism that selectively clears the *virB* mutant must be able to act on *virB* mutant bacteria but not on wild-type bacteria in the same animal. If mice lacked a component of immunity involved in clearing the *virB* mutant, then the *virB* mutant would be expected to be recovered in higher numbers than from control mice after mixed infection. The results of these experiments showed that inactivation of specific immune mechanisms in mice led to different effects on the ability of the *virB* mutant and wt to persist in the spleen (Table 1), suggesting that different immune mechanisms may contribute to limiting persistence of wild-type *B. abortus* and strains lacking the T4SS.

IFN-γ production is elicited in *B. abortus*-infected mice and was shown to begin between 3 and 7 days postinfection (9), suggesting that in vivo, IFN-γ might activate infected cells to kill the *virB* mutant selectively. However, our results (Fig. 3) show that the *Ifng*$^{-/-}$ mutation does not lead to a selective rescue of the *virB* mutant. These data are in agreement with our previous findings that clearance of *virB* mutants from mice is not dependent on inducible nitric oxide synthase or NADPH oxidase (30), which are elicited by IFN-γ. Similarly, a $\beta_2m^{-/-}$ mutation affecting CD8$^+$ T-cell development also failed to rescue the *virB* mutant (Fig. 5).

In contrast, a *Rag1*$^{-/-}$ mutation increased the ability of the *virB* mutant to persist in the spleen (Fig. 4). Since *Rag1*$^{-/-}$ mice lack mature B and T cells, this result suggested that the T4SS may enable *B. abortus* to evade adaptive immunity mediated by these cell types. *Rag1*$^{-/-}$ mice have been reported to be defective in controlling infection with *B. melitensis* (13). However, although the *Rag1*$^{-/-}$ mice did not clear the *virB* mutant as well as C57BL/6 mice, at 14 and 21 days postinfection approximately 10-fold more of wild-type than *virB* mutant *B. abortus* was recovered from the spleens of the *Rag1*$^{-/-}$ mice. Thus, the *Rag1*$^{-/-}$ mice retained some ability to limit persistence of the *virB* mutant by mechanisms that do not depend on B and T cells.

Both *Cd4*$^{-/-}$ and *Igh6*$^{-/-}$ mice were less able to eliminate the *virB* mutant from the spleen than control mice after mixed infection (Fig. 6A to D and 8A to D). These results suggested that B cells and/or CD4$^+$ T cells may be involved in clearance of the *virB* mutant. While it is tempting to speculate that the T4SS may enable *B. abortus* to evade immunity mediated by these cell types, an important consideration in the interpretation of these data is that the knockout mice used in this study may have additional defects or compensatory developmental changes in their immune systems that may affect their ability to clear the *virB* mutant. For example, CD4$^+$ T-cell help is required for antibody responses, and *Cd4*$^{-/-}$ mice have been shown to generate an antibody response to a model antigen that was only 10% of that elicited in control mice (27). Conversely, B cells have been shown to play a role in CD4$^+$ T helper cell function during murine malaria infection (17). Therefore, additional approaches will be required to narrow down the exact defects in these knockout mice that permit prolonged colonization with *B. abortus* strains defective in the T4SS. It should be noted that *Cd4*$^{-/-}$ mice were better able than control mice to control replication of wild-type *B. abortus*, which may be related to a lack of CD4$^+$ CD25$^+$ regulatory T cells in the mutant mice.

An *Igh6*$^{-/-}$ mutation leading to an absence of B cells rescued the survival defect of the *virB* mutant. The decreased ability of *Igh6*$^{-/-}$ mice to limit persistence of the *virB* mutant did not appear to be the result of a reduced capacity of their macrophages to control intracellular replication of the *virB* mutant, as bone marrow-derived, splenic, or resident peritoneal macrophages isolated from these mice were indistinguishable from control mice in their ability to limit intracellular growth of the *virB* mutant in vitro (Fig. 9). Therefore, the inability of these mice to prevent persistence of the *virB* mutant appears to involve a defect that can only be modeled in vivo. The macrophage model has yielded important information on the function of the T4SS in the cell, namely, the subversion of intracellular trafficking to create the endoplasmic reticulum-associated replicative compartment (3, 4, 5). Thus, this function is also expected to be important for T4SS-mediated survival of *Brucella* in vivo.

One possible interpretation of our results was that wild-type *B. abortus* and the *virB* mutant may differ in their ability to survive in macrophages after Fc receptor-mediated phagocytosis and that the absence of specific antibody in the *Igh6*$^{-/-}$ mice could render them more permissive for growth of the *virB* mutant. Since it has been shown that *B. abortus* 2308 opsonized with immune serum can survive and replicate in human monocytes (2), we tested this possibility and showed that in vitro, opsonization with immune serum did not affect intracellular survival of the *virB* mutant.

An alternative interpretation of our data is that additional immune components, such as cytokines or cell populations other than macrophages, may function during infection to

modulate the ability of macrophages to clear the *virB* mutant. The former idea is supported by the finding that human Vγ9Vδ2 T cells are able to limit intracellular growth of *B. suis* within autologous macrophages (25). Thus, one function of the T4SS may be to interfere with recognition of infected macrophages by other immune cells. A second possibility is that the *virB* mutant *B. abortus* may persist in the spleens of $Igh6^{-/-}$ and $CD4^{-/-}$ mice within a cell population that has a reduced ability to kill the *virB* mutant, or that the *virB* mutant may be present extracellularly. Location of bacteria in a different cell population or in an extracellular location may also explain why the T4SS is not required for survival in the spleen during the first 3 days after infection. Additional experiments will be required to distinguish between these different possibilities and to define the role of the T4SS in immune evasion in vivo. We anticipate that the results will give us further insights into how the T4SS enables *B. abortus* to cause persistent infection.

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