INFECTION AND IMMUNITY, July 2009, p. 3004–3013 Vol. 77, No. 7
0019-9567/09/$08.00+0 doi:10.1128/IAI.01114-08

Natural Antibody Contributes to Host Defense against an Attenuated *Brucella abortus virB* Mutant[▽]

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Received 5 September 2008/Returned for modification 22 October 2008/Accepted 29 March 2009

Brucella abortus **is an intracellular pathogen that persists within phagocytic cells of the reticuloendothelial system. To identify in vivo interactions between *B. abortus* and the host that lead to persistent infection, we studied the persistence of *B. abortus* and an isogenic *virB* mutant deficient in the VirB type IV secretion system (T4SS) in knockout mice. In contrast to control mice, mice lacking B cells (*$Igh6^{-/-}$*) were permissive for infection with the attenuated *virB* mutant. To determine the basis for this phenotype, we characterized immune functions of *$Igh6^{-/-}$* mice in the context of *B. abortus* infection. *$Igh6^{-/-}$* mice had greater numbers of extracellular bacteria in the spleen and increased early expression of proinflammatory cytokines during *B. abortus* infection. Further, a *virB* mutant, despite its wild-type level of survival, failed to elicit microgranuloma formation in the spleens of *$Igh6^{-/-}$* mice, suggesting a requirement for the T4SS to elicit this pathological change. Passive transfer of immunoglobulin G from naïve mice restored the ability of *$Igh6^{-/-}$* mice to control the persistence of the *virB* mutant by a complement-independent mechanism. Further, adoptive transfer of $CD11b^{+}$ cells from C57BL/6 mice to *$Igh6^{-/-}$* mice restored the ability of the knockout mice to limit the replication of the *virB* mutant in the spleen, suggesting that the *$Igh6^{-/-}$* mutation affects phagocyte function and that phagocyte function can be restored by natural antibody.**

Human brucellosis is a febrile disease resulting from the transmission of *Brucella abortus*, *B. suis*, *B. melitensis*, or *B. canis* from its respective zoonotic reservoir in cattle, swine, goats and sheep, or dogs (47). These pathogens are endemic in many areas of the world, including Central and South America, the Mediterranean, and Central Asia, and are responsible for an estimated 500,000 new brucellosis cases each year (1). In human brucellosis, as well as in the zoonotic reservoir species, bacteria may persist for long periods of time in the reticuloendothelial system (7). This aspect of infection can be modeled in the mouse, which has been used to identify and characterize the virulence factors involved in the systemic persistence of *Brucella* spp. (2, 22).

One essential virulence factor of the human pathogenic *Brucella* species is the type IV secretion system (T4SS) encoded by the *virB* locus on chromosome II (18, 29, 40). The T4SS of *Brucella* spp., similar to those of other bacterial pathogens, mediates the translocation of proteins into host cells; however, the functions of two newly identified *B. abortus* T4SS substrates, VceA and VceC, is not yet known (12, 25, 39, 45, 46). In cultured macrophages and dendritic cells (DC), the T4SS is essential for the intracellular replication and persistence of *B. abortus* (13, 36, 40). The T4SS mediates exclusion of late endosomal/lysosomal markers from the *Brucella*-containing vacuole and targeting of *B. abortus* to exit sites of the endoplasmic reticulum, where replication occurs (5, 6, 12, 41), suggesting that T4SS effectors are involved in this function. After intraperitoneal (i.p.) inoculation of mice, the T4SS is required not for the initial systemic dissemination of *B. abortus* but rather for persistence in the reticuloendothelial system (31, 34). In order to better understand the interactions between the host and *B. abortus* that lead to the persistence of wild-type (WT) strains and the eventual clearance of *virB* mutants, we examined the immune mechanisms required for clearance of the *virB* mutant. Unexpectedly, mice lacking B cells (*$Igh6^{-/-}$*) were permissive for the splenic persistence of the *virB* mutant, while the persistence of WT *B. abortus* was not increased. When cultured ex vivo, macrophages from *$Igh6^{-/-}$* mice behaved identically to macrophages from control mice in their ability to control the intracellular replication of the *virB* mutant while permitting the replication of WT *B. abortus* (34).

In this study, we attempted to pinpoint the defect in *$Igh6^{-/-}$* mice that renders them permissive for persistent infection by the *virB* mutant. Our results show that nonspecific antibody can reverse the defect of these mice in controlling *virB* mutant replication without affecting WT *B. abortus*. These results suggest that the T4SS mediates the evasion of a natural antibody-dependent immune clearance function by *B. abortus* during persistence in vivo.

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▽ Published ahead of print on 13 April 2009.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. The bacterial strains used in this study were *B. abortus* vaccine strain RB51, WT stain 2308, and its isogenic mutant BA41, 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 and is polar on the expression of downstream genes in the *virB* operon. 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. For cultures of strain BA41, kanamycin was added to the culture medium at 100 mg/liter. All work with live *B. abortus* was performed in a biosafety level 3 facility.

Infection of mice. Female 6- to 8-week-old B6.129S2.*Igh*-6^{tm1Cgn} (*Igh6*$^{-/-}$) (21) mice carrying a targeted knockout of the gene that encodes the immunoglobulin mu chain and age- and sex-matched C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Female 6- to 8-week-old B6.129P2-*FceR1g*^{tm1Rav} N12 (*FcR*$\gamma^{-/-}$) (42) mice deficient in the gamma chain subunit of the FcγRI, FcγRIII, and FcεRI receptors and age- and sex-matched C57BL/6 mice 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 5 to 10 knockout mice or 5 to 10 C57BL/6 mice were inoculated i.p. with 0.1 ml of phosphate-buffered saline (PBS) containing 5×10^4 CFU of a 1:1 mixture of WT and *virB* mutant *B. abortus*. For single infections, four mice per group were infected with 5×10^4 CFU of WT or *virB* mutant *B. abortus*. Twenty-one days after infection, the mice were euthanized by CO_2 asphyxiation and their 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 kanamycin for enumeration of CFU. All animal experiments were approved by the University of California at Davis Laboratory Animal Care and Use Committee and were conducted in accordance with institutional guidelines.

Histopathology and histomorphometry. Spleen samples were fixed in buffered formalin, processed according to standard procedures for paraffin embedding, sectioned at 5 μm, and stained with hematoxylin and eosin. A veterinary pathologist (M.N.X.) examined the slides by using blinded sample analysis. Volumetric proportions of splenic tissue components, including red pulp, lymphoid tissue, microgranulomas, and perifollicular macrophages, were measured with a 25-point circular grid (Zeiss KPL ocular 6.3× with 25 points) over 10 randomly selected microscopic fields (20× objective) for a total of 250 points.

Immunization of mice. C57BL/6 mice were inoculated i.p. with 0.1 ml of PBS containing 5×10^4 CFU of *B. abortus* 2308. Ten weeks later, serum and splenocytes were collected to perform transfer experiments. Age- and sex-matched mice were treated with 0.1 ml PBS alone, and at 10 weeks, serum and splenocytes were also collected.

Splenocyte isolation and transfer. Spleens were obtained from immunized or naïve C57BL/6 mice 10 weeks after infection. After gently teasing apart the spleens, cells were passed through a 70-μm cell strainer and treated 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. Cells were washed with PBS (Gibco) containing 1% bovine serum albumin (PBS-BSA). Cells were counted, and 1×10^6 viable cells were transferred intravenously into *Igh6*$^{-/-}$ mice. One group received PBS-BSA alone. Two days later, the *Igh6*$^{-/-}$ mice were infected with a 1:1 mixture of *B. abortus* WT strain 2308 or the *virB* mutant, and the CFU in their spleens were enumerated 21 days later.

Ex vivo Gm protection assay. Spleens were obtained 21 days after infection from groups of four C57BL/6 or *Igh6* mice infected with *B. abortus* 2308 or the *virB* mutant and gently teased apart to obtain single-cell suspensions. The splenocyte suspension of each spleen was divided in two. One half was treated with gentamicin (Gm, 50 μg/ml) for 4 h, and the other half was left untreated. After Gm treatment, supernatants were removed and plated (extracellular fraction) while splenocytes were lysed with 0.5% Tween 20 and plated (intracellular fraction). The fraction of intracellular or extracellular bacteria in the spleens of C57BL/6 or *Igh6* mice infected with either *B. abortus* 2308 or the *virB* mutant was calculated as the percentage of the CFU of 2308 or *virB* mutant bacteria recovered in the intracellular or extracellular fraction divided by the total number of CFU of strain 2308 or the *virB* mutant.

Serum transfer. Blood was collected from sex- and age-matched immunized or naïve C57BL/6 mice, as well as naïve *Igh6*$^{-/-}$ mice, and serum was obtained. Serum was diluted 1:10 in PBS and transferred intravenously into *Igh6*$^{-/-}$ mice at days 5, 11, and 17 after infection. Spleens were collected at day 21 postinfection, and CFU were enumerated.

IgG purification and transfer. Immunoglobulin G (IgG) from naïve or immunized C57BL/6 mice was purified by using Hi-Trap protein G HP columns (Amersham Biosciences, Piscataway, NJ) and following the manufacturer's instructions. Briefly, serum was passed through the column and washed three times with binding buffer (20 mM sodium phosphate, pH 7.0). IgG was eluted with 0.1 M glycine-HCl (pH 2.7) and dialyzed twice against PBS (pH 7.4). After checking the concentration of purified IgG, 75 μg was transferred intravenously three times into *Igh6*$^{-/-}$ mice at days 5, 11, and 17 after infection. Spleens were collected at day 21 postinfection, and CFU of *B. abortus* were enumerated.

For transfer of Fc and Fab fragments, purified IgG, Fc fragment, and Fab fragment were purchased from Sigma (Saint Louis, MO). Equal molar amounts of IgG, Fc fragment, or Fab fragment were transferred intravenously into *Igh6*$^{-/-}$ mice at days 5, 11, and 17 postinfection. Spleens were collected at day 21 postinfection, and CFU were enumerated.

Complement-antibody sensitivity assay. *B. abortus* 2308 or RB51 or the *virB* mutant was incubated in the presence of naïve or *Brucella*-specific serum supplemented with 30% fresh or heat-inactivated (56°C for 1 h) complement for 24 h at 37°C as described by Corbeil et al. (10). The numbers of CFU in triplicate cultures were determined by plating serial dilutions.

CD11b$^+$ cell isolation and transfer. Macrophages (CD11b$^+$ cells) were isolated from the spleens of naïve C57BL/6 or *Igh6*$^{-/-}$ mice with a MACS CD11b MicroBeads magnetic cell sorting kit from Miltenyi Biotech (Auburn, CA) by following the manufacturer's instructions. Briefly, after gently teasing apart the spleens, cells were passed through a 70-μm cell strainer and treated 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. Cells were washed with PBS-BSA, counted, and incubated with CD11b MicroBeads. Cells were applied to a magnetic column, washed, eluted, and counted. Viable cells (1×10^6) were transferred intravenously into *Igh6*$^{-/-}$ mice. One group received PBS-BSA alone. Two days later, the *Igh6*$^{-/-}$ mice were infected with a 1:1 mixture of *B. abortus* WT strain 2308 or the *virB* mutant, and 21 days later, spleens were collected and CFU were enumerated.

Bone marrow-derived macrophages. Bone marrow-derived macrophages were differentiated from bone marrow precursors of C57BL/6, *Igh6*$^{-/-}$, or *FcR*$\gamma^{-/-}$ mice by following a previously published procedure (34). *Igh6*$^{-/-}$ and C57BL/6 bone marrow macrophages were also differentiated in medium containing no or very little IgG. Ultra-low-IgG fetal bovine serum (Invitrogen) containing less than 5 μg of IgG/ml was further depleted of IgG by passage through a Hi-Trap protein G HP column (Amersham Biosciences, Piscataway, NJ) three times.

Macrophage infection. To evaluate the intracellular survival and growth of *B. abortus*, we used a previously described Gm protection assay (34). Briefly, 24-well microtiter plates were seeded with macrophages at a concentration of 2×10^5/well and incubated overnight at 37°C. Approximately 2×10^7 bacteria in 0.5 ml of RPMIsup or RPMIsup-IgG, containing *B. abortus* 2308 (wild type) or its isogenic *virB* mutant, were added to each well of macrophages. After the addition of Gm (50 μg/ml), wells were sampled at various time points after infection to determine intracellular CFU. Each infection was performed in triplicate, and C57BL/6 macrophages were always assayed together with *Igh6*$^{-/-}$ or *FcR*$\gamma^{-/-}$ macrophages.

RNA isolation. RNA from spleens of C57BL/6 or *Igh6* mice infected with *B. abortus* 2308 or the *virB* mutant for 1 or 3 days postinfection was isolated with Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH) by following the manufacturer's instructions. Briefly, spleen samples were homogenized in 1 ml of Tri-Reagent and incubated for 5 min at room temperature. RNA was extracted by adding 0.2 ml of chloroform and centrifuging the samples at 10,000 rpm for 15 min at 4°C. RNA was precipitated with 0.5 ml of isopropanol and resuspended in H_2O.

cDNA. One microgram of RNA was transcribed to cDNA with TaqMan reverse transcription reagents (Applied Biosystems, Branchburg, NJ). The RNA was mixed with 5 μl of 10× buffer, 11 μl of 20 mM $MgCl_2$, 10 μl of deoxynucleoside triphosphates, 2.5 μl of random hexamers, 1 μl of reverse transcriptase, and H_2O to a final volume of 50 μl. The PCR profile used to transcribe the samples required an incubation step performed at 25°C for 10 min, followed by the reverse transcription step done at 48°C for 30 min and an inactivation step performed at 95°C for 5 min.

Real-time PCR. Reverse-transcribed cDNA was amplified with published primer sets for mouse interleukin-6 (IL-6), IL-10, IL-12p40, chemokine CXCL1 (KC), MIP-2, gamma interferon (IFN-γ), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (30) with SYBR green PCR master mix (Applied Biosystems) and an ABI PRISM 7900HT detection system (Applied Biosystems) by following the manufacturer's instructions. *n*-Fold induction of mRNA was determined from the threshold cycle (C_T) values normalized for GAPDH expression and then normalized to the value derived from the mock-infected C57BL/6 control mice.

Statistical analysis. For determination of the statistical significance of differences between experimental groups, a Student *t* test was performed on the data after logarithmic conversion. A P value of <0.05 was considered significant. For histomorphometry, volumetric proportion data expressed as percentages were submitted to analysis of variance after angular transformation and comparison by

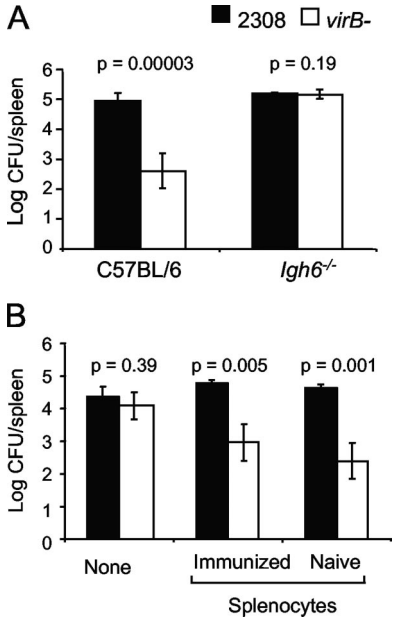

FIG. 1. (A) Bacterial loads in the spleen 21 days after infection of C57BL/6 or *Igh6*$^{-/-}$ mice with a mixed inoculum containing *B. abortus* 2308 (WT) and the *virB* mutant. Mice ($n = 5$) were inoculated i.p. with a 1:1 mixture of WT and *virB* mutant bacteria. The number of CFU of each bacterial strain was determined by differential plating. The significance of differences ($P < 0.05$) between the *virB* mutant and the WT was determined with the Student *t* test. (B) Bacterial loads in the spleens of *Igh6*$^{-/-}$ mice after splenocyte transfer. Splenocytes from immunized or naïve C57BL/6 mice were transferred intravenously into 10 *Igh6*$^{-/-}$ mice per group 2 days before they were inoculated i.p. with a 1:1 mixture of WT and *virB* mutant bacteria. Numbers of CFU from the spleens were determined 21 days after infection. The significance of differences ($P < 0.05$) between the *virB* mutant and the WT was determined by Student's *t* test.

the Tukey test. Analysis of variance was used to analyze the significance of differences between real-time PCR data.

RESULTS

Adoptive transfer of splenocytes from WT mice restores the ability of *Igh6*$^{-/-}$ mice to control the *virB* mutant. *B. abortus* 2308 is able to disseminate from the injection site and establish persistent infection of the spleen after i.p. inoculation of C57BL/6 mice. In contrast, a *virB* mutant can disseminate to the spleen and persist only briefly (5 days) before its numbers begin to decline (34). We reported previously that *Igh6*$^{-/-}$ mice, which do not produce mature B cells due to a targeted inactivation of the IgM heavy chain (21), permit the persistence of a *virB* mutant at the same level as WT *B. abortus* for at least 21 days, suggesting that the T4SS encoded by the *virB* locus contributes to the evasion of an immune mechanism that is defective in *Igh6*$^{-/-}$ mice (Fig. 1A) (34). Based on a time course experiment showing the greatest difference in the *virB* mutant colonization of *Igh6*$^{-/-}$ mice and control mice at 21 days postinfection, we chose this time point for subsequent experiments designed to reconstitute the immune defect of the *Igh6*$^{-/-}$ mice (34). To determine whether this defect could be restored by adoptive transfer, we transferred 1×10^6 splenocytes from either naïve mice or mice that had been infected 10 weeks previously with *B. abortus* 2308. Two days after the adoptive transfer of splenocytes, mice were infected with a 1:1 mixture of *B. abortus* 2308 and the *virB* mutant. Both naïve and immune splenocytes transferred the ability to control the persistence of the *virB* mutant but did not affect the number of WT *B. abortus* CFU recovered from the spleen at 21 days after i.p. infection (Fig. 1B).

***B. abortus* in the spleens of *Igh6*$^{-/-}$ mice are more susceptible to killing by Gm.** Our previous work showed that primary macrophages from *Igh6*$^{-/-}$ mice infected ex vivo were as bactericidal as macrophages from control mice against both WT *B. abortus* and the *virB* mutant, suggesting that increased survival of the *virB* mutant in these mice was not the result of defective macrophage function (34). However, a previous report showed that after uptake by epithelial cells in vitro, a nonpolar *virB10* mutant was recycled to the surface (9). This raised the possibility that in *Igh6*$^{-/-}$ mice the *virB* mutant might persist in an extracellular location, since antibody, which is absent in the *Igh6*$^{-/-}$ mice, might contribute to uptake and killing of extracellular bacteria in vivo. To test this possibility, C57BL/6 or *Igh6*$^{-/-}$ mice were inoculated with either *B. abortus* 2308 or the *virB* mutant BA41. At day 21, when equal numbers of both strains were present in the spleens of *Igh6*$^{-/-}$ mice, the spleens were excised and single-cell suspensions were prepared by gently teasing apart the spleens. This suspension was either treated for 4 h with Gm to kill extracellular bacteria or left untreated to determine the total number of bacteria (Fig. 2A). Data in Fig. 2B show that in C57BL/6 mice, approximately one-third of the *B. abortus* WT bacteria were in a Gm-sensitive or likely extracellular location, while two-thirds were resistant to Gm killing, which is indicative of intracellular residence. As expected, no *virB* mutant bacteria were recovered from C57BL/6 mouse spleens. In *Igh6*$^{-/-}$ mice, the ratio of Gm-susceptible to Gm-resistant bacteria was reversed. Approximately two-thirds of the bacteria were killed by Gm, suggesting an extracellular location, and one-third were Gm resistant or likely to be intracellular. This relationship of intracellular to extracellular bacteria was the same for both the WT and the *virB* mutant, suggesting that the increase in extracellular bacteria is a characteristic of *Igh6*$^{-/-}$ mice and not of the *virB* mutant strain.

***Igh6*$^{-/-}$ mice respond to *B. abortus* infection with expression of proinflammatory cytokines and chemokines.** A possible mechanism underlying the permissive phenotype of *Igh6*$^{-/-}$ mice for the *virB* mutant is a deficient innate immune response. We therefore tested the ability of these mice to respond to *B. abortus* infection with early (1 to 3 days) transcriptional activation of the proinflammatory chemokines KC and MIP-2 and the cytokines IFN-γ, IL-6, tumor necrosis factor alpha, and IL-12/23p40, which are produced early in *Brucella* infection of mice (33, 35, 38, 48). *Igh6*$^{-/-}$ mice expressed higher baseline levels of KC, MIP-2, IFN-γ, IL-12/23p40, and IL-6 than did control (C57BL/6) mice (Fig. 3). Similarly, at day 1 postinfection, *Igh6*$^{-/-}$ mice had higher levels of KC, MIP-2, IFN-γ, and IL-12/23p40 than control mice did. This result suggested that the cytokine milieu in the spleen very early in infection differs between *Igh6*$^{-/-}$ and control mice in that slightly higher levels of proinflammatory cytokines and chemokines are expressed in *Igh6*$^{-/-}$ mice. By day 3 postinfection, both *Igh6*$^{-/-}$ and control mice had similar responses to both WT *B. abortus* and the *virB* mutant (Fig. 3).

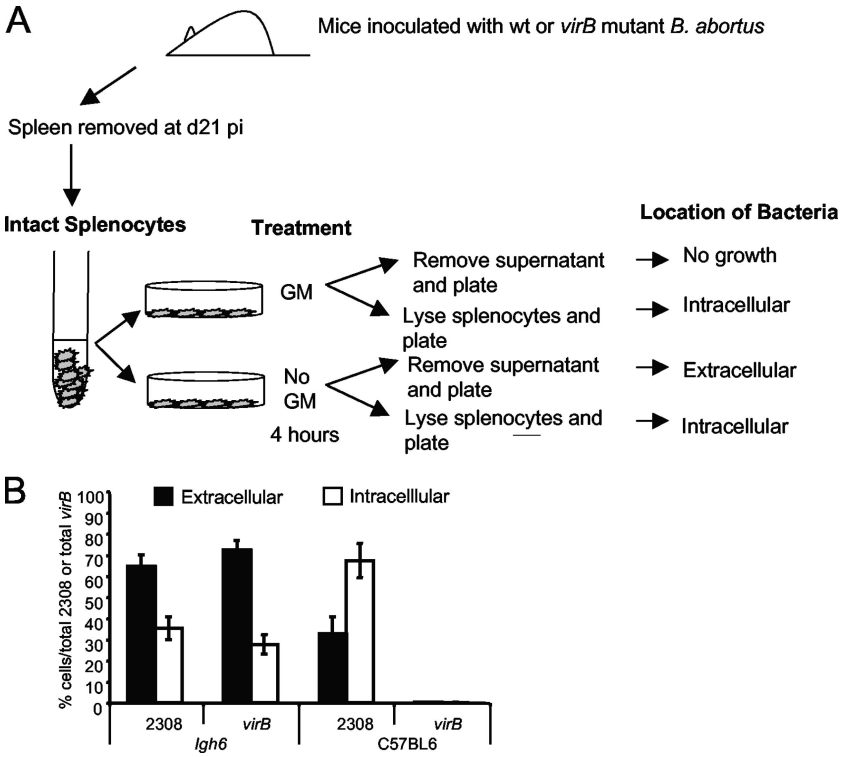

FIG. 2. Ex vivo Gm protection assay of intracellular *B. abortus* 2308 (WT) or the *virB* mutant in the spleens of C57BL/6 mice or *Igh6*$^{-/-}$ mice. (A) Schematic representation of the experiment. Groups of four C57BL/6 or *Igh6*$^{-/-}$ mice were inoculated with either *B. abortus* 2308 or the *virB* mutant. At day 21 postinfection (d21 pi), spleens were removed and teased apart to generate a cell suspension. Each splenocyte suspension was divided into two parts. One half was treated with Gm for 4 h, and the other half was left untreated. After Gm treatment, supernatants of treated and untreated cells were removed and plated to enumerate extracellular (Gm-sensitive) bacteria, while splenocytes were lysed with 0.5% Tween 20 and plated to count intracellular (Gm-protected) bacteria. (B) Percentages of intracellular (white bars) or extracellular (black bars) bacteria in the spleens of C57BL/6 or *Igh6*$^{-/-}$ mice infected with either *B. abortus* 2308 or the *virB* mutant were calculated as the percentage of the total CFU (intracellular or extracellular)/total CFU for both strain 2308 and the *virB* mutant.

The VirB T4SS is required for microgranuloma formation in the spleens of *Igh6*$^{-/-}$ mice. *B. abortus* infection is associated with the formation of focal granulomatous lesions in the spleen, liver, and lymphoid tissues of both humans and rodents, starting at 2 to 3 weeks postinfection (14, 15). To determine whether these later immune responses to *B. abortus* infection may be altered in *Igh6*$^{-/-}$ mice, we performed histopathologic and morphometric analyses of spleen tissue from mice infected for 21 days with either WT or *virB* mutant *B. abortus*. These data are summarized in Fig. 4. As expected, *Igh6*$^{-/-}$ mice did not have well-organized lymphoid follicles, unlike parental strain C57BL/6 mice, as B cells are required for this process (16, 17). Infection with WT *B. abortus* resulted in the formation of microgranulomas in the spleens of both C57BL/6 and *Igh6*$^{-/-}$ mice, which was associated with a decrease in the volumetric proportion of lymphoid follicles. Conversely, the *virB* mutant did not cause marked morphological changes in the spleens of either C57BL6 or *Igh6*$^{-/-}$ mice, with absence of microgranulomas. Interestingly, in spite of its ability to persist at WT levels in *Igh6*$^{-/-}$ mice, the *virB* mutant does not induce microgranuloma formation in these mice. Importantly, WT *B. abortus* is capable of inducing microgranulomas in *Igh6*$^{-/-}$ mice, indicating that the absence of microgranulomas in *Igh6*$^{-/-}$ mice infected with the *virB* mutant is likely to be due to a nonfunctional T4SS rather than the absence of mature B cells.

Passive transfer of natural antibody restores the ability of *Igh6*$^{-/-}$ mice to limit the persistence of the *virB* mutant. *Igh6*$^{-/-}$ mice lack mature B cells and are therefore unable to produce antibodies (21). To test the idea that antibody deficiency may underlie the increased susceptibility of these mice to persistent infection with the *B. abortus virB* mutant, we transferred serum from C57BL/6 mice that were either naïve or had been infected 10 weeks previously with *B. abortus* 2308. To mimic the development of an antibody response during infection, we transferred the serum to *Igh6*$^{-/-}$ mice on days 5, 11, and 17 after inoculation with *B. abortus*. Figure 5A shows that transfer of either naïve or immune serum restored the ability of *Igh6*$^{-/-}$ mice to control the persistence of the *virB* mutant but did not affect the persistence of WT *B. abortus* 2308. Serum from *Igh6*$^{-/-}$ mice had no effect on the persistence of the *virB* mutant (Fig. 5A), suggesting that antibody may be the serum component responsible for this activity. Following up on this observation, we purified serum IgG from either naïve or *Brucella*-immune mice with protein G columns and administered it to *Igh6*$^{-/-}$ mice 5, 11, and 17 days after i.p. inoculation with a 1:1 mixture of *B. abortus* 2308 and the *virB* mutant (Fig. 5B). A similar trend was observed as in the pre-

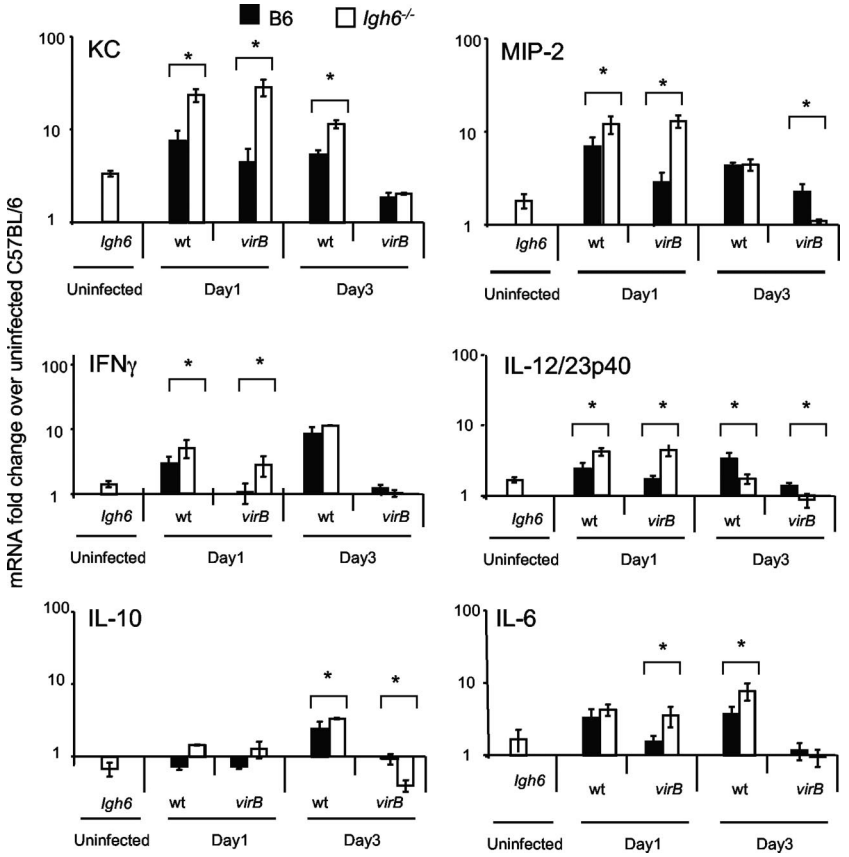

FIG. 3. Quantification of proinflammatory gene transcripts in the spleens of *Igh6*$^{-/-}$ and C57BL/6 mice infected with *B. abortus* 2308 or the *virB* mutant. Transcript levels in spleens from groups of four mice infected with either *B. abortus* 2308 (black bars) or the *virB* mutant (white bars) were assayed by real-time reverse transcriptase PCR at different time points postinoculation. Increases in transcript levels were calculated by normalizing C_T values for each of the gene levels to C_T values for GAPDH levels and to C_T values for each of the gene levels measured in a group of mock-infected C57BL/6 mice. Asterisks indicate significant differences ($\alpha = 0.05$) between *Igh6*$^{-/-}$ and control mice as calculated by analysis of variance.

vious experiment, with IgG from both naïve and *B. abortus*-infected mice conferring the ability to reduce the colonization levels of the *virB* mutant. These results were surprising and suggested that a lack of immunoglobulin per se, and not the ability to mount an adaptive antibody response, contributed to the reduced ability of *Igh6*$^{-/-}$ mice to limit the persistence of *virB* mutant *B. abortus*.

Natural IgG does not affect complement-mediated killing of the *virB* mutant. The previous results showed that passive transfer of IgG mediates increased control of *virB* mutant replication in the spleens of *Igh6*$^{-/-}$ mice. One function mediated by immunoglobulin is fixation of complement, with the Fab portion binding to the bacterium and complement fixation occurring at the Fc end of the molecule. Since *Igh6*$^{-/-}$ mice are deficient in antibody but not in complement, it was possible that passive transfer of natural antibodies could lead to increased complement fixation and/or killing of the *virB* mutant, compared to WT *B. abortus*. To test this idea, we compared the susceptibilities of *B. abortus* 2308, its isogenic *virB* mutant BA41, and rough vaccine strain RB51 to complement-mediated killing. As expected, incubation of bacteria with serum from naïve C57BL/6 mice and fresh rabbit complement reduced the number of CFU of RB51 by >2 orders of magnitude but only led to a fivefold reduction in the number of viable WT *B. abortus* CFU (10). No difference was observed between the killing of *B. abortus* 2308 and that of the *virB* mutant, showing that evasion of complement-mediated killing does not contribute to the increased survival of the *virB* mutant in *Igh6*$^{-/-}$ mice (Fig. 6).

Transfer of CD11b$^+$ cells into *Igh6*$^{-/-}$ mice restores their ability to limit the persistence of the *virB* mutant. *B. abortus* has been shown to localize to macrophages of the reticuloendothelial system during infection (19, 23, 28). Therefore, we considered defective phagocyte function as a possible reason for the inability of *Igh6*$^{-/-}$ mice to eliminate the *virB* mutant from the spleen. Previously, we showed that macrophages isolated from the spleen or peritoneal cavity or differentiated from the bone marrow of *Igh6*$^{-/-}$ mice behaved like those isolated from WT mice in that they permitted intracellular replication of WT *B. abortus* but killed the *virB* mutant (34). Therefore, we considered that our ex vivo culture conditions for macrophages may not completely reflect in vivo conditions. One possible difference was that the macrophages of *Igh6*$^{-/-}$ mice are not exposed to antibody in vivo, but ex vivo they were cultured in the presence of bovine serum, which contains IgG. To more closely mimic the in vivo conditions in *Igh6*$^{-/-}$ mice,

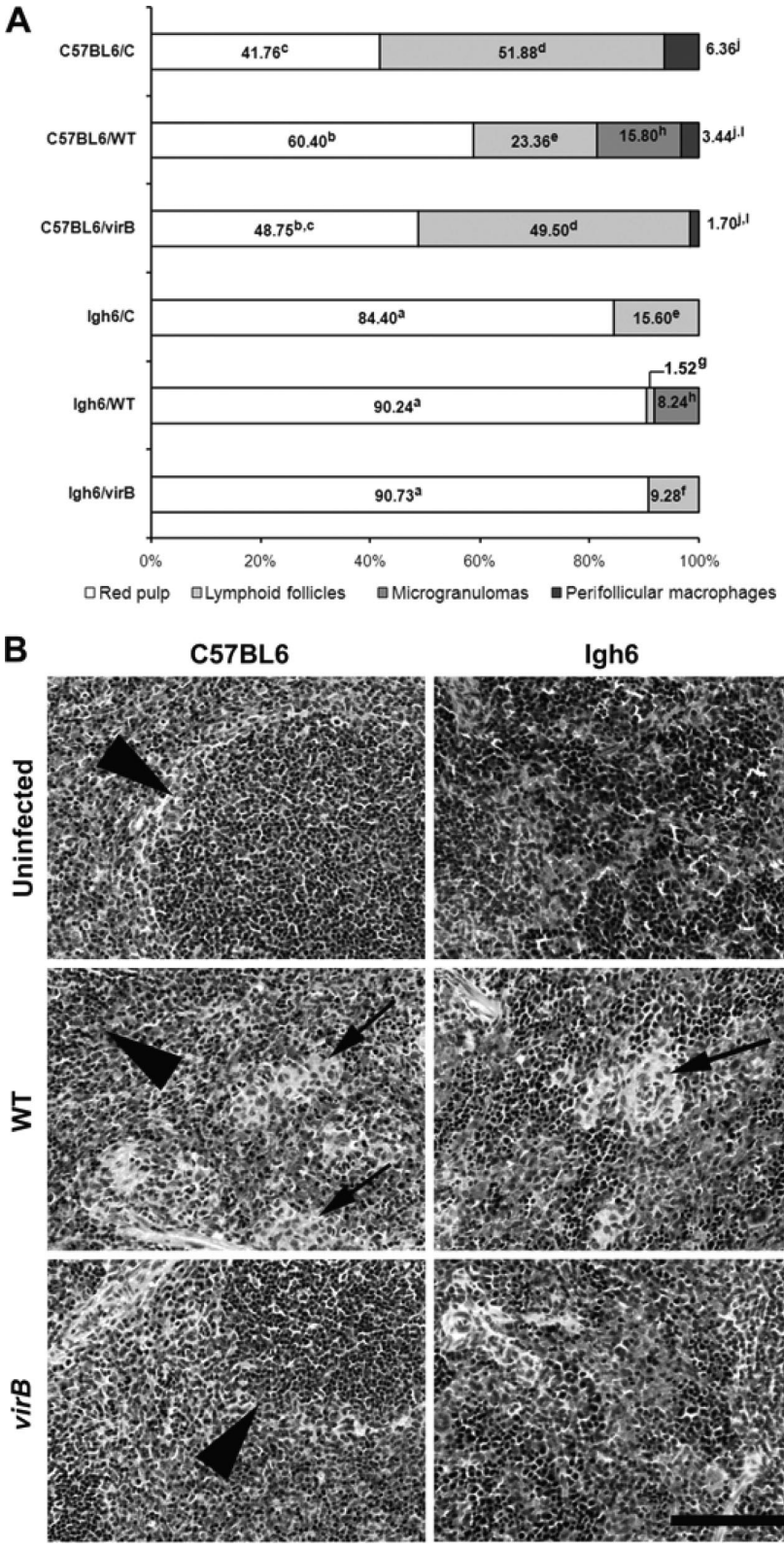

FIG. 4. (A) Morphometric analysis of spleens from C57BL/6 and *Igh6*$^{-/-}$ mice. Columns indicate volumetric proportions of tissue components ($n = 5$ per group). Different superscript letters in the same component indicate statistically significant differences ($P < 0.05$). (B) Representative micrographs of hematoxylin-and-eosin-stained sections of spleen tissue from C57BL/6 and *Igh6*$^{-/-}$ mice infected with WT *B. abortus* or the *virB* mutant or from uninfected controls, as indicated. Arrows indicate microgranulomas, and arrowheads indicate lymphoid tissue. Bar = 100 μm.

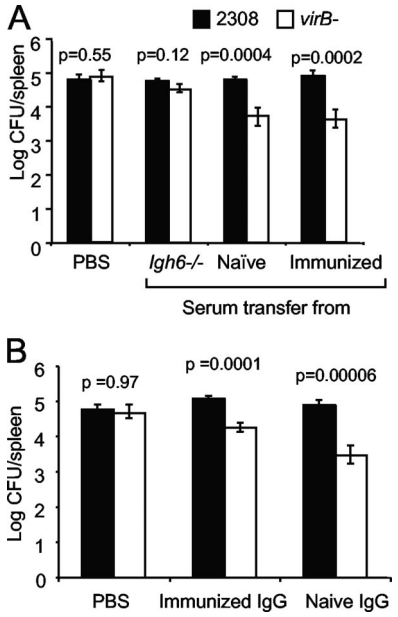

FIG. 5. (A) Bacterial loads in the spleens of $Igh6^{-/-}$ mice after serum transfer. $Igh6^{-/-}$ mice were inoculated i.p. with a 1:1 mixture of WT and *virB* mutant bacteria. Serum from naive $Igh6^{-/-}$ mice, naïve C57BL/6, or immunized C57BL/6 mice was transferred intravenously into 10 $Igh6^{-/-}$ mice per group at days 5, 11, and 17 after infection. CFU were recovered from the spleens 21 days after infection. The significance of differences ($P < 0.05$) between the *virB* mutant and the WT was determined by Student's *t* test. (B) Bacterial loads in the spleens of $Igh6^{-/-}$ mice after IgG transfer. $Igh6^{-/-}$ mice were inoculated i.p. with a 1:1 mixture of WT and *virB* mutant bacteria. IgG purified from naïve or immunized C57BL/6 mice was transferred intravenously into five $Igh6^{-/-}$ mice per group at days 5, 11, and 17 after infection. CFU from the spleens were enumerated 21 days after infection. The significance of differences ($P < 0.05$) between the *virB* mutant and the WT was determined by Student's *t* test.

we cultured macrophages in serum depleted of IgG; however, IgG-depleted serum did not support the growth of either primary cells or J774 macrophages (data not shown). To avoid culturing macrophages ex vivo, we tested their function by enriching macrophages from spleens of C57BL/6 or $Igh6^{-/-}$ mice with anti-CD11b magnetic beads and IgG-free buffers and adoptively transferring them to $Igh6^{-/-}$ mice. These mice were subsequently inoculated i.p. with a 1:1 mixture of WT and *virB* mutant *B. abortus*, and the number of CFU of each strain in the spleen was determined at 21 days after infection (Fig. 7). Adoptive transfer of $CD11b^+$ cells from $Igh6^{-/-}$ mice led to a slight reduction in the recovery of the *virB* mutant from the spleens of the mice, but this was not significant. However, mice that received $CD11b^+$ cells from C57BL/6 mice reduced the load of the *virB* mutant by more than 10-fold relative to that of WT *B. abortus*, suggesting that macrophages from $Igh6^{-/-}$ mice have a reduced function in vivo.

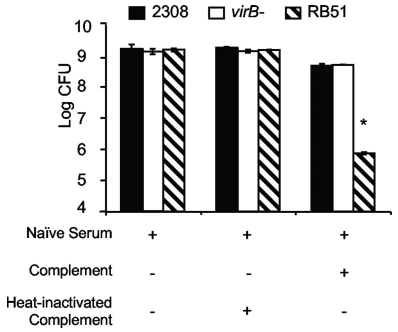

FIG. 6. Susceptibility of *B. abortus* 2308 or RB51 or the *virB* mutant to complement-antibody-mediated killing. Bacteria were treated with fresh or heat-inactivated complement and naïve serum, and CFU were subsequently determined. Bars represent the mean ± standard deviation of a single experiment that was representative of duplicate experiments performed. The asterisk denotes a statistically significant difference ($P < 0.05$) determined with the Student *t* test.

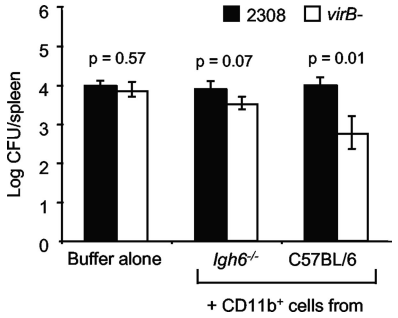

FIG. 7. Bacterial loads in the spleens of $Igh6^{-/-}$ mice after adoptive transfer. $CD11b^+$ cells from $Igh6^{-/-}$ or C57BL/6 mice were transferred intravenously into five $Igh6^{-/-}$ mice per group 2 days before i.p. infection with a 1:1 mixture of WT and *virB* mutant bacteria. CFU of WT and mutant bacteria in the spleens were enumerated 21 days after infection. The statistical significance of differences ($P < 0.05$) between the *virB* mutant and the WT was determined with the Student *t* test.

Role of Fc receptors in limiting *virB* mutant persistence in the spleen. Nonspecific IgG is known to have immunomodulatory properties and is used to treat human autoimmune diseases (8, 20, 43). Evidence has been presented for mechanisms of action mediated by both IgG binding to autoantigens via the Fab domain (3) and binding of the Fc portion to a receptor for the Fc portion of IgG, FcγRII (37). To determine whether one of these mechanisms could contribute to the effect of IgG on $Igh6^{-/-}$ mice, we treated mice with purified fragments of IgG (Fig. 8A). As shown above (Fig. 5), treatment with intact IgG led to a significant reduction in the number of *virB* mutant CFU in the spleens of $Igh6^{-/-}$ mice at 21 days postinfection. Treatment with equimolar amounts of the Fc or Fab portion of IgG did not significantly reduce the persistence of the *virB* mutant.

These results suggested that reduced colonization of the *virB* mutant mediated by passive transfer of IgG requires both Fc and Fab portions of the molecule; however, since Fab fragments are cleared from the circulation more rapidly than intact IgG (11), we could not exclude the possibility that reduced stability of Fc and Fab fragments led to an underestimation of their activity in our experiments. Since receptors for the Fc portion of IgG are present on DC and macrophages, two cell types in which *Brucella* survive intracellularly (4, 36), we reasoned that in normal mice, Fc receptors may have low-affinity interactions with IgG that contribute to cellular functions involved in killing the *virB* mutant. To test this idea, we obtained $FcR\gamma^{-/-}$ mice lacking the Fc receptor gamma chain required for FcγRI, the high-affinity receptor for IgG; FcγRIII, the

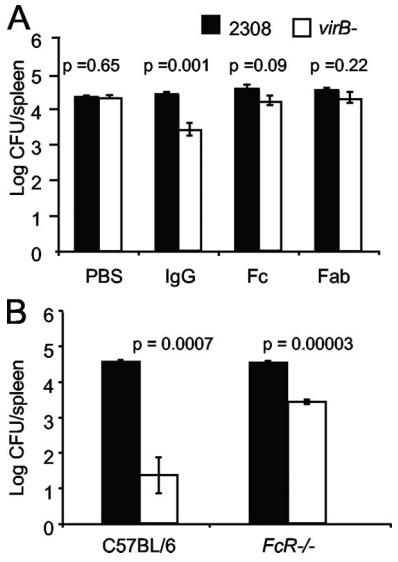

FIG. 8. (A) Bacterial loads in the spleens of *Igh6*$^{-/-}$ mice after passive transfer of IgG, IgG Fc fragment, or IgG Fab fragment. *Igh6*$^{-/-}$ mice were inoculated i.p. with a 1:1 mixture of WT and *virB* mutant bacteria. Equimolar amounts of purified IgG, Fc fragment, or Fab fragment were transferred intravenously into five *Igh6*$^{-/-}$ mice per group at days 5, 11, and 17 after infection. CFU were recovered from the spleens 21 days after infection. (B) Bacterial loads in the spleens of C57BL/6 or *FcR*$^{-/-}$ mice 21 days after infection with a mixed inoculum containing *B. abortus* 2308 (WT) and the *virB* mutant. Mice (four per group) were inoculated i.p. with a 1:1 mixture of WT and *virB* mutant bacteria. The number of CFU of each bacterial strain was determined by differential plating. The significance of differences ($P < 0.05$) between the *virB* mutant and the WT was evaluated with the Student *t* test.

low-affinity IgG Fc receptor; and FcγRIV. These mice were used to determine whether IgG-Fc receptor interactions are required for controlling the systemic persistence of the *virB* mutant (Fig. 8B). For these experiments, FcRγ$^{-/-}$ mice and C57BL/6 controls obtained from the same supplier were inoculated with a 1:1 mixture of *B. abortus* 2308 and the *virB* mutant as described above. At 21 days postinfection, absence of FcγR did not affect the ability of WT *B. abortus* to persist in vivo, since both C57BL/6 and *FcR*γ$^{-/-}$ mice had equal splenic loads of WT bacteria. However, there were 100-fold more of the *virB* mutant bacteria in the spleens of *FcR*γ$^{-/-}$ mice, suggesting that these mice are deficient in limiting the splenic persistence of the *virB* mutant. This defect was not as severe as that of *Igh6*$^{-/-}$ mice (Fig. 5A), which permitted the persistence of the *virB* mutant at the same level as that of WT *B. abortus*, but was significantly different from C57BL/6 mice. Taken together, these results suggested that both natural IgG and Fc receptors are required for limiting the persistence of the *virB* mutant.

DISCUSSION

This study was undertaken to define interactions between *B. abortus* and the host that result in persistent infection. Previously, we characterized several knockout mice lacking components of adaptive immunity to identify a mouse in which the persistence of WT *B. abortus* was unaffected but in which the attenuated phenotype of a *virB* mutant was rescued. We hypothesized that this strategy would enable us to identify immune functions that are evaded by *B. abortus* during infection by using the T4SS. *Igh6*$^{-/-}$ mice, which lack functional B cells and therefore do not make antibody (21), allowed the *virB* mutant to survive in the spleen at WT levels after inoculation with either individual strains or a mixed inoculum of the WT and the *virB* mutant (34), suggesting that this phenotype was not due to rescue of the *virB* mutant by WT *B. abortus*, as proposed based on in vitro studies (26). The finding that *virB* mutants were rescued in *Igh6*$^{-/-}$ mice but the survival of WT *B. abortus* was unaffected suggested that the T4SS contributes to the evasion of a response that depends either directly or indirectly on the function of B cells.

Since *B. abortus* has been localized to phagocytic cells in tissues, one possible explanation for the increased survival of the *virB* mutant in *Igh6*$^{-/-}$ mice was that their phagocytes were defective in killing the *virB* mutant. However, ex vivo, splenic, peritoneal, and bone marrow-derived macrophages exhibited no defects in killing either WT or *virB* mutant *B. abortus* (34). We therefore asked whether *B. abortus* may be extracellular in these mice. Since evidence of intracellular localization in vivo is based on microscopy, it is not known to what extent individual bacteria may be in an intracellular or extracellular location during infection. Ex vivo Gm protection assays showed that in control mice, the majority (65%) of bacteria were in a Gm-resistant, or likely intracellular, location (Fig. 2). However, in *Igh6*$^{-/-}$ mice, the majority of both WT and mutant bacteria (65 to 70%) were killed by Gm. The finding of any extracellular bacteria in either mouse strain was unexpected, since *B. abortus* is considered to be an intracellular pathogen. However, it is possible that a portion of the bacteria within the host is extracellular or that we observed a transient extracellular phase of infection. Although we cannot exclude with our experimental design the possibility that, in *Igh6*$^{-/-}$ mice, *B. abortus* was in a cell type that is more easily lysed during the preparation of splenocyte suspensions, this result suggested that one possible reason for *virB* mutant survival in these mice is that it is predominantly in an extracellular location, where WT *B. abortus* would have no survival advantage over the *virB* mutant.

Study of early host responses to *B. abortus* revealed that very early (1 day) postinfection, *Igh6*$^{-/-}$ mice expressed higher levels of proinflammatory chemokines and cytokines than did control mice. Although this difference disappeared by day 3, it is possible that these early differences in the cytokine milieu also affect the survival of the *virB* mutant in *Igh6*$^{-/-}$ mice, either by affecting polarization of the immune response to infection or by affecting the ability of phagocytic cells to take up and/or restrict the replication of the *B. abortus virB* mutant (Fig. 3). Interestingly, at a later stage of infection (21 days), the VirB T4SS was required to elicit microgranuloma formation in the spleens of *Igh6*$^{-/-}$ mice, a phenotype that could not be observed previously because of the rapid clearance of the mutant from tissues of control mice. Together with our previous observation that the T4SS is required to elicit proinflammatory responses in the spleen during *B. abortus* infection (35), this observation suggests that the T4SS is required to elicit responses required for microgranuloma formation. This response is independent of bacterial numbers, since both WT

and *virB* mutant *B. abortus* bacteria were present in equal numbers in the spleens of these mice.

To examine the mechanistic basis for the rescue of the *virB* mutant phenotype in *Igh6*$^{-/-}$ mice, we used several approaches to reconstitute B-cell-dependent functions in *Igh6*$^{-/-}$ mice. Surprisingly, we found that the ability to limit the persistence of the *virB* mutant could be rescued by passive transfer of IgG purified from *B. abortus*-naïve mice (Fig. 5). Further, the effect of *B. abortus*-immune IgG was the same as or less than that of naïve IgG. This result suggested that the defect of *Igh6*$^{-/-}$ mice that rescues the *virB* mutant is not in adaptive antibody responses to *B. abortus* per se but rather in an innate mechanism (natural antibody) that contributes either directly or indirectly to the control of bacterial infection. An analysis of the effector functions of natural antibody showed that this effect is not related to opsonization of bacteria or to complement-mediated killing of *B. abortus* (Fig. 6 and reference 34). However, it did involve the activity of a population of CD11b^{+} cells, since transfer of these cells to *Igh6*$^{-/-}$ mice restored their ability to eliminate the *virB* mutant from the spleen (Fig. 7). Therefore, our results suggest that, as a result of antibody deficiency, *Igh6*$^{-/-}$ mice have a defect in a population of CD11b^{+} cells.

How could natural antibody affect phagocytic cell function? In human medicine, intravenous IgG, a preparation of pooled IgG, is used therapeutically to treat autoimmune disease (8). Two mechanisms for the function of intravenous IgG have been proposed: one is binding of monomeric IgG to Fc receptors on phagocytic cells, resulting in activation of signal transduction cascades in the cell, and the second is activation of phagocytic cell function by binding via the Fab portion to autoantigens (27, 37). Fc receptors for IgG differ in their distribution on different cell types, in their activity, and in their affinity for different IgG isotypes (32). FcγRI on macrophages binds monomeric IgG2a at high affinity, and under physiologic conditions in vivo, most of the Fc receptors are occupied by monomeric IgG2a (44). Therefore, it is likely that passive transfer of IgG into *Igh6*$^{-/-}$ mice resulted in the binding of monomeric IgG2a to FcγRI, since we observed an effect of naïve serum that was equal to or greater than that of immune serum. This binding could trigger signaling events that either directly activate antibacterial functions of the phagocyte or, alternatively, act indirectly by stimulating the production of cytokines.

To test the first possibility, we determined whether mice defective in Fc receptors for IgG could rescue the *virB* mutant. This rescue was only partial, suggesting that activation of phagocyte function to kill *virB* mutants via IgG-Fc interaction may contribute to the effect of IgG on *Igh6*$^{-/-}$ mice (Fig. 8). An example of the second case is the genetic disease X-linked agammaglobulinemia, in which a lack of DC maturation has been reported. Addition of natural IgG and of autoreactive antibodies to DC from these patients results in their maturation, as assessed by upregulation of major histocompatibility complex class II and costimulatory molecules (3). *B. abortus* resides in DC in vivo, and mouse DC are able to kill *virB* mutants (4, 36). Since DC do not develop normally in *Igh6*$^{-/-}$ mice (24), it is possible that defective DC function is an additional factor that contributes to the persistence of the *virB* mutant. Our results do not allow us to define the relative contributions of Fc receptor signaling and binding of autoantigens to the reconstitution of *Igh6*$^{-/-}$ mice by natural IgG.

Taken together, our results suggest that multiple defects in *Igh6*$^{-/-}$ mice contribute either independently or in concert to the increased persistence of an attenuated *B. abortus virB* mutant. First, residence of increased numbers of bacteria in an extracellular location allows them to avoid phagocyte killing. Second, natural IgG is able to abolish rescue of the *virB* mutant, suggesting that IgG either activates phagocyte functions or contributes to intracellular localization of the bacteria. However, our previous results showing no increase in bacterial uptake by macrophages after opsonization with naïve serum (34), as well as results shown here that resistance to *virB* mutant infection can be adoptively transferred by CD11b^{+} cells, favor the former hypothesis. Together, our findings confirm the importance of phagocytic cells in vivo in restricting the growth of attenuated *B. abortus* strains and point to a novel role for antibody in maintaining the function of these cells.

ACKNOWLEDGMENTS

We thank members of the Tsolis lab for critical reading of the manuscript, as well as A. Bäumler, N. Baumgarth, and C. Baldwin for helpful discussions of these results.

This work was supported by PHS award AI050553 to R.M.T. R.L.S. and M.N.X. are recipients of fellowships from CNPq (Brazil).

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Editor: J. B. Bliska