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Inactivation of the Type IV Secretion System Reduces the Th1 Polarization of the Immune Response to *Brucella abortus* Infection▽

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The *Brucella abortus* type IV secretion system (T4SS), encoded by the *virB* operon, is essential for establishing persistent infection in the murine reticuloendothelial system. To gain insight into the in vivo interactions mediated by the T4SS, we compared host responses elicited by *B. abortus* with those of an isogenic mutant in the *virB* operon. Mice infected with the *B. abortus virB* mutant elicited smaller increases in serum levels of immunoglobulin G2a, gamma interferon (IFN-γ), and interleukin-12p40 than did mice infected with wild-type *B. abortus*. Despite equal bacterial loads in the spleen, at 3 to 4 days postinfection, levels of IFN-γ were higher in mice infected with wild-type *B. abortus* than in mice infected with the *virB* mutant, as shown by real-time PCR, intracellular cytokine staining, and cytokine levels. IFN-γ-producing $CD4^+$ T cells were more abundant in spleens of mice infected with wild-type *B. abortus* than in *virB* mutant-infected mice. Similar numbers of IFN-γ-secreting $CD8^+$ T cells were observed in the spleens of mice infected with *B. abortus* 2308 or a *virB* mutant. These results suggest that early differences in cytokine responses contribute to a stronger Th1 polarization of the immune response in mice infected with wild-type *B. abortus* than in mice infected with the *virB* mutant.

Brucella abortus is an intracellular pathogen that resides mainly in phagocytic cells and causes disease in animals and humans (3, 43). The infection in both natural and incidental host species is characterized by bacterial persistence in the reticuloendothelial system. This persistent phase of the infection can be studied in the mouse model, which has been used to identify virulence mechanisms used by *Brucella* spp. to establish persistence (15, 17, 22, 31, 32).

Protection against *B. abortus* infection is thought to be mediated primarily by a Th1 type of immune response (1, 44). *B. abortus* triggers host antigen-presenting cells to release interleukin-12 (IL-12), which causes Th0 cells to differentiate into gamma interferon (IFN-γ)-secreting Th1 cells that are capable of activating macrophage microbicidal mechanisms (25, 44). In vitro and in vivo studies using mouse or murine macrophages have shown that infection with *Brucella* spp. triggers the production of IL-6, IL-1, and tumor necrosis alpha, whereas in humans, IL-1, IL-6, and IL-8, but not tumor necrosis alpha, are produced during infection (5, 11, 28). While IFN-γ and IL-12 promote the control of *Brucella* replication in the mouse, IL-10 decreases the ability of mice to control infections with *B. abortus* (12, 33).

The type IV secretion system (T4SS) encoded by the *virB* operon is essential for establishing persistent infection by *Brucella* spp. in mice (10, 17, 35, 38). In addition, it has been shown to contribute to intracellular survival in in vitro models of infection by allowing the vacuole containing *Brucella* spp. to exclude lysosomal proteins and associate with the exit sites of the endoplasmic reticulum (6–9). This endoplasmic reticulum-associated compartment appears to be the preferred niche for the intracellular replication of *Brucella* spp. Although it is widely thought that the formation of the replicative niche for *Brucella* requires the translocation of effector proteins into the host cell that interfere with vacuolar trafficking, no secreted effectors of the T4SS have been identified to date.

Our knowledge about how *Brucella* spp. persist in the host in the face of an active immune response to the bacteria is limited. In particular, the relationship between T4SS-mediated intracellular survival and replication in vitro and persistence in the reticuloendothelial system in vivo is unclear. *B. abortus* mutants lacking an intact T4SS are initially able to colonize the spleens of mice during the first 3 days after infection at the same levels as those of wild-type bacteria (31, 32). Furthermore, while *B. abortus* infection results in the early activation of host genes involved in inflammation and immunity, mutants lacking a functional T4SS do not trigger this response (32). To gain further insight into how the T4SS affects the host response to infection, we characterized serum antibody and cytokine responses to *B. abortus* 2308, a wild-type strain, and an isogenic *virB* mutant. The results of these studies showed that while infection with wild-type *B. abortus* elicits a Th1 type of immune response, this polarization is decreased in mice infected with a mutant lacking a functional T4SS.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. Bacterial strains used in this study were *Brucella abortus* wild-type strain 2308 and its isogenic mutant strain BA41 (17), which has an insertion of mTn5Km2 at nucleotide 1232 of the *B.*

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abortus virB locus (GenBank accession number AF226278). This insertion is located 59 bp downstream of the *virB1* gene and is polar upon the expression of downstream genes in the *virB* operon (39). Strains were cultured on tryptic soy agar (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 tryptic soy agar plus 5% blood (2). For cultures of strain BA41, kanamycin (Km) was added to the culture medium at 100 mg/liter. All work with live *B. abortus* cells was performed at biosafety level 3.

Infection of mice. Female C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and were used at the age of 6 to 8 weeks. Mice were held in microisolator cages with sterile bedding and water and irradiated feed in a biosafety level 3 facility. Groups of 5 to 10 mice were inoculated intraperitoneally (i.p.) with 0.1 ml of phosphate-buffered saline (PBS) containing 5×10^5 cells of a *B. abortus* wild-type strain or an isogenic *virB* mutant. All animal experiments were approved by the Texas A&M University and University of California Laboratory Animal Care and Use Committees and were conducted in accordance with institutional guidelines.

Multiplex cytokine assays. Detection of specific cytokines in the serum or spleens of C57BL/6 mice was performed using Multi-Plex cytokine assays (4) (Bio-Rad, Hercules, CA). Groups of five C57BL/6 mice were infected i.p. with 5×10^5 cells of either the wild type or the *virB* mutant, and serum was obtained from the saphenous vein at days 1, 4, and 7 postinfection. Cytokine detection was performed according to the instructions provided by the kit's manufacturer. Briefly, serum was filtered twice through a 0.2-μm filter and then diluted 1:3 in Multi-Plex sample diluent buffer, added to the plate, and incubated at room temperature for 30 min. The samples were washed several times, and 25 μl of the detection antibody solution was added to each well. Washes were monitored by incubation at room temperature for 30 min. The samples were washed, and 50 μl of streptavidin-phycoerythrin was added and incubated at room temperature for 10 min. The samples were washed and resuspended in 125 μl of assay buffer (proprietary formula) and read using the Luminex 100 instrument (Bio-Rad).

For the detection of specific cytokines in the spleen, groups of five C57BL/6 mice were infected i.p. with 5×10^5 cells of either wild-type or *virB* mutant *B. abortus*, and spleens were obtained at days 1, 4, and 7 postinfection. Spleens were homogenized in 3 ml of PBS and passed through a 100-μm cell strainer. The cells were centrifuged for 10 min at 1,000 rpm. The supernatant was passed twice through a 0.2-mm filter and cultured to ensure that no viable bacteria were present. Cytokines were concentrated by using Millipore 5K filter devices and stored at −80°C. Cytokine detection was performed as described above.

ELISA. The presence of antibody specific for *Brucella abortus* in the serum samples from 10 C57BL/6 mice infected with *B. abortus* 2308 and 10 mice infected with the *B. abortus virB* mutant was determined by indirect enzyme-linked immunosorbent assay (ELISA). MaxiSorp plates from Qiagen (Valencia, CA) were coated with 100 μl formalin-killed whole *B. abortus* cells (1 μg/ml) in carbonate buffer (pH 9.6), and plates were incubated at 4°C overnight. After washing with PBS and 0.05% Tween 20, the serum samples were diluted 1:100 in PBS containing 1% bovine serum albumin and incubated at room temperature for 1 h. After washing with PBS and 0.05% Tween 20, the reactivity was measured using horseradish peroxidase-conjugated anti-mouse immunoglobulin M (IgM), IgG, IgG1, IgG2a, or IgG3 (1:1,000; BD Pharmingen, San Diego, CA) by incubating the plates at 37°C for 1 h. The reaction was developed with Sigma Fast *o*-phenylenediamine dihydrochloride tablet sets. The resulting color was read at 410 nm with an ELISA microplate reader (MR5000; Dynatech). Data points are the averages of duplicate dilutions, with each measurement being performed twice.

RNA isolation. RNA was isolated using Tri reagent (Molecular Research Centre, Inc., Cincinnati, OH). 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 using TaqMan reverse transcription reagents (Applied Biosystems, Branchburg, NJ). The RNA was mixed with a solution containing 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 in a final volume of 50 μl. Samples were incubated at 25°C for 10 min, followed by reverse transcription at 48°C for 30 min and inactivation at 95°C for 5 min.

Real-time PCR. Reverse-transcribed cDNA was amplified with previously reported primer sets (26) for mouse IFN-γ (forward primer TCA AGT GGC ATA GAT GTG GAA GAA and reverse primer TGG CTC TGC AGG ATT TTC ATG) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward primer TGT AGA CCA TGT AGT TGA GGT CA and reverse primer AGG TCG GTG TGA ACG GAT TTG) using Sybr green PCR master mix (Applied Biosystems) and an ABI Prism 7900HT detection system (Applied Biosystems) according to the manufacturer's instructions. 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 naive controls (23).

Intracellular cytokine staining. Intracellular staining for IFN-γ was performed in splenocytes from groups of four C57BL/6 mice infected for 3 days with *B. abortus* 2308 or a *virB* mutant. Splenocytes from each mouse were analyzed individually using four-color flow cytometry. Briefly, after passing the spleen 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, splenocytes were washed with PBS (Gibco) containing 1% bovine serum albumin (fluorescence-activated cell sorter [FACS] buffer). Cells were incubated for 6 h in RPMI 1640–5% fetal bovine serum with 10 μg/ml brefeldin A (Sigma). Cells were stained individually or with a cocktail of Pacific Blue-conjugated rat anti-mouse CD4 (L3T4), Alexa Fluor 700-conjugated rat anti-mouse CD8a (Ly-2), and allophycocyanin II-Alexa Fluor 750 rat anti-mouse CD3e (eBioscience). The cells were washed with FACS buffer and fixed with 4% formaldehyde for 30 min at 4°C, followed by permeabilization for 30 min using a saponin-based buffer and staining with phycoerythrin-conjugated anti-mouse IFN-γ for 30 min at 4°C. Finally, samples were washed in FACS buffer containing saponin. Flow cytometry analysis was performed using an LSRII apparatus (Becton Dickinson, San Diego, CA), and data were collected for over 1 million cells/mouse. The CD3 cells were gated (R1) based on side scatter and CD3 expression. $CD4^+$ or $CD8^+$ T cells were gated (R2) based on CD3 expression and CD4 or CD8 expression, respectively.

Statistical analysis. For the determination of statistical significance between experimental groups, a Student's *t* test was performed on the data after logarithmic conversion. A *P* value of <0.05 was considered to be significant.

RESULTS

The T4SS mutant and the wild type elicit different antibody responses in infected mice. To compare host responses to wild-type *B. abortus* and a *virB* mutant, anti-*B. abortus* IgG levels in serum from infected mice were measured by ELISA. The results of this experiment (Fig. 1A) revealed that between days 7 and 21, the *virB* mutant elicited a higher *B. abortus*-specific IgG response than strain 2308, while after day 56, mice infected with wild-type *B. abortus* exhibited higher IgG titers. This trend was also observed when IgM was assayed in the same serum samples (data not shown). The differences in titer early during infection may reflect increased killing and presentation of antigen from the *virB* mutant, which started to be cleared from spleens of infected mice by 5 days after infection, while CFU of the wild-type strain did not decline (31). To determine whether these IgG responses are qualitatively similar, we assayed the same serum samples for IgG1, IgG2a, and IgG3 isotypes. Both strains elicited similar levels of IgG1 (Fig. 1B) and IgG3 (Fig. 1D), but levels of IgG2a (Fig. 1C) were significantly higher in mice infected with *B. abortus* 2308 between 35 and 70 days postinfection. In an identical experiment performed using BALB/c mice (data not shown), we obtained the same results, showing that this difference is not related to differences between the two mouse strains in their responses to *Brucella* infection (25).

The *virB* mutant elicits reduced levels of circulating IFN-γ and IL-12 in comparison with those of wild-type *B. abortus*. In order to determine whether the higher levels of IgG2a elicited by wild-type *B. abortus* reflected a greater Th1 polarization of the early immune response, we determined the levels of the Th1-associated cytokines IFN-γ and IL-12 after infection with wild-type *B. abortus* or the *virB* mutant. For these experiments,

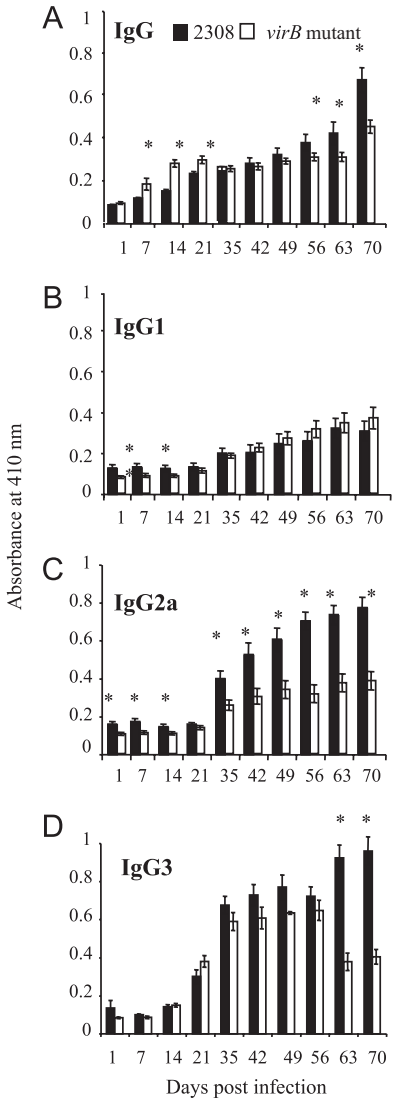

FIG. 1. Measurement of antibody levels in sera from mice infected with *B. abortus* 2308 or the *virB* mutant by ELISA. Samples from groups of 10 mice inoculated i.p. with 5×10^5 cells of either *B. abortus* 2308 or the *virB* mutant were taken at different times postinoculation. The significance of differences between arithmetic means of data for wild-type and mutant mice at each time point was determined using a Student's *t* test. Asterisks indicate significant differences between immunoglobulin levels in mice infected with 2308 and those in mice infected with the *virB* mutant ($P < 0.05$).

mice were infected with either wild-type *B. abortus* or the *virB* mutant, and serum was obtained at days 1, 4, and 7 postinfection. The detection of IFN-γ and IL-12p40 was performed using BioPlex cytokine assays (Fig. 2). Since the cytokine levels in naive serum varied among mice, the increase in the cytokine concentration in infected serum samples over that in uninfected serum samples was calculated (Fig. 2). Results of the cytokine assays showed that on days 4 and 7 postinfection, levels of IFN-γ and IL-12p40 remained unchanged in the sera of mice infected with *B. abortus virB*, whereas in mice infected with the *B. abortus* wild-type strain, the levels of IL-12p40 and IFN-γ increased significantly above preinfection levels.

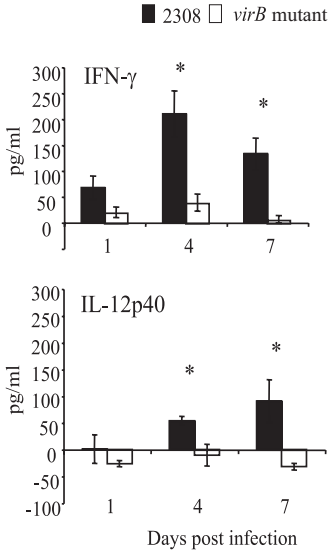

FIG. 2. Quantification of cytokine levels in sera of mice infected with *B. abortus* 2308 or the *virB* mutant. Samples from groups of 10 mice infected i.p. with either *B. abortus* 2308 or the *virB* mutant were taken at different times postinoculation, and cytokine concentrations were determined by Multi-Plex assays. Data shown are increases in cytokine concentrations over preinfection levels. The significance of differences for wild-type and mutant mice at each time point was determined using a paired Student's *t* test. Error bars represent standard deviations. Asterisks indicate significant differences between the wild type and the mutant ($P < 0.05$).

Splenocytes of mice infected with the *Brucella* wild-type strain produce higher levels of IFN-γ than do splenocytes of mice infected with the T4SS mutant. Since *B. abortus* grows and persists in tissues of the reticuloendothelial system, we determined whether the difference in the level of circulating IFN-γ correlates with changes in IFN-γ production in splenic tissue when the CFU levels of 2308 and the *virB* mutant are similar. RNA from spleens isolated from mice infected with *B. abortus* 2308 or the *virB* mutant was used to quantify IFN-γ transcripts by quantitative real-time PCR. The results shown in Fig. 3A are normalized to levels of GAPDH and to levels in uninfected mice using the $2^{-\Delta\Delta CT}$ formula. Figure 3B (bottom) shows increases in IFN-γ protein levels measured by a BioPlex assay in concentrated supernatants of spleen homogenates. Significantly greater increases in both IFN-γ transcripts and protein levels were observed at day 4 postinfection in mice infected with *B. abortus* 2308 than in mice infected with the *virB* mutant, which is in agreement with the levels of IFN-γ measured in the serum (Fig. 2).

Splenic $CD4^+$ T cells, but not $CD8^+$ T cells, produce more IFN-γ when mice are infected with wild-type *B. abortus* than when they are infected with the *virB* mutant. We decided to determine the main source of IFN-γ in splenic T cells after infection with wild-type *Brucella* or the *virB* mutant (Fig. 4 and 5). Three days after infection, when the bacterial burdens of wild-type *B. abortus* and the *virB* mutant were similar (31, 32; data not shown), mice infected with wild-type *B. abortus* had more numerous IFN-γ-producing $CD4^+$ T cells (9.7% ± 0.6%) than did *virB* mutant-infected mice (2.3% ± 0.5%) (Fig. 6). However, no difference in the frequencies of IFN-γ-pro-

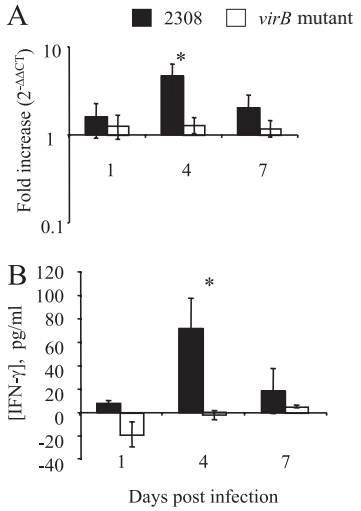

FIG. 3. Quantification of IFN-γ levels in spleens of mice infected with *B. abortus* 2308 or the *virB* mutant. (A) Transcript levels in spleens from groups of five mice infected with either *B. abortus* 2308 (black bars) or the *virB* mutant (white bars) were assayed by real-time reverse transcription-PCR at different times postinoculation. Increases ($2^{-\Delta\Delta CT}$) in transcript levels were calculated by normalizing C_T values for IFN-γ levels to C_T values for GAPDH levels and to C_T values for IFN-γ levels measured in a group of mock-infected mice. (B) Cytokine measurement of IFN-γ in splenic tissue by Multi-Plex assay was performed with groups of five mice infected with either *B. abortus* 2308 or the *virB* mutant at different times postinoculation. Data shown are increases in cytokine concentrations over preinfection levels. The significance of differences for wild-type and mutant mice at each time point was determined using a paired Student's *t* test. Error bars represent standard deviations. Asterisks indicate significant differences between the wild type and the mutant ($P < 0.05$). Results shown at the top and bottom are from two independent experiments.

ducing $CD8^+$ T cells in wild-type and mutant-infected mice was observed (2.7% ± 0.6% for 2308 and 2.6% ± 0.5% for the *virB* mutant) (Fig. 6). This result shows that $CD4^+$ T cells are the source of increased levels of IFN-γ elicited by *B. abortus* and that infection with the *virB* mutant elicits reduced IFN-γ production by splenic $CD4^+$ T cells.

DISCUSSION

This study was undertaken to investigate the in vivo effects of the *B. abortus* T4SS on the host response to infection. We showed previously that during the infection of mice with *B. abortus* wild-type isolate 2308 and its isogenic *virB* mutant, both strains are present in similar numbers for the first 5 days postinfection (31). By 5 days postinfection, colonization of the spleen by the T4SS mutant starts to decrease relative to that of the wild-type, which persists at a constant level (31). The finding that both the *virB* mutant and the wild type are initially able to colonize the spleen at similar levels following i.p. inoculation suggests that during the first few days after infection, the *virB* mutant is able to resist immune clearance as well as does wild-type *B. abortus*. However, despite equal survival rates in tissues during this early period (3 to 4 days) after infection, wild-type *B. abortus* and *virB* mutant *B. abortus* elicited very different transcriptional responses in host splenocytes. While infection with wild-type *B. abortus* resulted in the upregulation of genes involved in inflammation and immunity, including IFN-γ and genes induced by both IFN-γ and IFN-α/β, the *virB* mutant used in the present study did not (32). To determine whether these differences in early host transcriptional responses in the spleen correlate with differences in global immune responses to both *Brucella* strains, we examined serum

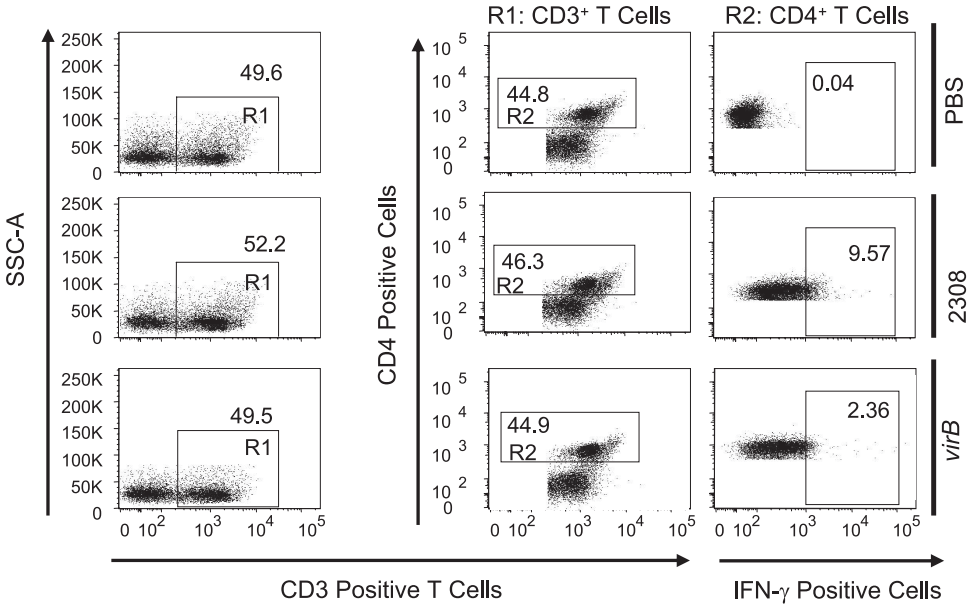

FIG. 4. IFN-γ-producing $CD4^+$ T cells in spleens of mice after infection with *B. abortus* 2308 or the *virB* mutant. C57BL/6 mice (four mice per group) were injected i.p. with PBS or 5×10^5 CFU of *B. abortus* 2308 or the *virB* mutant. Mice were sacrificed 3 days after infection, and splenocytes were stained with a cocktail of Pacific Blue-conjugated anti-mouse CD4 (L3T4), Alexa Fluor 700-conjugated anti-mouse CD8a (Ly-2), APC-Alexa Fluor 750 anti-mouse CD3e, and phycoerythrin-conjugated anti-mouse IFN-γ. One million cells/sample were acquired with an LSRII apparatus. Numbers indicate the percentages of cells in each gate. Data for one mouse per group are shown as a representative of the group. SSC, side scatter.

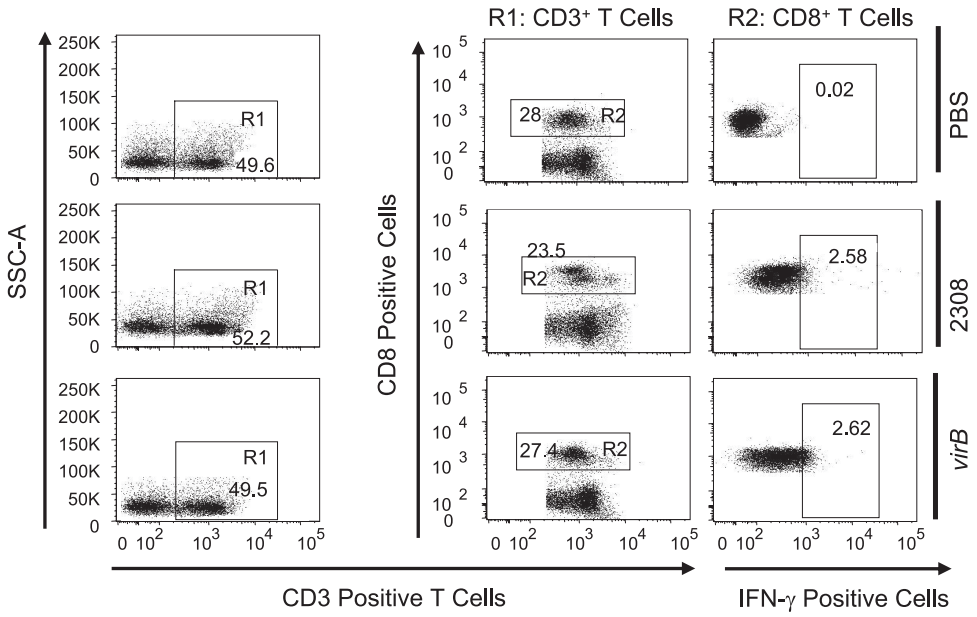

FIG. 5. IFN-γ-producing $CD8^+$ T cells in spleens of mice after infection with *B. abortus* 2308 or the *virB* mutant. C57BL/6 mice (four mice per group) were injected i.p. with PBS or 5×10^5 CFU of *B. abortus* 2308 or the *virB* mutant and sacrificed 3 days after infection. Splenocytes were stained as described in the legend of Fig. 4. One million cells/sample were acquired with an LSRII apparatus. Numbers indicate the percentages of cells in each gate. Data for one mouse per group are shown as a representative of the group. SSC, side scatter.

antibody and cytokine responses to wild-type *B. abortus* and the T4SS mutant.

In line with previous reports, infection with wild-type *B. abortus* preferentially elicited IgG2a, which is indicative of a Th1-type response (41). Interestingly, the increase in the level of IgG2a in sera of mice infected with the *virB* mutant over levels in sera of uninfected mice was only modest (Fig. 1). There are two possible explanations for this difference: one is a lack of antigen to stimulate the immune response in mice infected with the *virB* mutant, as by 56 days, very few of the *virB* mutant bacteria remained in the spleen (17, 31). Alternatively, wild-type *B. abortus* and the *virB* mutant could elicit different responses early during infection that could influence later development of different antibody isotype profiles. To test the second idea, we compared serum cytokine profiles elicited by infection with *B. abortus* 2308 and those elicited by infection with the *virB* mutant at 3 to 4 days postinfection, when similar numbers of both strains were present in the spleen (31). In agreement with previously published reports (13, 14, 37, 44), the levels of IL-12p40 and IFN-γ increased significantly above preinfection levels in mice infected with wild-type *B. abortus*. The *virB* mutant did not elicit IL-12 production, which is required for the stimulation of IFN-γ production and Th1 polarization (19), and also did not elicit IFN-γ production. We did not detect increases in serum levels of IL-4 (not shown), which would be indicative of Th2 polarization (40), in mice infected with either *B. abortus* strain. The weak Th1 polarization elicited by the *virB* mutant may contribute to its reduced protective properties as a vaccine compared with other attenuated strains of *Brucella* spp. (20, 21).

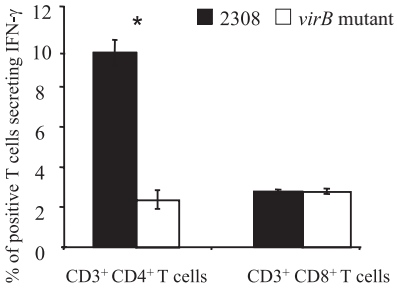

FIG. 6. Percentage of splenic $CD4^+$ T cells or $CD8^+$ T cells secreting IFN-γ after infection with *B. abortus* 2308 or the *virB* mutant. C57BL/6 mice (four mice per group) were injected i.p. with PBS or 5×10^5 CFU of *B. abortus* 2308 or the *virB* mutant. Mice were sacrificed 3 days after infection, and cells/mouse were stained for IFN-γ-producing cells. One million cells/sample were acquired with an LSRII apparatus. The asterisk represents statistical significance determined using a Student's *t* test on data after logarithmic conversion.

The cytokines present during T-cell responses influence the differentiation of Th cells into Th1 cells, which produce IFN-γ and IL-2, thereby stimulating cell-mediated responses, or Th2 cells, which produce IL-4, IL-5, IL-6, and IL-10 and mediate humoral responses. Although differences in methodology make it difficult to compare these results with those from previously published studies, the trend that we observed is in line with previous reports in which cytokine production could be elicited in splenocytes from *B. abortus*-infected mice by ex vivo stimulation with *B. abortus* antigens (18, 25, 27).

Effectors secreted by the T4SS play important roles in the interaction of bacterial pathogens with their hosts, and a number of them interfere with the immune response to infection (16, 30, 34, 36, 42). Furthermore, in *Helicobacter pylori* and *Legionella pneumophila*, there is evidence for the T4SS-mediated release of peptidoglycan (42) and flagellin (24, 29), respectively, into infected cells, and therefore, the T4SS-dependent induction of IFN-γ and IL-12 could result from either the

injection of effectors or the release of pathogen-associated molecular patterns into the host cell cytosol. Once type IV secreted *Brucella* molecules have been identified, it will be possible to determine whether they act directly or indirectly to trigger the T4SS-dependent host responses observed in this study. In summary, results presented here suggest that the T4SS is required to elicit early differences in cytokine responses to wild-type *B. abortus* and the *virB* mutant that contribute to the Th1 polarization of the immune response.

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