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

Hortensia García Rolán¹,² and Renée M. Tsolis¹,²*

Department of Medical Microbiology and Immunology, University of California at Davis, One Shields Avenue, Davis, California 95616,¹ and Department of Medical Microbiology and Immunology, Texas A&M University System Health Science Center, College Station, Texas 77843-1114²

Received 13 February 2008/Returned for modification 3 April 2008/Accepted 25 April 2008

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. abortus* *virB* operon.

**Expression of the *virB* operon.** The *virB* operon is encoded by the T4SS, which is required for *B. abortus* to promote the control of *Brucella* spp. to infection, we characterized serum antibody and cytokine responses to *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 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 BAA41, kanamycin (Km) was added to the culture medium at 100 μg/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 Multiplex 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 mon- itored for 10 min at room temperature for 30 min. The plates were washed, and 50 μl of streptavidin-phycocerythrin 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 Luminox 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 the wild type or the virB mutant 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 filter and then diluted 1:3 in PBS containing 1% bovine serum albumin 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 Nunc (Roskilde, Denmark) 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 the 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 mea- sured using horseradish peroxidase-conjugated anti-mouse immunoglobulin M (IgM), 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 492 nm with an ELISA microplate reader (MR5000; Dynatech). Data points are the averages of duplicate dilutions, with each measurement being performed twice.

**DNA 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 H2O.

**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 MgCl2, 10 μl of deoxynucleoside triphosphates, 2.5 μl of random hexamers, 1 μl of reverse transcriptase, and H2O in a final volume of 50 μl. Samples were incu- bated at 25°C for 10 min, followed by reverse transcription at 40°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 TCG TGC AGG ATT TTC ATG) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward primer TGT AGA CCA TGT AGT TGA GCT A and reverse primer AGG TCG GTG TGA ACG GAT TTG) by Sybr green PCR master mix (Applied Biosystems) and an ABI Prism 7000HT detection system (Applied Biosystems) according to the manufacturer’s instructions. Induction of mRNA was deter- mined from the threshold cycle (Ct) 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 storing the samples at −20°C, cells were washed twice with PBS and re-suspended in 1 ml formalin-killed whole Brucella (10^5 cells/ml) in RPMI 1640–5% fetal bovine serum with 100 μg/ml Brefeldin A (Sigma). Cells were stained individually or with a cocktail of Pacific Blue-conjugated anti-mouse IgD CD4 (L3T4), Alexa Fluor 700-conjugated rat anti-mouse CD8a (Ly-2), and allophycocyanin IFluor Alex Fluor 705 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 loga- rithmic 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 presen- tation 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 sim- ilar, 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 be- tween 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,
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/H9253 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/H9253 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.

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-
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...
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).

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.

ACKNOWLEDGMENTS

This investigation was conducted in a facility constructed with support from Research Facilities Improvement Program grant C06 RR01251-01 from the National Center for Research Resources, National Institutes of Health. Work in the laboratory of R.M.T. is funded by NIH grants AI50553 and U41 AI07156.

We thank L. Sower and A. Recinos III for assistance with use of the BioPlex Instrument in the WRCE Proteomics Facility. We are also grateful to A. Bämmer and N. Baumgart for critical comments on the manuscript.

REFERENCES

24. Microb. 867–75.
29. Microb. 867–75.

Editor: J. B. Bliska