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*J Immunol* 2002; 168:2433-2440; doi: 10.4049/jimmunol.168.5.2433
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Susceptibility of IFN Regulatory Factor-1 and IFN Consensus Sequence Binding Protein-Deficient Mice to Brucellosis

Jinkyung Ko,* Annette Gendron-Fitzpatrick,† and Gary A. Splitter²*

IFN-γ is a key cytokine controlling Brucella infection, and the diverse functions of this cytokine are mediated by IFN regulatory factors (IRFs) such as IRF-1, IRF-2, and IFN consensus sequence binding protein (ICSBP). However, the roles of these three IRFs in Brucella infection have not been investigated. The infection of each IRF-deficient mouse strain provides an opportunity to determine not only the significance of each IRF molecule but also the crucial immune components necessary for host defense during in vivo infection, because respective IRF-deficient mouse strains contain unique immunodeficient phenotypes. Brucella abortus S2308-infected IRF-1⁻/− mice were dead within 2 wk postinfection, while IRF-2⁻/− mice contained less splenic Brucella CFU than wild-type mice at the early stage of infection. Infected ICSBP-deficient mice possessed a plateau of splenic Brucella CFU throughout the infection. Additional infection of IL-12p40-, NO synthase 2-, and p48⁻/⁻-deficient mice indicates that these immune components are crucial for Brucella immunity and may contribute to the susceptibility of IRF-1⁻/− and ICSBP⁻/⁻ mice. Immunologic and histopathological analyses of infected IRF-1⁻/− mice indicate that the absence of IL-12p40 induction and serious hepatic damage are involved in the death of IRF-1⁻/− mice. These results indicate that 1) IRF-1 and ICSBP are essential transcriptional factors for IFN-γ-mediated protection against Brucella; 2) IL-12, reactive nitrogen intermediates, and reactive oxygen intermediates are crucial immune components against Brucella, and their absence may contribute to the susceptibility of IRF-1⁻/− and ICSBP⁻/⁻ mice; and 3) hepatic damage caused by Brucella virulence contributes to the death of IRF-1⁻/− mice. The Journal of Immunology, 2002, 168: 2433–2440.

The Gram-negative facultative intracellular bacterium Brucella abortus causes brucellosis with pathological manifestations of arthritis, endocarditis, and meningitis in humans, and spontaneous abortion in cattle (1, 2). This airborne pathogen not only resists killing by neutrophils but also replicates inside macrophages (3). Neither virulence factors that allow intracellular survival of Brucella nor the specific factors that induce such manifestations have been clearly elucidated (4).

One of the crucial cytokines involved in Brucella immunity is IFN-γ, a Th-1 cytokine produced by activated CD4⁺ and CD8⁺ T cells (5, 6). The major function of IFN-γ in Brucella immunity is the stimulation of bactericidal activity in macrophages, host cells of Brucella spp. However, the function of IFN-γ is more diverse than the induction of bactericidal function and includes the stimulation of Ag presentation through class I and class II MHC molecules, the orchestration of leukocyte-endothelium interactions, the effects on cell proliferation and apoptosis, as well as stimulation and repression of a variety of genes whose functional significance remains obscure (7). The implementation of pleiotropic effects by a single cytokine is accomplished by complex patterns of cell-specific gene regulation, and additionally several IFN-γ-regulatory genes are themselves components of transcription factors that create a complex picture for IFN-γ functions.

IFN regulatory factor (IRF) is a family of secondary transcriptional factors containing the unique tryptophan cluster DNA-binding region located at the N terminus and includes IRF-1, IRF-2, IRF-3, IRF-4, IRF-5, IRF-6, IRF-7, IFN consensus sequence binding protein (ICSBP; IRF-8), and p48 (IRF-9) (8, 9). IRFs can be induced by IFN-αβ and/or IFN-γ, and among the nine IRFs, the expressions of IRF-1, IRF-2, ICSBP, and p48 are induced by IFN-γ. Thus, to understand the roles of IRFs induced by IFN-γ in brucellosis in vivo, currently available IRF-1, IRF-2, and ICSBP gene knockout (KO) mice were introduced. Intriguingly, respective KO mice contain unique multiple immunodeficient phenotypes (Table I). For example, IRF-1⁻/⁻ mice are deficient in the induction of inducible NO synthase (iNOS), IL-12 p40, CD8⁺ T cells, and NK cells as well as IFN-γ, while IRF-2⁻/⁻ mice possess a deficiency in NK cells and the dysregulation of IL-12p40 induction (10–13). ICSBP⁻/⁻ mice are deficient in the induction of IL-12p40 and IRF-2 as well as the production of reactive oxygen intermediates (ROI) (14–16). Therefore, the infection of these three IRF-deficient mouse strains provides a unique opportunity to determine not only the significance of each IRF molecule induced by IFN-γ but also the crucial immune components during the infection. For instance, IRF-1⁻/⁻ mice were susceptible to Mycobacterium bovis (13), Leishmania major (17), and Toxoplasma gondii (18), due to the absence of iNOS induction and/or IL-12p40 induction, but were resistant to Listeria monocytogenes infection (14). IRF-2⁻/⁻ mice were susceptible to L. monocytogenes (14) and L. major (10) infection. ICSBP⁻/⁻ mice were susceptible to T. gondii (15), L. major (16), and L. monocytogenes (14) infection.

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Received for publication July 27, 2001. Accepted for publication January 9, 2002.

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1 This work was supported by National Institutes of Health Grant R01AI48490, U.S. Department of Agriculture Grant 98-35204-6760, and Binational Agricultural Research and Development Fund Grants US-2781-96 and US-2968-98C.

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Abbreviations used in this paper: IRF, IFN regulatory factor; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase; ICSBP, IFN consensus sequence binding protein; iNOS, inducible NO synthase; KO, knockout; p.i., postinfection; RNI, reactive nitrogen intermediate; ROI, reactive oxygen intermediate.
due to the absence of IL-12p40 induction or ROI production. Thus, to additionally elucidate the immune components that may contribute to the susceptibility of respective IRF-deficient mice, IL-12p40−/−, NOS2−/−, and gp91phox−/− mice were also infected with Brucella.

The aim of the current study was to elucidate the functions of IRFs induced by IFN-γ during Brucella infection in vivo. Here, we report 1) the susceptibility of Brucella infection in IRF-1+/− and ICSBP−/− mice, 2) the involvement of IL-12, reactive nitrogen intermediates (RNI), and ROI, which may contribute to the susceptibility of IRF-1+/− and ICSBP−/− mice, in Brucella immunity, and 3) the correlation between the level of hepatic damage and acute death of IRF-1+/− mice induced by virulent B. abortus strains. These results suggest the significant roles of IRF-1 and ICSBP during Brucella infection and the ascription of hepatic damages to the death of IRF-1+/− mice infected with virulent B. abortus strains.

Materials and Methods

Bacterial strains

*Brucella abortus* S2308, the virulent wild-type strain, was provided by Dr. B. Martin (Veterinary Services, National Animal Disease Center, Ames, IA). RB51, the current live attenuated U.S. vaccine strain derived from S2308 deficient in O-chain LPS, was a gift from Dr. G. G. Schurig (Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA) (19).

*Mice*

Breeding pairs of IRF-1+/− and IRF-2+/− mice on the C57BL/6 background were provided by Dr. T. W. Mak (Amgen Institute, Ontario Cancer Institute, University of Toronto, Toronto, Canada) (20). Breeding pairs of ICSBP+/− mice on the DBA/2J background were donated by Dr. H. C. Morse III (National Institute of Allergy and Infectious Diseases, Bethesda, MD) (16). These three strains were heterozygously bred, and genotypes were determined by PCR (20, 21). Whenever possible, IRF-1+/−, IRF-2+/−, and ICSBP+/− mice derived from heterozygous littermates were used as controls; otherwise, C57BL/6 (H-2b) or DBA/2J (H-2d) mice were used (The Jackson Laboratory, Bar Harbor, ME). Breeding pairs of IL-12p40−/− (22), NOS2−/− (23), and gp91phox−/− (24) on the C57BL/6 background (The Jackson Laboratory) were homozygously bred, and C57BL/6 mice were used as controls. The founders of double KO NOS2−/−/ICSBP−/−, NOS2−/−/IRF-2−/−, and wild-type mice on the C57BL/6 × DBA/2J background were generated by cross-breeding F1 (NOS2−/−/ICSBP−/−) mice and were used for breeding. Mice were genotyped by PCR if necessary, and 6- to 9-wk-old mice were used for experimental infection. All the mice were bred at the Department of Animal Health and Biomedical Sciences animal care facilities, and mice infected with *B. abortus* were housed in the Biosafety Level 3 facilities in the School of Veterinary Medicine. Infected IRF-1−/− mice were monitored daily for survival.

Infection and enumeration of Brucella from spleens and livers

Mice were infected i.p. with *B. abortus* S2308 or RB51 at 5 × 10^5 CFU in 200 μl PBS. To count residual Brucella CFUs in the spleens or livers of mice, five mice from each group were examined at each sampling period. Spleens or livers were homogenized in plastic bags with 10 ml sterile PBS using a Stomacher Lab Blender (Tekmar, Cincinnati, OH). To enumerate viable Brucella, homogenized spleen or liver samples were serially diluted 10-fold with PBS and plated on Brucella agar (Difco, Detroit, MI). Brucella colonies were counted after a 3-day incubation at 37°C with 5% CO₂. CFU numbers <100/organ could not be detected using this method.

Bioactive rIL-12p70 injection

IRF-1−/− mice were i.p. injected with mouse rIL-12p70 (Sigma-Aldrich, St. Louis, MO) for the initial 8 days of infection at a concentration of 0.33 mg/mouse in 200 μl PBS daily beginning at the time of challenge with *B. abortus* S2308. This concentration of IL-12 has been shown previously to be effective in significantly reducing the mortality of IRF-1−/− or wild-type mice due to acute parasite infection (18, 25). Medication of iNOS inhibitor

C57BL/6 and IL-12p40−/− mice were medicated with an iNOS inhibitor, #67, and t-arginine analogs (Dr. P. T. Manning, Searle, St. Louis, MO) in the drinking water (1 mg/ml) from 3 days before infection throughout the completion of the experiment (26). The solution was completely replaced three times per week.

GOT, GPT, and albumin assays

Mice were exsanguinated from the orbital sinus, and sera were collected using Microtainer (BD Biosciences, Rutherford, NJ). Glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) activities as well as albumin concentration were quantitatively measured by colorimetric determination with diagnostic transaminase reagents and albumin reagent (*M. bovis*), respectively, following the manufacturer’s directions (Sigma-Aldrich). The activities of GOT and GPT were measured with 1/5 diluted sera with PBS.

Histology

Mice infected with 5 × 10^5 CFU *B. abortus* S2308 or RB51 and were killed 12 days postinfection (p.i.). Organs were immersed in 10% formalin, embedded, microsectioned, mounted on microscopic slides, and stained with H&E. The H&E-stained slides were microscopically observed.

Statistical analysis

Data from infected mice were analyzed independently and compared using ANOVA and the generalized Wilcoxon rank sum test (LIFETEST) in the SAS program (SAS Institute, Cary, NC). Infection of IRF-1−/− mice was performed twice independently, and then the results were pooled to perform the data analysis. Values of *p* < 0.05 were considered significant.

Results

IRF-1−/−, IRF-2−/−, and ICSBP−/− mice infected with *B. abortus*

All IRF-1−/− mice infected with *B. abortus* wild-type strain S2308 at 5 × 10^5 CFU were dead within 2 wk p.i., and 50% of IRF-1−/− mice were dead at 9 days p.i. (Fig. 1A). However, no death occurred in S2308 (see Fig. 3) or RB51 (data not shown)-infected IRF-1−/− mice or in RB51-infected IRF-1−/− mice throughout 4 wk p.i., suggesting that the death of IRF-1−/− mice is caused by the reciprocal relation between the immunocompromised phenotypes of IRF-1−/− mice and the level of virulence in *Brucella* strains (Fig. 1A). The death of IRF-1−/− mice caused by *B. abortus* wild-type strain infection is notable, because even immunocompromised SCID (27) or nude (28) mice control *Brucella* infection. Dead IRF-1−/− mice previously infected with the virulent *B. abortus* strain S2308 contained ascitic fluids, suggesting that hepatic damage might have occurred. IRF-2−/− mice contained fewer splenic S2308 CFUs than IRF-2−/− mice during the initial 2 wk p.i., and ICSBP−/− mice maintained a plateau of splenic S2308 CFU throughout the experiment similar to IL-12p40−/− mice (Fig. 1B). IL-12 was not detected by ELISA in IRF-1−/− and ICSBP−/−

Table I. Phenotypes of IRF-1−/−, IRF-2−/−, and ICSBP−/− mice

<table>
<thead>
<tr>
<th>Immune Components</th>
<th>IRF-1−/− Mice (Ref.)</th>
<th>IRF-2−/− Mice (Ref.)</th>
<th>ICSBP−/− Mice (Ref.)</th>
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<tr>
<td>IL-12p40 induction</td>
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<td>Dysregulation (10)</td>
<td>Deficiency (14, 15)</td>
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<td>Deficiency (12)</td>
<td>Normal (13)</td>
<td>Normal (13)</td>
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<td>ROI production</td>
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<td>Normal (13)</td>
<td>Deficiency (13)</td>
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<tr>
<td>NK cells</td>
<td>Deficiency (11)</td>
<td>Deficiency (10)</td>
<td>Normal (14, 15)</td>
</tr>
<tr>
<td>CD8+ T cells</td>
<td>Deficiency (20)</td>
<td>Normal (20)</td>
<td>Normal (14, 15)</td>
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mice during the initial 5 days p.i. (data not shown). IRF-2−/− mice had very low amounts of IL-12 on day 3 p.i. RNI and ROI were measured in peritoneal macrophages obtained from uninfected KO mice treated with LPS and/or IFN-γ. ROI was deficient in ICSBP−/− mice, and RNI was defective in IRF-1−/− mice. However, IRF-2−/− mice were normal in RNI and ROI activities (data not tabulated). These results suggest that the roles of IRF-1 and ICSBP in Brucella immunity are critical, and the death of IRF-1−/− mice correlates with the level of virulence in B. abortus strains.

IL-12p40−/−, NOS2−/−, and gp91^phox−/− mice infected with B. abortus S2308

IRF-1, IRF-2, and ICSBP-deficient mouse strains possess unique immunodeficient phenotypes (Table I). However, the roles of these defective immune components in Brucella immunity in vivo have not been fully elucidated. The importance of IL-12 and CD8+ T cells in Brucella immunity in vivo has been tested using anti-IL-12 Ab-injected mice (29) and MHC class I-deficient β2-microglobulin−/− mice (30), respectively; however, IL-12-deficient mice have not been tested in brucellosis. Additionally, although the significance of ROI has been delineated in vitro (31), the roles of iNOS and ROI have not been investigated during Brucella infection in vivo. The NK cell-depleted mice could control Brucella infection as wild-type mice do, suggesting that the role of NK cells is minor (32). Thus, to demonstrate a definitive role of each immune component during the Brucella infection in vivo, IL-12p40−/−, NOS2−/−, and gp91^phox−/− mouse strains were infected.

IL-12 is a 70-kDa heterodimeric cytokine formed by two covalently linked glycosylated chains of p35 and p40 and produced mostly by phagocytic cells in response to intracellular bacteria (33). In vivo IL-12 induces IFN-γ production from NK and T cells during early infection and favors Th-1 cell differentiation by promoting CD4+ T cells for high IFN-γ production. The crucial role of IL-12 in other intracellular pathogen infections was demonstrated using IL-12p40−/− mice (34, 35). IL-12p40−/− mice infected with B. abortus S2308 could not control infection and maintained a high plateau of bacteria for the duration of the experiment, suggesting that the Th-1 immune response is required to control Brucella infection (Fig. 2).

Chemically reactive micromolecules, RNI and ROI, are two major antimicrobial products of phagocytes (36, 37). NOS2 is iNOS dominantly expressed in phagocytes and produces a high output of NO. NADPH oxidase, the major source of pathogen-triggered ROI production in phagocytes, becomes activated upon translocation of several cytosolic proteins (i.e., gp47^phox, gp47^phox, gp67^phox, and the Rho family GTPase, Rac2) to the membrane-bound complex carrying cytochrome c (gp91^phox, gp22^phox−Rap1a). Thus, NOS2-deficient mice or NADPH oxidase component-deficient mice, such as gp91^phox and p47^phox-deficient mice, have been infected with selected pathogens to elucidate the roles of RNI and ROI during the infection. The roles of RNI and ROI in Brucella immunity were initially demonstrated in vitro using a specific inhibitor (31), and ROI was considered an important innate immune component involved in bactericidal function in macrophages. However, recent in vitro results provide additional evidence that RNI may be involved in Brucella killing in macrophages (38, 39). Thus, to test the significance of ROI or RNI in vivo, respective immunodeficient mouse strains were infected. Splenic B. abortus CFU from NOS2−/− and gp91^phox−/− mice demonstrated a delayed control of infection (Fig. 2). At 2 wk p.i. splenic CFU reaches a peak, and a 100-fold CFU difference was observed between C57BL/6 mice and NOS2−/− or gp91^phox−/− mice. However, after 2 wk p.i. a dramatic reduction of CFU was observed for the last 2 wk, indicating that other compensatory bactericidal mechanisms are probably activated to control the infection in NOS2−/− and gp91^phox−/− mice. In fact, NOS2/gp91^phox double-KO mice showed delayed control of splenic Brucella similar to NOS2 or gp91^phox single-KO mice, suggesting that other sources
of ROI and RNI besides NADPH oxidase and NOS2, respectively, are involved in a Brucella killing mechanism (data not shown). However, death did not occur in any of these immunodeficient mouse strains, confirming that death in IRF-1−/− mice is unusual. Taken together, these results suggest that IL-12, RNI, and ROI are crucial innate immune components involved in Brucella immunity in the early stage of infection, and the absence of these immune components may contribute to the susceptibility of IRF-1−/− and ICSBP−/− mice.

Bioactive rIL-12p70-supplemented IRF-1−/− mice infected with B. abortus S2308

The death of IRF-1−/− mice from Brucella virulence is an unusual feature in brucellosis. Thus, we hypothesized that the death of IRF-1−/− mice results from the multiple immunodeficiencies required for immunity to intracellular pathogens. The persistent Brucella infection occurring in IL-12p40−/− mice indicated the significance of IL-12 in B. abortus infection in vivo. Therefore, we tested whether the absence of IL-12p40 induction contributes to the death of IRF-1−/− mice by administering IL-12. Bioactive rIL-12p70 (0.33 mg/mouse in 200 μl PBS) was injected i.p. to IRF-1−/− mice for the initial 8 days of infection. rIL-12 administered to IRF-1−/− mice delayed death, indicating the absence of IL-12p40 induction is involved in the death of IRF-1−/− mice as well as confirming the significance of IL-12 in Brucella immunity (Fig. 3).

Phenotypic NOS2/IL-12p40 double-immunodeficient mice infected with B. abortus S2308

The results obtained from NOS2−/− and IL-12p40−/− mice indicate the importance of iNOS and IL-12 in B. abortus immunity; however, this single immunodeficient mice with high splenic Brucella CFUs failed to die (Fig. 2). In addition, the delayed death of IRF-1−/− mice supplemented with rIL-12 indicated that the absence of IL-12 contributed to the death of IRF-1−/− mice (Fig. 3). Thus, we hypothesized that multiple immunodeficiencies including IL-12p40 induction contribute to the death of IRF-1−/− mice. Therefore, mice deficient in both iNOS and IL-12p40 were tested for their ability to control Brucella infection (Fig. 4). Because the genes encoding NOS2 and IL-12p40 are located on the same chromosome, NOS2/IL-12p40 double-KO mice are difficult to generate at the gene level. Therefore, double-immunodeficient phenotype mice, iNOS and IL-12p40 deficiency, were generated by mediating IL-12p40−/− mice with an iNOS inhibitor, #67, followed by Brucella infection (Fig. 4A). The iNOS inhibitor-treated C57BL/6

![FIGURE 3. Bioactive rIL-12p70 supplemented IRF-1−/− mice infected with B. abortus strain S2308 (5 × 10⁷ CFU). Supplementation of rIL-12 for the initial 8 days of infection prolonged 50% survival in infected IRF-1−/− mice to 35 days p.i. (p < 0.0001), indicating that the absence of IL-12p40 contributes to the death of IRF-1−/− mice.](http://www.jimmunol.org/)

![FIGURE 4. Phenotypic NOS2/IL-12p40 double-immunodeficient mice infected with B. abortus strain S2308 (5 × 10⁷ CFU). A, iNOS inhibitor, #67, medicated IL-12p40−/− and C57BL/6 Brucella-infected mice. Delayed control of infection was observed in medicated C57BL/6 mice (M-C57BL/6), and increased splenic CFUs were detected in medicated IL-12p40−/− mice (M-IL-12p40−/−). Death was not observed. B, NOS2/ICSBP double-immunodeficient mice infection. NOS2/ICSBP double-immunodeficient mice (C57BL/6 × DBA/2J) as well as control mice on the same background were infected with B. abortus S2308 (5 × 10⁷ CFU). These double-immunodeficient mice did not die, suggesting that the death of IRF-1−/− mice was caused by other immunodeficiencies.](http://www.jimmunol.org/)
mice were delayed in controlling Brucella infection similar to NOS2−/− mice, with nearly a 1-log CFU increase at 2 wk p.i. Also, treated IL-12p40−/− mice had more splenic Brucella CFU than nontreated IL-12p40−/− mice. These results suggest both NO and IL-12 play important roles, but this double immunodeficiency was not sufficient to explain the death of IRF-1−/− mice. Also, the reduction of splenic CFU in treated C57BL/6 mice, but not in treated IL-12p40−/− mice, suggests that the control mechanism after 2 wk p.i. in NOS2-deficient mice is IL-12 dependent (Fig. 2).

Alternatively, ICSBP−/− mice that are immunodeficient in IL-12p40 induction were cross-bred with NOS2−/− mice to generate the NOS2/IL-12p40 double-immunodeficient phenotype. The expected phenotypes with deficiencies in peritoneal macrophage NO production or serum IL-12 production were confirmed from NOS2/ICSBP double-immunodeficient (C57BL/6 × DBA/2J) as well as single-immunodeficient and wild-type mice (data not shown), and these mice were infected with B. abortus S2308 (5 × 10^5 CFU; Fig. 4B). Each single-immunodeficient mouse strain on the C57BL/6 × DBA/2J background demonstrated an infection similar to those in NOS2−/− mice on the C57BL/6 background and ICSBP−/− mice on the DBA/2J background. NOS2/ICSBP double-immunodeficient mice infected with B. abortus did not die, and the growth of Brucella CFU in spleens was similar to that in IL-12p40−/− mice treated with the iNOS inhibitor. CFUs from double-immunodeficient mice were 1.5 log higher than those in ICSBP−/− mice and 2.5 log higher than those in wild-type and NOS2−/− mice. Taken together, the cause of death in IRF-1−/− mice may result from a multiple immunodeficiencies, and NOS2/IL-12p40 double immunodeficiency was not sufficient to induce the death of infected mice.

Increased B. abortus CFUs in the livers of IRF-1−/− mice

The level of systemic infection in murine brucellosis is detectable by enumerating the number of residual Brucella CFU in liver and spleen (6). Thus, IRF-1−/+ and IRF-1−/− mice were infected with B. abortus virulent S2308 and avirulent RB51 strains, and hepatic and splenic CFUs were counted every third day for investigating the cause of death in IRF-1−/− mice (Fig. 5).

The number of hepatic CFU detected from IRF-1−/+ and IRF-1−/− mice infected with 5 × 10^5 CFU of S2308 maintained nearly a 10-fold difference until 9 days p.i. (Fig. 5A). From day 6 p.i. IRF-1−/+ mice began to control S2308 CFU in the liver. However, IRF-1−/− mice failed to control hepatic B. abortus, and at 12 days p.i. IRF-1−/− mice had 2.5-log higher gastrointestinal CFUs than IRF-1−/− mice (p < 0.005). Unlike S2308-infected mice, IRF-1−/+ and IRF-1−/− mice infected with 5 × 10^5 CFU of RB51 had a parallel pattern of hepatic CFU clearance. Although a delay of bacterial clearance was detected from IRF-1−/− mice compared with IRF-1−/+ mice, ultimately RB51 was cleared from the livers of both mouse strains within 12 days p.i. These results indicate that a virulence-dependent bacterial hepatic burden in IRF-1−/− mice that induces hepatic damage contributed to death in this mouse strain.

The spleens from IRF-1−/+ and IRF-1−/− infected mice had a similar plateau of S2308 CFUs for the 12 days p.i., except for 3 and 6 days p.i. where 0.5 log higher CFU was detected from IRF-1−/− mice (Fig. 5B). The splenic RB51 CFUs from IRF-1−/+ and IRF-1−/− mice demonstrated a delay of clearance in IRF-1−/− mice. At 9 days p.i., >100-fold CFU difference was detected in IRF-1−/− mice compared with IRF-1−/+ mice, but splenic RB51 CFUs in IRF-1−/− mice declined by day 12.

Taken together, the hepatic Brucella CFU difference between IRF-1−/+ and IRF-1−/− mice was dramatically increased in S2308-infected mice, but not in RB51-infected mice. Thus, a certain level of bacterial virulence is required to induce such a hepatic bacterial burden, or the basal immunity in IRF-1−/− mice can control the infection caused by RB51 virulence. This continual hepatic bacterial burden in IRF-1−/− mice may lead to fatal hepatic damage.

Histological analysis of IRF-1−/− mice infected with B. abortus

Brucellosis is a chronic infection inducing granulomatous formation in the livers and spleens of humans and mice (1, 28, 40). To determine whether differences in the levels of hepatic Brucella CFUs detected in IRF-1−/+ and IRF-1−/− mice affect the degree of hepatic damage, livers from infected IRF-1−/+ and IRF-1−/− mice were examined histologically. Compared with RB51 infection, S2308 induced more granulomas in the livers of IRF-1−/+ and IRF-1−/− mice at 12 days p.i. Also, more extensive hepatic granulomas were detected in IRF-1−/− mice compared with IRF-1−/+ mice. Thus, IRF-1−/− mice infected with S2308 showed the greatest hepatic granuloma formation (Fig. 6A), while RB51-infected IRF-1−/− mice had fewer and less extensive microfocal granulomas (Fig. 6B). These results indicate that the virulence of B. abortus correlates qualitatively and quantitatively with granuloma formation in IRF-1−/− mice and can be interpreted as the level of bacterial hepatic burden correlates with the level of liver damage in IRF-1−/− mice based on the previous results (Fig. 5A). Additional analyses with brain, lung, heart, kidney, spleen, as well as genital and gastrointestinal organs did not show significant histopathological manifestations (data not shown).
Biochemical analysis of hepatic damage in IRF-1<sup>−/−</sup> mice

Histological analyses identified different levels of hepatic granuloma formation in the IRF-1<sup>+/+</sup> and IRF-1<sup>−/−</sup> mice infected with Brucella strains containing different degrees of virulence. Thus, to quantify the level of liver damage, sera GOT and GPT activities were measured from the infected mice. Both uninfected IRF-1<sup>+/+</sup> and IRF-1<sup>−/−</sup> mice maintained normal GOT and GPT activities in serum (37 and 19 IU/l, respectively; Fig. 7, A and B) (41). Also, RB51-infected IRF-1<sup>+/+</sup> and IRF-1<sup>−/−</sup> mice did not possess dramatically increased GOT and GPT activity in sera. In contrast, S2308-infected IRF-1<sup>+/+</sup> and IRF-1<sup>−/−</sup> mice possessed increased GOT and GPT activities, indicating that acute hepatitis occurred in both mouse strains. However, the extent of liver damage between the two mouse strains was discernable. Serum GOT activity in S2308-infected IRF-1<sup>+/+</sup> and IRF-1<sup>−/−</sup> mice was 81 and 166 IU/l, and GPT activity was 39 and 81 IU/l, respectively, indicating that 2- and 4-fold increased liver damage had occurred in each mouse strain. These results indicate that there is virulence-dependent hepatic damage in both mouse strains, and the level of hepatic damage in IRF-1<sup>−/−</sup> mice was more severe than that in IRF-1<sup>+/+</sup> mice.

The existence of ascitic fluids (6–10 ml/mouse) in IRF-1<sup>−/−</sup> mice infected with B. abortus S2308 implied that the concentration of albumin in sera may be low, because this protein is produced by the liver as an osmotic regulator (42). Both uninfected and RB51-infected IRF-1<sup>+/+</sup> and IRF-1<sup>−/−</sup> mice maintained normal levels of serum albumin (3.4 g/dl; Fig. 7C) (41). S2308-infected IRF-1<sup>+/+</sup> and IRF-1<sup>−/−</sup> mice had decreased albumin, indicating that dysosmoregulation occurred in both mouse strains. Serum albumin in S2308-infected IRF-1<sup>+/+</sup> mice and IRF-1<sup>−/−</sup> mice was 2.5 and 1.8 g/dl, respectively, indicating that 23 and 47% albumin reduction had occurred in each mouse strain. The albumin concentration in ascitic fluids in IRF-1<sup>−/−</sup> mice was 1.5 g/dl, suggesting that a 56% reduction occurred compared with albumin in serum (data not shown). Additional analyses of the ascitic fluid obtained from S2308-infected IRF-1<sup>−/−</sup> mice at 12 days p.i. determined that the ascitic pellet was 90–95% macrophages with the remaining cells consisting of small lymphocytes, neutrophils, as well as a rare plasma cell, and 1.6 × 10<sup>5</sup>–5.9 × 10<sup>5</sup> CFU/ml S2308 bacteria were present in the ascitic fluid, providing evidence of peritonitis (data not shown).

As a result, GOT and GPT as well as albumin assays identified serious liver damage and dysosmoregulation in IRF-1<sup>−/−</sup> mice infected with the B. abortus virulent S2308 strain, suggesting that the cause of death in IRF-1<sup>−/−</sup> mice involved liver damage, and that a certain level of bacterial virulence is required to induce liver damage.

Discussion

Typical host immunity to Brucella is a Th-1-dependent cellular immune response like other intracellular bacteria (5, 6). IL-12-polarized and -activated T cells such as CD4<sup>+</sup> and CD8<sup>+</sup> T cells secrete Th-1-type cytokines, e.g., IFN-γ, and IFN-γ activates phagocytic functions such as ROI and RNI in macrophages to kill intracellular bacteria. However, the actual functions induced by IFN-γ to control the intracellular infection are diverse, and these various implementations are facilitated by secondary transcriptional factors such as IRFs (7). Thus, to investigate the roles of
IFN-γ during Brucella infection, the roles of IRFs induced by IFN-γ such as IRF-1, IRF-2, ICSBP, and p48 require elucidation. Currently, IRF-1, IRF-2, and ICSBP gene-disrupted mice are available, and these individual gene KO mouse strains are useful tools to determine the function of respective factors during the infection in vivo. Unlike other gene KO mice that are deficient in cytokines or innate immune components such as IL-12p40, iNOS, or gp91phox, these three transcriptional factor-deficient mice possess multiple immunodeficient phenotypes (Table I). Thus, to understand the immune responses in IRF-deficient mice during Brucella infection, the roles of individual immune components deficient in respective mouse strains also need to be elucidated.

IRF-1−/− mice infected with B. abortus virulent strains died within 12 days p.i. IRF-2−/− mice contained less splenic Brucella CFU than IRF-2+/+ mice during the initial 2 wk p.i. ICSBP−/− mice maintained a plateau of splenic Brucella CFU throughout the experiment. These results clearly indicate that the roles of IRF-1 and ICSBP are essential for Brucella immunity. However, the defective immune components in IRF-1−/− and ICSBP−/− mice that contribute to Brucella susceptibility are still unknown. The lower splenic CFU detected in IRF-2−/− compared with IRF-2+/+ mice for the first 2 wk p.i. was unexpected, because the dysregulation of IL-12p40 induction in IRF-2−/− mice was anticipated to prolong bacterial clearance (Fig. 1B) (10), and very low concentrations of IL-12 were detected by ELISA from the sera of B. abortus S2308-infected IRF-2−/− mice during the initial 5 days p.i. (data not shown). However, IRF-2−/− mice are phenotypically different from IL-12p40−/− mice. For example, IRF-2 functions to down-regulate the genes activated by IRF-1. Thus, IRF-2−/− mice may have increased activation of the genes induced by IRF-1 that play an important role in controlling Brucella infection. In contrast to our result, IRF-2−/− mice were highly susceptible to L. monocytogenes (14) and L. major (10) infection, suggesting that the immune response in respective IRF-deficient mice may be pathogen specific. However, IRF-2−/− (9) and ICSBP−/− (21) mice demonstrate an autoimmune and a chronic myelogenous leukemia-like syndrome later in life, respectively, suggesting that the progression of these manifestations in even young mice may influence the in vivo results of Brucella infection obtained from these mouse strains.

IL-12p40, NOS2-, or gp91phox-deficient mice were also susceptible to Brucella infection, suggesting the significance of IL-12, RNI, and ROI in Brucella immunity that may contribute to the susceptibility of IRF-1−/− and ICSBP−/− mice. We hypothesize that the combination of these immune components produced the susceptibility of IRF-1−/− and ICSBP−/− mice to Brucella infection. In fact, IL-12-supplemented IRF-1−/− mice infected with B. abortus survived much longer than PBS-injected IRF-1−/− mice, indicating that the absence of IL-12 contributes to the death of IRF-1−/− mice. This result is coincident to the result obtained after T. gondii infection that suggests the induction of an IFN-γ-independent mechanism of protection against this opportunistic pathogen by IL-12 (18).

In contrast to the rapid death of Brucella-infected IRF-1−/− mice (Fig. 1A), others have recently reported that IFN-γ−/− mice have increased Brucella replication compared with wild-type mice, but survive for 6–10 wk (43). This difference in IRF-1−/− mouse susceptibility to Brucella suggests that multiple immune components contribute to IRF-1−/− mouse susceptibility. In addition, the fact that immunocompromised SCID (27) and nude (28) mice control Brucella infection significantly better than IRF-1−/− mice indicates the possibility of an IFN-γ and T cell-independent mechanism(s) for controlling brucellosis. TNF-α, produced by macrophages and NK cells in SCID, nude, or IFN-γ KO mice, may induce bactericidal mechanisms in macrophages that are T cell independent. The significant role of TNF-α in murine brucellosis has been delineated (44). Therefore, IRF-1−/− mice defective in IFN-γ and TNF-α production (45) would lack IFN-γ-dependent as well as independent mechanisms to activate the phagocytic function of macrophages to control Brucella infection. Thus, more severe symptoms would be anticipated in Brucella-infected IRF-1−/− mice than in SCID, nude, and IFN-γ KO mice.

The critical question regarding hepatic damage is why B. abortus virulent strains were not cleared and possessed a hepatotropism phenotype in IRF-1−/− mice, while the attenuated strains were controlled in the liver (Fig. 5A). Future experiments are required to address the hepatotropic growth of virulent B. abortus strains in IRF-1−/− mice. Kupffer cells may be unable to control virulent infection in IRF-1−/− mice because the levels of IFN-γ and TNF-α in IFN-1−/− mice are much lower than those in wild-type mice (45). Thus, dysregulation of these cytokines as well as insufficient IFN-γ signaling in IRF-1−/− mice may not activate the phagocytic activity of Kupffer cells. Thus, Kupffer cells in IRF-1−/− mice would be a niche for B. abortus proliferation in livers.

To understand the pathogenic mechanisms in IRF-1−/− mice, immunological and histopathological analyses were performed with mice infected with diverse B. abortus strains containing different levels of virulence. The contribution of defective IL-12p40 induction to the death of IRF-1−/− mice was demonstrated by rIL-12p70 administration (Fig. 3). However, phenotypic NOS2/IL-12p40 double-immunodeficient mice did not die of Brucella infection, suggesting that other immunodeficient combinations may contribute to death (Fig. 4). Histopathological analysis demonstrated that the level of hepatic damage correlated with the degree of virulence of the B. abortus strain (Fig. 6). One manifestation of brucellosis is acute hepatitis (46). Most animals infected with Brucella overcome this acute hepatitis and maintain a chronic infection, and this transition is dependent on the degree of virulence in Brucella strains (1). This shared symptom of hepatitis between natural hosts and IRF-1−/− mice supports the role of IRF-1−/− mice as a relevant animal model for detecting Brucella virulence. In addition, the level of hepatic bacterial burden (Fig. 5A) correlated with the level of granuloma formation (Fig. 6) as well as increasing activities of GOT and GPT and decreasing albumin concentration in sera (Fig. 7), indicating that hepatic damage contributes to the death of IRF-1−/− mice. IRF-1−/− mice were susceptible to M. bovis (13), L. major (17), and T. gondii (18), but resistant to L. monocytogenes (14) infection, suggesting that pathogen-specific host immune mechanisms are required for protection. The death of IRF-1−/− mice occurred with T. gondii, but liver damage was not described (18). Thus, the death of IRF-1−/− mice with liver damage and ascitic fluids may be a unique feature of brucellosis. Also, the acute death of IRF-1−/− mice that manifest hepatic damage is dependent on the virulence of Brucella (Fig. 1A). These results suggest that the rapidity of death in IRF-1−/− mice may be an alternative yardstick to determine the level of Brucella virulence. In fact, IRF-1−/− mice were infected with 5 × 10^9 CFU of diverse B. abortus strains containing different degrees of virulence, and the correlation between the rapidity of death in IRF-1−/− mice and the level of virulence was confirmed.4

In summary, using IRF-1−, IRF-2−, and ICSBP-deficient mice, we demonstrated that 1) the roles of IRF-1 and ICSBP are essential for Brucella immunity; 2) IL-12, RNI, and ROI, whose absence may contribute to the susceptibility of IRF-1−/− and ICSBP−/− mice, are important innate immune components in brucellosis; 3) the absence of IL-12p40 and hepatic damage contribute to the death of IRF-1−/− mice; and 4) the level of hepatic damage in

IRF-1−/− mice is dependent on the bacterial burden in livers and \( B.\) abortus virulence.

Acknowledgments
We thank Dr. Tak W. Mak (University of Toronto) and Dr. Herbert C. Morse (National Institute of Allergy and Infectious Disease) for providing immunodeficient mouse strains, and Dr. Pamela T. Manning (Searle) for providing iNOS inhibitor 687, as well as Dr. Peter S. MacWilliams (University of Wisconsin) for performing the cytology assay of ascitic fluids in IRF-1−/− mice and discussing serum transaminase and albumin assays.

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