REGULAR ARTICLE

Innate Lymphoid Cells and Interferons Limit Neurologic and Articular Complications of Brucellosis

Charles R. Moley,* Catherine A. Chambers,† Alexis S. Dadelahi,† Bárbara Ponzilacqua-Silva, Mostafa F.N. Abushahba,‡ Carolyn A. Lacey,‡ Craig L. Franklin,* and Jerod A. Skyberg*†‡

From the Department of Veterinary Pathobiology,* College of Veterinary Medicine, and the Laboratory for Infectious Disease Research,† University of Missouri, Columbia, Missouri; and the Department of Zooneses,‡ Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt

Accepted for publication May 18, 2023.

Address correspondence to Jerod A. Skyberg, Department of Veterinary Pathobiology, College of Veterinary Medicine, University of Missouri, Columbia, MO 65211. E-mail: skybergj@missouri.edu.

Brucellosis is a globally significant zoonotic disease. Human patients with brucellosis develop recurrent fever and focal complications, including arthritis and neurobrucellosis. Here, the role of innate lymphoid cells (ILCs) in the pathogenesis of focal brucellosis caused by Brucella melitensis was investigated. After footpad infection, natural killer cells and ILC1 cells both limited joint colonization by Brucella. Mice lacking natural killer cells, and in particular mice lacking all ILCs, also developed marked arthritis after footpad infection. Following pulmonary infection, mice lacking adaptive immune cells and ILCs developed arthritis, neurologic complications, and meningitis. Adaptive immune cells and ILCs both limited colonization of the brain by Brucella following pulmonary infection. Transcriptional analysis of Brucella-infected brains revealed marked up-regulation of genes associated with inflammation and interferon responses, as well as down-regulation of genes associated with neurologic function. Type II interferon deficiency resulted in colonization of the brain by Brucella, but mice lacking both type I and type II interferon signaling more rapidly developed clinical signs of neurobrucellosis, exhibited hippocampal neuronal loss, and had higher levels of Brucella in their brains than mice lacking type II interferon signaling alone. Collectively, these findings indicate ILCs and interferons play an important role in prevention of focal complications during Brucella infection, and that mice with deficiencies in ILCs or interferons can be used to study pathogenesis of neurobrucellosis. (Am J Pathol 2023, ■: 1–15; https://doi.org/10.1016/j.ajpath.2023.05.006)

Supported by NIH grants R21AI153074 (J.A.S.), R01AI150797 (J.A.S.), and 2T32 OD011126-44 (C.R.M.).

Disclosure: C.A.L. now works for AbbVie. This article is composed of the authors’ work and ideas and does not reflect the ideas of AbbVie. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Current address of C.A.C., University Research Animal Resources, University of Georgia, Athens, GA; of C.A.L., AbbVie, Chicago, IL.

Brucellosis, a bacterial disease caused by the Brucella genus, is one of the eight neglected zoonoses according to the World Health Organization.1 Brucella melitensis, Brucella abortus, and Brucella suis are the most common human pathogens in this genus, infecting small ruminants, cattle, and swine, respectively.2 Brucellosis causes significant agricultural and public health problems worldwide, especially in endemic areas, such as the Mediterranean Basin, Middle East, and Central Asia.3 Presenting as late-term abortion in affected livestock species, brucellosis causes devastating financial losses within endemic countries.4 High rates of human disease correlate with lambing season in these countries, as the disease is transmitted to humans through the ingestion of unpasteurized dairy products or direct contact (ingestion, inhalation, and wound infection) with contaminated tissues.5 Brucellosis accounts for >500,000 recorded human cases per year, but many believe the actual number of annual infections is 10 to 20 times higher as most cases go undiagnosed.6 The disease in humans is characterized by an undulating fever and
systemic symptoms, including lethargy, chills, arthralgia, and headaches.

Multiple focal complications can develop during brucellosis, the most common being arthritis. Previous studies have shown that interferon (IFN)-γ prevents Brucella-induced arthritis by both limiting infection as well as by inhibiting excessive inflammatory activation through the induction of nitric oxide. The most morbid complication, however, is neurobrucellosis, which involves muscular, neurological, and brainstem symptoms, including lethargy, chills, arthralgia, and headaches.

**Materials and Methods**

**Bacterial Strains and Growth Conditions**

All experiments with live *B. melitensis* were performed in biosafety level 3 facilities. *Brucella melitensis* 16 M, obtained from Montana State University (Bozeman, MT), was grown on *Brucella* agar at 37°C (Becton Dickinson, Franklin Lakes, NJ). Colonies were picked from *Brucella* agar plates, and cultured in *Brucella* broth (Becton Dickinson) overnight at 37°C with shaking. The overnight *Brucella* concentration was estimated by measuring the OD at 600 nm, and the inoculum was diluted to the appropriate concentration in sterile phosphate-buffered saline (PBS). Actual viable titer was confirmed by serial dilution of the *B. melitensis* inoculum onto *Brucella* agar plates.

**Mice**

Experiments were conducted using 6- to 12-week-old age- and sex-matched mice on a C57BL/6 background. C57BL/6J wild-type (WT), Rag2^−/−, Ifng^−/−, Rag2^−/−/Ifng^−/−, and Terc^−/−, *Tbx21^−/−*, *Ifngr1^−/−*, *Ifngr1^−/−/Ifnar1^−/−*, and *Tcrα1^−/−* mice were originally obtained from Jackson Laboratory (Bar Harbor, ME). Rag2^−/− mice were intercrossed with *Rorc^−/−* and *Tbx21^−/−* mice to generate Rag2^−/−*/Rorc^−/−* and Rag2^−/−*/Tbx21^−/−* mice. Rag2^−/−*/Ifng^−/−* mice were from Taconic (Germantown, NY). For footpad infection, mice were infected in both rear footpads with 50 μL of PBS containing 1 × 10^6 colony-forming units (CFUs) of *Brucella*. For intranasal infections, mice were first anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine, and 20 μL of PBS containing 1 × 10^8 CFUs of *Brucella* was placed onto the anterior nares. To neutralize IFN-γ, mice were treated intraperitoneally with 0.5 mg anti–IFN-γ (clone XMGI1.2; BioXCell, Lebanon, NH) 1 day before and 3 days after infection. Mice were treated with 0.2 mg of anti-NK1.1 (clone PK136; Leinco, St. Louis, MO) or anti-CD90.2 (clone 30H12; Leinco), on days 1, 2, 5, 8, and 11 in relation to infection, to deplete these mice of NK cells or ILCs, respectively. Control mice received rat IgG (Southern Biotech, Birmingham, AL) as an isotype control. Flow cytometry was performed to confirm depletion of NK cells or ILCs (Supplemental Figure S1). Rag2^−/−* mice were treated intraperitoneally with 0.3 mg SR3335 (Cayman, Ann Arbor, MI) daily for 7 days to inhibit Rorγ. All animal experiments were approved by the University of Missouri (Columbia, MO) Animal Care and Use Committee.

**Measurement of Bacterial Burdens and Cytokines in Tissues**

At 7, 15, 23, 27, 30, 39, or 42 days after infection (as described in figure legends), mice were euthanized, and blood, spleens, lungs, brains, and/or joints (following removal of skin) were harvested. Tissues were homogenized mechanically in sterile PBS. A series of 10-fold dilutions were performed in triplicate in sterile PBS and plated onto *Brucella* agar. Plates were incubated 3 to 4 days at 37°C/C5% CO2, colonies were enumerated, and the number of CFUs/tissue was calculated (brains and blood were incubated up to 7 days). For measurement of cytokines, homogenized tissues were centrifuged at 2000 × g for 5 minutes, and supernatants were filter sterilized (0.2 μm) and stored at −70°C before analysis. Cytokines were measured with a Luminex (Austin, TX) MagPix instrument using Milliplex magnetic reagents, according to manufacturer’s instructions (MilliporeSigma, Burlington, MA). Luminox data were analyzed with Milliplex Analyst Software version 5.1 (MilliporeSigma).

**Assessment of Joint Pathology**

Following footpad infection, joint swelling was evaluated as described previously. Briefly, ankle swelling was measured at 7 or 15 days after infection by collective measurements of both tibiotarsal joints. The difference of the recorded measurement from the basal measurement was reported as mean...
joint swelling. Following pulmonary infection, clinical arthritis scores were determined using the following scale per paw: 0, no swelling or redness; 1, mild redness or swelling of a single digit; 2, swelling of ankle or wrist with erythema; and 3, severe swelling and erythema, with a maximum clinical score of 12 in an individual mouse. For histology of mouse ankles, skin was removed, and ankles were fixed in 10% buffered zinc formalin, decalcified in 15% formic acid, rinsed in tap water, dehydrated with an alcohol gradient and clearing agent, and then embedded in paraffin. Tissue sections (5 μm thick) were mounted onto glass slides, and serial sections were stained with hematoxylin and eosin and covered with a coverslip with aqueous mounting medium. Tissues were evaluated for arthritis within the metatarsophalanegal joint through the examination of macrophagic infiltration into associated structures (synovial membrane, bone marrow, tendons, and skeletal muscle), severity of proliferative synovitis, and extent of bony resorption and myositis within and around the joint.

Assessment of Brain Pathology

Before removal of brain, mice were exsanguinated, and 2 to 3 mL of PBS was perfused through the left ventricle to reduce blood contamination. For brain histology, brains were either cut transversely, and sections were taken from the cranial cerebrum, caudal cerebrum, midbrain, and cerebellum, or cut sagittally, with one hemisphere being harvested in the cranial cerebrum, caudal cerebrum, midbrain, and cerebellum, and the other sectioned transversely as described above. Samples were rehydrated in xylene and graded alcohols rinsed with distilled water. Antigen retrieval was performed by immersing sections in either citrate buffer, pH 6.0 (to detect Brucella), or Diva (to detect myeloperoxidase (MPO), heating under pressure for 15 minutes, followed by cooling to room temperature while immersed in buffer. All subsequent incubations were performed at room temperature. Slides intended for enzyme-mediated histochemistry were treated with Peroxidazed 1 and rinsed. All slides were blocked with casein for 15 minutes and rinsed before incubation for 60 minutes with the following primary antibodies: anti-MPO (1:500 dilution of rabbit monoclonal antibody ab208670; Abcam, Waltham, MA) or anti-Brucella (1:2000 of rabbit polyclonal antibody bs222929; Bioss, Woburn, MA). Sections were then washed and incubated for 60 minutes with a rabbit-on-canine horseradish peroxidase polymer (RC542; Biocare Medical, Concord, CA). Slides were then developed with diaminobenzidine (SignalStain DAB; Cell Signaling Technology, Danvers, MA), lightly counterstained with hematoxylin, and coverslipped with permanent mounting medium.

RNA Extraction and RNA Sequencing

Before removal of brain, mice were exsanguinated, and 2 to 3 mL of PBS was perfused through the left ventricle. Brains were then homogenized in TRIzol Reagent (ThermoFisher, Waltham, MA), and RNA was isolated according to manufacturer's instructions. RNA was further purified on an RNeasy column (Qiagen, Valencia, CA). Poly A–enriched stranded mRNA libraries were generated, which were then sequenced on a NovaSeq 6000 (Illumina, San Diego, CA). RNA-sequencing analysis was performed using OneTopRNAseq. Specifically, FastQC version 0.11.5 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc) and MultiQC were used for raw read quality control, and QoRTs was used for postalignment quality control. Reads were aligned to the reference genome assembly mm10 with star_2.7.5a and annotated with gencode. vM25. Aligned exon reads were counted toward gene expression with featureCounts_2.0. With default settings, differential expression analysis was performed with DESeq2_1.28.1. Heat maps were generated with heatmap (https://github.com/raivokolle/heatmap). Gene set enrichment analyses were performed. The data discussed in this publication have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo; accession number GSE217496).

Quantitative Reverse Transcription PCR

RNA was extracted as described above. cDNA was generated using the Superscript III First Strand Synthesis System (ThermoFisher) with oligo dT primers. The quantitative reverse transcription PCR was set up in duplicate, and data...
were collected on an Applied Biosystems (Waltham, MA) StepOne Real-Time PCR System. mRNA in relation to glyceraldehyde-3-phosphate dehydrogenase was quantified by measuring SYBR Green (ThermoFisher) incorporation. The following primers from Integrated DNA Technologies (Coralville, IA) were used: Gapdh, 5′-GTGGACCTGGACCATCATC-3′ (forward) and 5′-GGGTGCAGCTTCCGCCTGCA-3′ (reverse); Prl, 5′-TCACTCAATAGC-CTGCCCACCT-3′ (forward) and 5′-CAGAAGAAACCTCCTCCGGAGGGA-3′ (reverse); Ccr2, 5′-AGCAGTGGTGTGAAATCCTGCT-3′ (forward); Cxcr2, 5′-CAGTTCAACCAGCCTGTG-GA-3′ (forward) and 5′-GGGTAGCTGCAATGG-3′ (reverse).

**Open-Field Behavior Testing**

Mice were placed in a 40 by 40-cm opaque, acrylic, open-top chamber with the floor equally divided into nine sections. Mice were placed in the center section of the chamber, exploratory behavior was filmed for 5 minutes, and the number of quadrant crossings was recorded. To record a quadrant crossing, all four feet of the mouse had to leave one quadrant and enter another. Following each 5-minute recording, the box was cleaned with 70% ethanol before placing the next mouse in the chamber.

**Statistical Analysis**

Statistical analysis of the difference between two mean values was conducted using a two-tailed t-test with significance set at P ≤ 0.05, whereas comparisons of three or more mean values were done using analysis of variance, followed by the Tukey test, with significance set at P ≤ 0.05. In figures, significance is denoted as follows: *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. The incidence of arthritis or neurologic signs was analyzed by log-rank analysis on incidence curves. All error bars display SD, and n values and the number of experimental repeats are provided in figure legends.

**Results**

**NK Cells and Other ILCs Limit Joint Infection and Inflammation following Footpad Infection with *B. melitensis***

Although adaptive immune cells partially control *Brucella* infection in the joint, innate sources of IFN-γ, including ILCs, also contribute to control of infection and suppression of *Brucella*-induced arthritis. Here, the role of individual ILC subsets in control of infection and inflammation during articular brucellosis was investigated. *Rag1−/−* mice (B- and T-cell deficient) were infected in the rear footpads with *B. melitensis* and treated with isotype control, anti-NK1.1 (to deplete NK cells), or anti-CD90 (to deplete all ILCs, including NK cells). At 7 days after infection, depletion of NK cells increased *Brucella* levels in the joint but did not affect joint swelling or splenic *Brucella* burdens (Figure 1A–C). In addition, joint IFN-γ levels were significantly decreased in mice lacking NK cells (Figure 1D). Similar to a previous report, at 7 days after infection, ILC depletion enhanced joint swelling, but actually decreased *Brucella* levels in the joint and spleen relative to mice depleted of NK cells alone (Figure 1E–G). At 15 days after infection, depletion of NK cells or all ILCs markedly increased joint bacterial burdens, and in contrast to what was found 7 days after infection, mice depleted of all ILCs had slightly higher joint CFU levels than joints from mice depleted of NK cells alone (Figure 1H). In addition, depletion of all ILCs, but not NK cell depletion, enhanced *Brucella* levels in the spleen (Figure 1I). Although depletion of NK cells did enhance joint swelling 15 days after infection, joint swelling in mice depleted of all ILCs was more than two times higher (Figure 1J). To further investigate the role of ILCs on inflammation, joints were assessed via histopathology. In isotype-treated animals, there was mild to moderate macrophagic inflammation, consisting primarily of macrophages and fewer numbers of neutrophils, within the metatarsophalangeal joint and associated structures. Proliferative synovitis, myositis within peripheral skeletal muscle, and bony resorption of the navicular bone was noted; however, the severity of these lesions was mild to moderate (Figure 1K). On examination of joints from NK cell–depleted mice, increased recruitment of macrophagic cells into the joint space, synovial membrane, and adjacent periosteum was noted, as well as significantly worsened synovitis, myositis, and bony resorption of the talus, navicular bone, and distal tibia (Figure 1K). Similarly, depletion of all ILCs resulted in severe osteoarthritis with the added effects of significant synovial hyperplasia and extension of macrophagic inflammation into adjacent musculature and edema, leading to worsened joint swelling. In addition, mice lacking all ILCs displayed the most extensive bone resorption (with inflammatory cells filling approximately 90% of the bone marrow cavity in certain areas) when comparing the three groups (Figure 1K). Because of the extreme joint swelling and inflammation in ILC-depleted mice, footpad infections were not extended past day 15.

**ILCs Are Required for IFN-γ Production and Limit Production of Other Inflammatory Cytokines within the Joint following Footpad Infection with *B. melitensis***

To determine how ILCs were limiting infection and inflammation, cytokine levels were investigated at day 15...
within the joints of *B. melitensis*-infected Rag1−/− mice receiving treatment with isotype control, anti-NK1.1, or anti-CD90 via Luminex instrument. Mice lacking all ILCs displayed significantly reduced levels of IFN-γ within the joint when compared with those with intact ILCs or lacking just NK cells (Figure 2A). Levels of IL-6, CXCL1, and chemokine (C-C motif) ligand 3 were significantly elevated in mice lacking all ILCs when compared with isotype control, anti-NK1.1, or treated with anti-CD90. Chemokine (C-C motif) ligand 2 and tumor necrosis factor-α levels were elevated in mice lacking NK cells or all ILCs relative to isotype control treated mice (Figure 2, E and F). Joint levels of IL-1β, IL-10, and CXCL9 did not significantly vary between groups (Figure 2, G–I). Collectively, these data suggest ILC deficiency leads to impaired IFN-γ production that, in turn, could result in enhanced levels of *Brucella* and proinflammatory cytokines within the joint.

**NK Cells Control Joint Infection in an IFN-γ-Dependent Manner following Footpad Infection with *B. melitensis***

To further examine the relationship between NK cells and IFN-γ in protection against bacterial colonization (Figure 1), Rag2−/− (B- and T-cell deficient) mice were footpad infected with *B. melitensis* and administered isotype control, anti–IFN-γ, or both anti–IFN-γ and anti-NK1.1. On day 7 after infection, joints and spleens were harvested and *Brucella* colonization was assessed. Mice that were administered anti–IFN-γ had joint *Brucella* loads 100-fold higher when compared with isotype control treated mice (Figure 3A), whereas splenic bacterial loads were similar between all groups (Figure 3B). In addition, mice lacking both IFN-γ and NK cells had bacterial levels similar to mice lacking only IFN-γ, indicating that NK cell-mediated protection against bacterial colonization within the joint is IFN-γ dependent (Figure 3A).

Next, the role of ILC subsets 1, 2, and 3 in articular brucellosis was investigated. To examine each individual cell type, the transcription factors T-bet (*Tbx21*; expressed by ILC1s),31 Rorγ (expressed by ILC2s),32 and Rorγt (*Rorc*; expressed by ILC3s)15 were targeted. In the first experiment examining these subtypes, Rag2−/− mice were compared with Rag2−/− mice lacking ILC1s (Rag2−/−/*Tbx21−/−*) and Rag2−/− mice lacking ILC3s (Rag2−/−/*Rorc−/−*). All mice were infected in the footpad with *B. melitensis*, and 15 days after infection, swelling was assessed and spleens and joints were harvested. Mean tibiotarsal joint swelling (Figure 3E) and bacterial colonization within the spleen (Figure 3D) showed no significant differences between groups. However, Rag2−/− mice lacking ILC1s displayed a significantly lessened bacterial burden in joints and spleens compared with Rag2−/− mice (Figure 3G, H). Additionally, Rag2−/− mice lacking ILC3s displayed a significantly lessened bacterial burden in joints and spleens compared with Rag2−/− mice (Figure 3I, J). These data indicate that ILC1s play a critical role in limiting joint bacterial colonization during brucellosis. Moreover, these data suggest ILC3 deficiency ameliorates joint bacterial load and decreases spleen bacterial load in a Rag2−/− background. Together, these data further support the critical role of ILC subsets 1, 2, and 3 in protection against *Brucella* infection.
Innate lymphoid cells (ILCs) are required for interferon (IFN)-γ production, and limit production of other inflammatory cytokines during Brucella-induced arthritis. Rag1−/− mice were footpad infected with 10⁵ colony-forming units of Brucella melitensis. Mice were also depleted of natural killer (NK) cells (anti-NK1.1), depleted of all ILCs (anti-CD90), or treated with isotype control. A–E: At 15 days after infection, cytokine levels in the joint were measured by LumineX instrument. Data are combined from two experiments, n = 8 to 9 Rag2−/− mice (A–E). *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. CCL, chemokine (C-C motif) ligand; TNF-α, tumor necrosis factor-α.

Figure 3 Natural killer (NK) cells control Brucella joint infection in an interferon (IFN)-γ–dependent manner. A and B: Rag2−/− mice were footpad infected with 10⁵ colony-forming units (CFUs) of Brucella melitensis. Mice were also treated with isotype control, neutralized of IFN-γ (anti–IFN-γ), or neutralized of IFN-γ and depleted of NK cells (anti-NK1.1). A and B: At 7 days after infection, Brucella levels in joint (A) and spleen (B) were measured. C and D: Rag2−/−, Rag2−/−/Rorc−/−, and Rag2−/−/Rag2−/− mice were footpad infected with 10⁵ CFUs of B. melitensis. C–E: At 15 days after infection, Brucella burdens in joint (C) and spleen (D) were determined along with tibiotarsal joint swelling (E). Data are combined from two experiments. n = 8 to 10 mice (A–D). *P < 0.05, ****P < 0.0001.
Although Rorγ is required for ILC2 development, its utility in long-term infection studies is limited as genetic deficiency of Rorγ results in development of a staggering phenotype in mice and death within 4 weeks. Therefore, in studies evaluating ILC2s, a chemical inhibitor of Rorγ, SR3335, was utilized. Although the differences were modest, SR3335 treatment reduced joint swelling and increased joint bacterial burdens following a 7-day footpad infection, whereas no significant effect was found on Brucella concentrations within the spleen (Supplemental Figure S3). Collectively, these data suggest a synergistic or compensatory relationship between ILC1s, ILC2s, and ILC3s throughout infection, rather than a single dominant non-NK cell ILC subtype responsible for mediating protection against brucellosis.

ILCs Limit Arthritis and Neurologic Complications of Brucellosis following Pulmonary Infection with B. melitensis

ILCs have been shown to be particularly abundant at mucosal barriers, and because inhalation is a natural route of Brucella infection, the role of ILCs following pulmonary infection was investigated. Although a previous study revealed that IFN-γ–deficient mice develop arthritis regardless of the route of Brucella infection, no clinical signs of arthritis were observed in Rag2−/−, Rag2−/−/Tbx21−/−, or Rag2−/−/Rorc−/− mice infected intranasally with B. melitensis over the course of a 6-week infection (data not shown). There were also no marked differences between Rag2−/−, Rag2−/−/Tbx21−/−, or Rag2−/−/Rorc−/− mice in the level of Brucella recovered from the spleen (Supplemental Figure S4A), and there were only slight differences in the lung (Supplemental Figure S4B). To examine the role of all ILCs in brucellosis following pulmonary challenge, ILC-deficient Rag2−/−/Il2rg−/− mice were employed. These mice possess a deletion of both the rag2 and the X-linked il2rg genes, which leads to a lack of T, B, and all innate lymphoid cells (including NK cells, ILC1s, ILC2s, and ILC3s). Following pulmonary infection, Rag2−/−/Il2rg−/− mice began showing signs of arthritis at day 27, and within the following 20 days, 90% of Rag2−/−/Il2rg−/− mice had developed arthritis (Figure 4A). In contrast, no Rag2−/− control mice developed signs of arthritis during the infection. The peak arthritis clinical scores were also significantly higher in Rag2−/−/Il2rg−/− mice when compared with Rag2−/− mice (Figure 4B).

Interestingly, during this infection, multiple Rag2−/−/Il2rg−/− mice unexpectedly began developing neurologic signs (head tilt, circling, diminished proprioception, and lethargy) starting at day 35, with 50% of Rag2−/−/Il2rg−/− mice developing these signs by day 49 after infection. In contrast, no Rag2−/− mice developed any signs of neurologic involvement during the 70-day long experiment (Figure 4C). Brains were harvested from neurologic Rag2−/−/Il2rg−/− mice at the time of clinical signs and sectioned in four separate areas (anterior cerebrum, posterior cerebrum, midbrain, and cerebellum). Hematoxylin and eosin staining revealed numerous multifocal areas of subdural perivascular cuffing and macrophagic cell infiltration throughout all sections (Figures 4D and 5A and Supplemental Figure S5A). In the retrosplenial region, the meninges were markedly expanded by high numbers of degenerate neutrophils and karyorrhectic debris (Figure 5A). Immunohistochemical analysis demonstrated a significant number of MPO-positive cells within these meningitic lesions, indicating macrophagic and neutrophil infiltration (Figure 5B). Immunohistochemistry also revealed abundant Brucella antigen within inflammatory lesions in the meninges and ventricles of infected Rag2−/−/Il2rg−/− mice, seemingly both within and around MPO-positive cells when comparing serial sections (Figure 5, B and C). No such
findings were noted in uninfected controls (data not shown). Brains were also harvested from Rag2−/− mice 70 days after infection. However, meningeal lesions were only detected in two of eight brains, each of which had a single inflammatory lesion (Figure 4D).

ILCs Limit Colonization of the Brain by *B. melitensis* following Pulmonary Infection

To determine if colonization of the brain was causing the observed neurologic signs, Rag2−/−/Il2rg−/− mice, along with groups of WT and Rag2−/− mice, were infected intranasally with *B. melitensis*. Beginning 1 month after infection, neurologic signs were observed in Rag2−/−/Il2rg−/− mice and, therefore, brains, blood, lungs, and spleens were collected and plated for quantification of bacterial levels. Infected Rag2−/−/Il2rg−/− mice showed significantly higher concentrations of *Brucella* within brain, blood, and spleen when compared with either WT or Rag2−/− mice (Figure 6, A–D), indicating ILCs confer protection to the host against *Brucella* following pulmonary challenge. Bacterial concentrations in the brain exhibited the most dramatic difference, as an approximately 1000-fold increase in *Brucella* was found in the brains of Rag2−/−/Il2rg−/− mice when compared with brains from Rag2−/− mice. In addition, the Rag2−/−/Il2rg−/− mice with the highest bacterial burdens in the brain were also the only three mice showing neurologic signs at the time of euthanasia, indicating that development of neurologic signs might be directly correlated with *Brucella* burden within the brain (Figure 6A). More important, although all Rag2−/−/Il2rg−/− mice were bacteremic, CFU levels in brain were >10 times higher than in blood, indicating the *Brucella* that was recovered from the brain was not due to potential contamination by blood (Figure 6, A and B). Although *Brucella* was detected in 50% of the brains from Rag2−/− mice, CFU levels were low and generally around the limit of detection (approximately 43 CFUs/brain). In addition, *Brucella* was not recovered from the brains of any wild-type mice. Cytokine levels were also quantitated in the brains of these animals via Luminex instrument. Although overall cytokine levels were typically low and near the limit of detection, CXCL1 was significantly increased in the brains of Rag2−/−/Il2rg−/− mice when compared with both Rag2−/− and WT mice, whereas chemokine
Enhanced Expression of Genes Related to Inflammation, and Decreased Expression of Genes Related to Neurologic Function, in the Brains of B. melitensis—Infected Mice

To uncover transcriptional changes occurring in Brucella-infected brains that might lead to neurologic complications, a group of Rag2<sup>−/−</sup>/Il2rg<sup>−/−</sup> mice was infected intranasally while another group of Rag2<sup>−/−</sup>/Il2rg<sup>−/−</sup> mice was mock infected with PBS. At the first observation of neurologic signs (which ranged from 39 to 54 days after infection), the brain of the infected mouse was harvested along with a brain of a mock-infected Rag2<sup>−/−</sup>/Il2rg<sup>−/−</sup> mouse, and then RNA was isolated from the entire brain. This was repeated until 80% of infected Rag2<sup>−/−</sup>/Il2rg<sup>−/−</sup> mice had displayed neurologic complications, and then RNA sequencing was performed. Differential gene expression analysis revealed that 1899 genes were up-regulated and 167 genes were down-regulated in the brains from infected animals (Figure 7A and Supplemental Table S1). Expression levels of genes encoding chemokine receptors involved in the recruitment of neutrophils (Cxcr2) and monocytes (Ccr2) were highly up-regulated in the brains of Brucella-infected animals (Supplemental Table S1 and Supplemental Figure S5B). Examining the gene ontology (biological process) of down-regulated pathways identified by gene set enrichment analysis, the pathways with the greatest net enrichment scores were related to nervous system function and development (Table 1). In contrast, up-regulated pathways in the brain during Brucella infection were associated with cytokine release and general inflammatory responses (Table 1).

Type I IFNs Limit Colonization of the Brain and Neuronal Loss following Pulmonary Infection with B. melitensis

The role of type I IFNs in brucellosis is nebulous, as macrophages deficient in the type I IFN receptor (Ifnar<sup>−/−</sup>) are...
more susceptible to *B. abortus* infection in vitro, whereas *Ifnar1*−/− mice control splenic *B. abortus* infection following systemic challenge better than control animals. As a marked up-regulation in genes involved in the type I IFN response in the brains of infected *Rag2*−/−/*Ifnar1*−/− mice was found, the role of type I IFNs in neurobrucellosis was investigated. Although *Brucella* does not consistently colonize the brain of WT animals (Figure 6A), *Brucella* reproducibly colonizes the brains of IFN-γ-deficient mice (Supplemental Figure S6). Therefore, WT, *Ifngr1*−/−, and *Ifngr1*−/−/*Ifnar1*−/− mice were infected intranasally with *B. melitensis* to study the role of type I IFNs in neurobrucellosis. Strikingly, by 14 days after infection, 50% of the *Ifngr1*−/−/*Ifnar1*−/− mice were displaying signs of overt neurologic disease (head tilt, circling, and diminished proprioception), whereas no WT or *Ifngr1*−/− mice were exhibiting such complications (Figure 8A and Supplemental Videos S1 and S2). On day 15, mice were sacrificed, and tissues were collected and plated for analysis of bacterial burden. *Ifngr1*−/−/*Ifnar1*−/− mice showed significantly higher levels of *Brucella* in the brain, blood, lung, and spleen when compared with WT animals, and >10-fold more bacteria in the brain, blood, and spleen when compared with *Ifngr1*−/− mice (Figure 8A, A–D). On histologic examination of the brains, there were significantly more inflammatory lesions within the meninges of the *Ifngr1*−/−/*Ifnar1*−/− mice when compared with WT mice (Figure 8E). However, the number of lesions overall was low and did not differ between *Ifngr1*−/− and *Ifngr1*−/−/*Ifnar1*−/− mice. Interestingly, there was significant neuronal loss noted in the hippocampus, specifically in the cornu ammonis 3 region, of 40% of *Ifngr1*−/−/*Ifnar1*−/− mice (all of which displayed neurologic signs at the time of necropsy). In contrast, only one *Ifngr1*−/− animal and no WT mice displayed marked neuronal loss within the hippocampus (Figure 8F).

**Discussion**

Although NK cells have previously been shown to be protective against intracellular bacteria, little is known about the role of other ILCs in bacterial diseases, brucellosis included. Here, depleting NK cells from B- and T-cell–deficient mice infected with *B. melitensis* enhanced bacterial colonization and swelling of the joint, indicating a clear protective role of these cells in articular brucellosis (Figure 1, H and J). Previous studies have shown that IFN-γ suppresses *Brucella*-induced arthritis. It has also been well documented that NK cells can play an important role in IFN-γ production during infection. Indeed, IFN-γ levels were significantly decreased in B- and T-cell–deficient mice depleted of NK cells (Figure 1D), and NK cell–mediated protection against *Brucella* colonization within the joint was found to be IFN-γ dependent (Figure 3A). Although depletion of NK cells from B- and T-cell–deficient mice enhanced susceptibility to colonization of the joint by *Brucella*, others have shown that depletion of NK cells from wild-type mice infected with *Brucella* does not enhance susceptibility to infection. This could indicate that NK cells play a compensatory role in controlling *Brucella* infection when B and T cells are absent. As T cells contribute to control of *Brucella* infection within the joint (Supplemental Figure S7), and can promote osteoclastogenesis, in the future, interactions between T cells and ILCs should be investigated in the pathogenesis of articular brucellosis.

Although NK cells contributed to control of *Brucella* within the joint, mice lacking all ILCs showed lower joint
ILCs and IFNs Prevent Neurobrucellosis

Table 1  Gene Ontology Terms of Pathways Differentially Regulated by Brucella Infection

<table>
<thead>
<tr>
<th>Pathway Description</th>
<th>NES</th>
<th>P value</th>
<th>FDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Down-regulated pathways in the brains of Brucella melitensis—infected Rag2&lt;sup&gt;−/−&lt;/sup&gt;/Il2rg&lt;sup&gt;−/−&lt;/sup&gt; mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GO_SYNAPTIC_SIGNALING</td>
<td>−10.79</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GO_NEUROGENESIS</td>
<td>−10.28</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GO_NEURON_DIFFERENTIATION</td>
<td>−10.09</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GO_NEURON_DEVELOPMENT</td>
<td>−9.71</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GO_CELL_CELL_SIGNALING</td>
<td>−9.27</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GO_CELL_PART_MORPHOGENESIS</td>
<td>−9.24</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GO_REGULATION_OF_NERVOUS_SYSTEM_DEVELOPMENT</td>
<td>−8.62</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GO_CELL_PROJECTION_ORGANIZATION</td>
<td>−8.61</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GO_CELL_MORPHOGENESIS_INVOLVED_IN_NEURON_DIFFERENTIATION</td>
<td>−8.43</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GO_REGULATION_OF_TRANS_SYNAPTIC_SIGNALING</td>
<td>−8.38</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GO_REGULATION_OF_IMMUNE_RESPONSE</td>
<td>10.37</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GO_RESPONSE_TO_CYTOKINE</td>
<td>13.27</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GO_CELL_ACTIVATION</td>
<td>12.4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GO_RESPONSE_TO_BIOTIC_STIMULUS</td>
<td>12.24</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GO_IMMUNE_EFFECTOR_PROCESS</td>
<td>12.07</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GO_DEFENSE_RESPONSE</td>
<td>11.89</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GO_REGULATION_OF_IMMUNE_SYSTEM_PROCESS</td>
<td>11.73</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GO_INFLAMMATORY_RESPONSE</td>
<td>11.22</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GO_INNATE_IMMUNE_RESPONSE</td>
<td>10.72</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GO_RESPONSE_TO_CYTOKINE</td>
<td>10.56</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GO_REGULATION_OF_IMMUNE_RESPONSE</td>
<td>10.37</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Up-regulated pathways in the brains of B. melitensis—infected Rag2&lt;sup&gt;−/−&lt;/sup&gt;/Il2rg&lt;sup&gt;−/−&lt;/sup&gt; mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GO_NEURON_DEVELOPMENT</td>
<td>10.09</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GO_NEURON_DIFFERENTIATION</td>
<td>10.28</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GO_REGULATION_OF_IMMUNE_SYSTEM_PROCESS</td>
<td>10.79</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GO_IMMUNE_EFFECTOR_PROCESS</td>
<td>10.28</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GO_REGULATION_OF_IMMUNE_SYSTEM_PROCESS</td>
<td>10.79</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GO_INFLAMMATORY_RESPONSE</td>
<td>10.28</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GO_RESPONSE_TO_CYTOKINE</td>
<td>10.79</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GO_REGULATION_OF_IMMUNE_RESPONSE</td>
<td>10.28</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Gene Ontology (biological process; http://www.geneontology.org) showing the top 10 pathways down-regulated and up-regulated in the brains of Brucella-infected mice (nominal P values and FDR q values are shown).

FDR, false discovery rate; NES, net enrichment score.

IFN-γ levels (Figure 2A), and significantly higher joint and splenic Brucella burdens (Figure 1, H and I), as well as worsened tarsal joint swelling relative to mice lacking NK cells alone (Figure 1J). ILC1s are known to produce IFN-γ, and can confer robust protection against other intracellular pathogens. Therefore, it was somewhat surprising that ILC1s only modestly contributed to control of Brucella infection within the joint (Figure 3C). Collectively, these findings indicate multiple ILC subsets play a compensatory role in protection against articular brucellosis.

When measuring joint cytokines, a pattern of increased cytokine levels was found in the joints of mice lacking all ILCs, especially for cytokines involved in the promotion of neutrophil chemotaxis, such as CXCR2 ligands (Figure 2), which were previously shown to mediate Brucella-induced arthritis. Increased levels of joint proinflammatory cytokines were also seen in mice lacking NK cells, albeit not to the same degree as in mice lacking all ILCs (Figure 2). This abundance of chemotactic cytokines in the absence of ILCs indicates that these cells may play an important regulatory role by controlling infection, preventing inflammation, and limiting the need for neutrophil recruitment to the joints of Brucella-infected mice.

Similar to what was observed during footpad infection, ILCs also limited the development of arthritis after pulmonary infection (Figure 4A). Surprisingly, beginning around day 35 after infection, Rag2<sup>−/−</sup>/Il2rg<sup>−/−</sup> mice began developing neurologic signs, including a head tilt, circling, diminished proprioception, and lethargy, whereas WT and Rag2<sup>−/−</sup> mice did not display these complications. Histologically, a primarily macrophagic cellular infiltration within the meninges and leptomeninges was noted throughout various sections of the brains (rostral cerebrum, caudal cerebrum, midbrain, and cerebellum), with severe perivascular cuffing noted (Figure 5 and Supplemental Figure S5). Detection of MPO by immunohistochemistry also indicated the infiltration of macrophages and neutrophils into the meninges of infected mice (Figure 5B). In addition, immunohistochemistry revealed significant amounts of Brucella antigen within these lesions, marking the first time Brucella has been visualized in the brains of mice (Figure 5C). Bacteria look to be located within MPO-positive inflammatory cells when comparing serial sections (Figure 5, B and C). These lesions appear to be similar to what occurs in human patients with neurobrucellosis, who display meningitis with heavy infiltration of inflammatory cells, particularly in the walls of blood vessels. Ceteacans with neurobrucellosis also display perivascular cuffing, which may include Brucella antigen. Astrogliosis and reactive microgliosis are also observed in the brains of human patients with neurobrucellosis. Astrogliosis is associated with enhanced expression of glial fibrillary acidic protein, and the gene encoding glial fibrillary acidic protein was up-regulated approximately threefold in the brains of Brucella-infected Rag2<sup>−/−</sup>/Il2rg<sup>−/−</sup> mice (Supplemental Table S1). In addition, kynurenine levels are elevated in
the cerebrospinal fluid of human patients with brucellosis,\textsuperscript{51} and multiple enzymes involved in kynurenine metabolism (Kynu, Haao, Kmo, and Qprt)\textsuperscript{52} were up-regulated in the brains of Rag2\textsuperscript{−/−}/Il2rg\textsuperscript{−/−} mice infected with \textit{Brucella} (Supplemental Table S1). Although neurobrucellosis has been well characterized in naturally infected dolphins\textsuperscript{47} and induced in experimentally infected macaques,\textsuperscript{3} this is the first study describing clinical and histologic signs of neurobrucellosis in a mouse model.

\textit{Brucella} can be detected in the brains of human patients with neurobrucellosis,\textsuperscript{53,55} and in other animals infected with \textit{Brucella}.\textsuperscript{33,55} The Rag2\textsuperscript{−/−}/Il2rg\textsuperscript{−/−} mice with the highest \textit{Brucella} concentrations within the brain were the same mice showing neurologic complications at the time of euthanasia 1 month after infection (Figure 6A), suggesting that the development of neurologic signs is directly related to the bacterial burden within the brain. In subsequent studies, \textit{Brucella} was found to be able to colonize the brains of Rag2\textsuperscript{−/−}/Il2rg\textsuperscript{−/−} mice within approximately 2 weeks of pulmonary infection (Supplemental Figure S8A), which likely leads to neurologic complications at later time points. A total of 50\% of Rag2\textsuperscript{−/−} mice had low levels of \textit{Brucella} in their brains at 1 month after infection, whereas \textit{Brucella} was not detected in the brains of WT mice (Figure 6A), indicating that both B/T cells and ILCs limit colonization of the brain by \textit{Brucella}. Colonization of the brains of Rag2\textsuperscript{−/−}/Il2rg\textsuperscript{−/−} mice by \textit{Brucella} does not appear to be dependent on the route of challenge, as \textit{Brucella} was also detected in the brains of Rag2\textsuperscript{−/−}/Il2rg\textsuperscript{−/−} mice infected intraperitoneally with \textit{B. melitensis} (Supplemental Figure S9A). However, Rag2\textsuperscript{−/−}/Il2rg\textsuperscript{−/−} mice infected intraperitoneally with \textit{Brucella} lost more weight than animals infected via the pulmonary route (Supplemental Figure S9E) and needed to be euthanized before the development of neurologic signs. This indicates that pulmonary infection may be preferable to systemic infection to study neurologic complications of brucellosis in Rag2\textsuperscript{−/−}/Il2rg\textsuperscript{−/−} mice. In addition, both \textit{B. melitensis} and \textit{B. suis} are able to colonize the brains of Rag2\textsuperscript{−/−}/Il2rg\textsuperscript{−/−} mice on a BALB/c background (Supplemental Figure S9F), suggesting ILCs might limit infection of the brain and neurologic complications of brucellosis regardless of \textit{Brucella} species or mouse background.

When identifying the transcriptional changes occurring in the brains of infected Rag2\textsuperscript{−/−}/Il2rg\textsuperscript{−/−} mice, a significant
up-regulation in the genes involved in the IFN-γ and type I IFN pathways was found (Figure 7B and Table 1). In contrast, a significant down-regulation in genes related to normal neuronal function, such as synaptic signaling, neurogenesis, and neuron development, was detected (Table 1). These changes suggest that in the presence of Brucella within the brain, a proinflammatory phenotype is adopted while basic brain functions are neglected. Thus, it is possible that these transcriptional changes are playing a role in the development of neurologic signs observed in the affected mice.

Previously, it has been demonstrated that both type I and type II IFN responses can play an integral role in preventing both viral and bacterial encephalitis and promoting clearance of bacteria from the brain.56,57 However, it has also been shown that Ifngr1−/− mice are less susceptible to pneumococcal meningitis, suggesting a potential detrimental role of type II IFN in certain diseases.58,59 Therefore, further research was necessary to determine the role of type I and II IFN responses in Brucella-induced meningitis. Ifngr1+/+/Ifnar1−/− mice were found to develop neurologic signs within 14 days following intranasal infection, whereas neither WT nor Ifngr1−/− mice developed such complications (Figure 8A and Supplemental Videos S1 and S2). These signs were similar to those seen in Rag2−−/II2rg−− mice; however, they developed more rapidly. Also, similar to what was observed in Rag2−−/II2rg−− mice, four of the five Ifngr1+/+/Ifnar1−/− mice with the highest bacterial burdens in their brains also displayed neurologic signs at the time of necropsy (Figure 8A), suggesting a correlation between bacterial burden within the brain and the development of behavioral abnormalities. Although type I IFNs promote control of Brucella infection in mice lacking IFN-γ signaling (Figure 8), others have shown that type I IFNs are deleterious to control of systemic Brucella infection in mice with an intact IFN-γ response.37 These data suggest the role of type I IFNs in controlling Brucella infection is context specific, and that type I IFNs may play a compensatory role in controlling Brucella infection when IFN-γ responses are impaired. However, future work will be needed to clarify the interactions of type I and type II IFNs in brucellosis.

After observing neurologic changes, behavior testing was performed on Brucella-infected mice. An open-field testing model was opted for as it was possible to conduct this testing in an animal biosafety level 3 setting within a biosafety cabinet50 and because this testing model has been shown to accurately quantify neurologic changes in mouse models of meningitis.50,60 Neurologic complications have been associated with increased61 or decreased60 exploratory behavior, depending on the study. However, although Brucella infection has been shown to alter the exploratory behavior of mice,62 no significant difference in exploratory behavior was observed between Brucella-infected WT, Ifngr1−/−, or Ifngr1−/−/Ifnar1−/− mice, or when comparing Ifngr1−/−/Ifnar1−/− mice that had neurologic signs with Ifngr1+/−/Ifnar1−/− animals without such complications (Supplemental Figure S10).

On evaluation of histology from neurologic Ifngr1+/−/Ifnar1−/− mice, cresyl violet-stained sections revealed significant neuronal death in the cornu ammonis 3 section of the hippocampus in 40% of Ifngr1−/−/Ifnar1−/− mice. Previous studies examining various animal models have demonstrated that the cornu ammonis 3 region of the hippocampus is a common anatomic landmark for identifying neuronal apoptosis/necrosis following toxin administration,63 induction of morphine addiction,64 and carbon monoxide and carbon disulfide exposure.65,66 In addition, in humans, this brain region is implicated in spatial processing and episodic memory development,67 and a decrease in neuronal density within this region can be observed in patients with Alzheimer disease.68 Therefore, neuronal death seen in the infected Ifngr1+/−/Ifnar1−/− mice could be contributing to the development of neurologic signs. Thus, measuring cornu ammonis 3 neuronal loss may be useful in the investigation and diagnosis of neurobrucellosis.

Collectively, these findings indicate ILCs and interferons play an important role in limiting both arthritis and meningitis during Brucella infection. In addition, this work details the first murine models of neurobrucellosis in which Brucella colonizes the brain, induces inflammation, and impairs neurologic function. In the future, these models can be employed to investigate multiple aspects of neurobrucellosis, such as invasion of the brain by Brucella, or therapeutics for treatment of neurologic complications of brucellosis.

Supplemental Data

Supplemental material for this article can be found at http://doi.org/10.1016/j.ajpath.2023.05.006.

References

29. Molely et al.