Osteoarticular tissue infection and development of skeletal pathology in murine brucellosis

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SUMMARY

Brucellosis, a frequent bacterial zoonosis, can produce debilitating chronic disease with involvement of multiple organs in human patients. Whereas acute brucellosis is well studied using the murine animal model, long-term complications of host-pathogen interaction remain largely elusive. Human brucellosis frequently results in persistent, chronic osteoarticular system involvement, with complications such as arthritis, spondylitis and sacroiliitis. Here, we focused on identifying infectious sites in the mouse that parallel Brucella melitensis foci observed in patients. In vivo imaging showed rapid bacterial dispersal to multiple sites of the murine axial skeleton. In agreement with these findings, immunohistochemistry revealed the presence of bacteria in bones and limbs, and in the lower spine vertebrae of the axial skeleton where they were preferentially located in the bone marrow. Surprisingly, some animals developed arthritis in paws and spine after infection, but without obvious bacteria in these sites. The identification of Brucella in the bones of mice corroborates the findings in humans that these osteoarticular sites are important niches for the persistence of Brucella in the host, but the mechanisms that mediate pathological manifestations in these sites remain unclear. Future studies addressing the immune responses within osteoarticular tissue foci could elucidate important tissue injury mediators and Brucella survival strategies.

INTRODUCTION

Brucellosis is caused by Brucella species, which infect a wide range of mammalian hosts. The World Health Organization considers brucellosis as one of the seven neglected zoonoses that contributes to the perpetuation of poverty (Maudlin and Weber-Mosdorf, 2006). Efforts to control brucellosis have been challenging, with recent disease re-emergence and new endemic foci posing significant risks to human health (Pappas et al., 2006). Management of individuals with brucellosis is extremely complex owing to its wide spectrum of clinical manifestations, resulting in high initial treatment failure and significant risk of relapse (Buzgan et al., 2010; Franco et al., 2007; Pappas et al., 2006). Osteoarticular complications from Brucella infection are the most common clinical manifestation, but symptoms also include debilitating neurological, heart and liver damage (Buzgan et al., 2010). In a recent study, as many as 46.5% of brucellosis patients experienced osteoarticular complications (Turan et al., 2011). Often, the challenges of clinical brucellosis are associated with its focal involvement; however, the pathophysiological manifestations in animal models of brucellosis remain poorly characterized.

Murine models have provided many cellular and molecular insights into brucellosis pathogenesis, such as the efficacy of vaccine candidates, nature of major virulence factors, and essential host immune responses (Baldwin and Goenka, 2006; Murphy et al., 2001). Pathology has been largely determined by the recovery of Brucella colony-forming units (CFU) in well-studied organs such as liver and spleen (Baldwin and Parent, 2002; Enright et al., 1990; I zadjoo et al., 2008; Mense et al., 2001; Silva et al., 2011; Young et al., 1979). Because of the wide tissue tropism observed in natural infections (Makdour, 1989), we hypothesized that murine brucellosis also has a multifocal presentation.

We chose to identify and characterize novel infectious foci in Brucella-infected BALB/c mice using in vivo bioluminescence imaging (BLI) in combination with guided histological characterization of the infected sites. BLI allows spatiotemporal identification of infectious foci in living hosts (Contag et al., 1995; Doyle et al., 2004). This technique allows us to decisively indicate the presence of metabolically active bacteria in deep tissues of mice (Hardy et al., 2009).

RESULTS

Brucella melitensis multifocal dispersion in the skeletal structure of the murine host

Brucella has a complex interaction with the host, and multiple organs and tissues can be affected in brucellosis patients, and frequently result in osteoarticular complications. Infection of mice has been widely used as a quantitative model of Brucella infection; however, the extent of peripheral murine tissue colonization is not well characterized. We evaluated bacterial dispersion in mice using a virulent, bioluminescent strain of Brucella melitensis, which we previously characterized (Rajashekara et al., 2005). BLI signals were monitored after intraperitoneal infection, and we observed rapid Brucella dissemination throughout the body (Fig. 1). Within days, BLI signals from the mouse body, mainly from peritoneum and liver (Fig. 1A), disseminated to peripheral sites, including the tail (Fig. 1B,C). Surprisingly, temporal resolution of infection in the peripheral sites was disproportionate and revealed a dispersion...
**TRANSLATIONAL IMPACT**

**Clinical issue**
Infection by *Brucella* spp results in brucellosis, a common, highly contagious zoonosis. *Brucella* can induce multiple effects in the host and, in humans, infection frequently results in persistent, chronic osteoarticular complications, including peripheral arthritis, spondylitis and sacroiliitis. In addition, the pathogen is able to persist in the bone marrow, resulting in chronic infection of the host. Although acute brucellosis has been extensively studied using murine models, the focal complications remain poorly understood because the extent of the infection in the many potential host sites (e.g. peripheral joints) cannot be easily evaluated in living animals.

**Results**
Here, the authors use a bioluminescent strain of *Brucella melitensis* to monitor dispersion of the bacteria in osteoarticular tissues of living BALB/c mice, followed by histological characterization of the infection sites. In vivo imaging demonstrated the rapid dispersion of bacteria to multiple sites in the skeletal structure of the murine host, following *Brucella* infection. Furthermore, the authors’ histological analysis showed that *Brucella*-positive cells were consistently located in the marrow regions of all bone types studied. Mice infected for a chronic period of 26 weeks exhibited multiple skeletal complications with inflammatory and non-inflammatory features.

**Implications and future directions**
Individuals with brucellosis display a wide spectrum of clinical manifestations. The high rate of treatment failure and relapse associated with the disease is often attributed to the intracellular survival of bacteria at the foci of infection. The identification of *B. melitensis* in murine bone marrow corroborates the theory that this site is an important niche for chronic persistence of bacteria in the host. It remains unclear whether infection at this site is facilitated by immune tolerance mechanisms, or, conversely, whether the immune responses cause inflammation and mediate the bone pathological manifestations observed. Therefore, future studies addressing the immune responses at the osteoarticular tissue foci will provide insight into the long-term survival strategies of *Brucella* and resulting chronic tissue injury in human patients. This study demonstrates the utility of the murine model for the exploration of pathophysiological mechanisms underlying chronic brucellosis.

pattern that is consistent with the segmented skeletal structure of the tail, with residual bacteria surviving for at least 2 weeks post-infection (Fig. 1A, day 6, day 13, arrow). Interestingly, these persistent foci were associated with defects in axial skeleton that were detected as dark spots in the tail (Fig. 1A, day 3). We further estimated the number of viable bacteria in caudal tissue segments (days 5 and 13 post-infection), and the BLI intensity from the caudal region strongly correlated with the bacterial CFU counts (Fig. 1D; \( R^2 = 0.84 \)). The CFU counts were directly proportional to the BLI, confirming that the observed luminescent pattern corresponded to the level of viable and replication-competent bacteria in the tissues. Although most infected animals did not display noticeable disease symptoms in the peripheral joints, some animals presented redness and swelling, with visible lesions accompanying the presence of bacteria, as shown by BLI (supplementary material Figs S1, S2, arrows).

Monitoring infection by BLI revealed initially the rapid bacterial dispersion dynamics throughout the mouse body followed by a punctuated luminescence pattern in the tail that matched the anatomical skeletal organization of vertebrae, suggesting direct colonization of osteoarticular tissue (Fig. 1B). Spatiotemporal evaluation of infectious foci revealed BLI signal in tails for at least 2 weeks after infection (Fig. 1). Interestingly, the BLI signals persisted at a time (day 14) that bacteria were rapidly being cleared from the region corresponding to spleen and liver (Fig. 1A,C). At this time point (2 weeks after challenge), mice infected under similar conditions had \( \sim 3 \times 10^5 \) CFU/spleen. Osteoarticular infections were identifiable and consistent in mice infected with a range of infectious inoculum doses (Fig. 1; supplementary material Fig. S1).

To investigate the mechanisms of bacteria dissemination, we sought first to characterize the relevant osteoarticular sites that parallel those affected in the disease in humans, possibly colonized by *Brucella*. Imaging at the higher BLI spatial resolution (4 binning, 10 cm field-of-view; see Materials and Methods) showed that *Brucella*-positive skeletal structures also include bones of the knee joints as well as smaller bones of the paws (Fig. 2). BLI patterns were remarkably symmetrical for the right and left knees, paws and ventral processes of the axial caudal skeleton, therefore highlighting the non-random pattern of bacteria dissemination routes in the body. Additionally, and as expected, the earliest and strongest BLI signal observed was from the liver (Fig. 2, ‘L’). Using high-resolution imaging, we identified five major locations of *Brucella*-associated cells in the mouse skeleton: (1) tibia and femur bones of the knee joint; (2) calcaneus; (3) metatarsophalangeal and metatarsatarsal articulations in paws; (4) ankle aspect of the tibia; (5) ventral processes of caudal vertebrae specifically in prezygapophyses, but not in transverse processes.

**Histological localization of Brucella in liver, spleen and skeletal tissues**
Brucellosis patients frequently display symptoms associated with bone and joint infection (Buzgan et al., 2010). After identifying potential skeletal infectious foci in mice using BLI, we sought to define the specific histological location of bacteria. To characterize the rheumatologic involvement of mice following *Brucella* infection, histopathological analysis was performed on small joints of hind and fore paws and larger knee and hip joints. Axial skeleton from the thoracic spine to caudal region, including the entire sacrum, was also examined.

To monitor *Brucella* dissemination at the tissue level, immunohistochemical (IHC) staining with antibodies to *Brucella* (Cat. # TC-7011, Tetracore Inc.) was used. Cross-reactivity of secondary antibodies with endogenous immunoglobulins could produce a considerable background, particularly when IHC of spleen and bone marrow tissues was performed. Specifically for this reason, we selected an alternative IHC detection approach for *Brucella*: primary antibodies were biotinylated and detected with streptavidin conjugated to horseradish peroxidase (St-HP). When primary and secondary antibodies are used, it is most effective to block tissues by using normal serum from the same host species as the labeled secondary antibody. Because secondary antibodies were not used for the detection in our protocol, the major concern was blocking endogenous biotin in spleen, bone and marrow tissues to prevent nonspecific staining with St-HP conjugate. Several sets of control staining were used to prevent false-positive signals. First, the complete IHC protocol was performed with tissues of mice that were not infected with *Brucella*, and produced no background staining. Second, primary goat antibodies were substituted with normal goat serum and then applied to St-HP detection (Fig. 3). Non-specific control staining was negative in liver (Fig. 3), bone
marrow (Fig. 4) and spleen (not shown). Also, St-HRP staining alone was used while omitting the primary antibodies, and staining was negative in all studied tissues (data not shown).

Liver infection with *B. melitensis* was associated with massive granuloma formation with fusion of individual granulomas as previously observed (Ko et al., 2002a; Ko et al., 2002b) (Fig. 3). The granuloma core consisted of round mononuclear cells with enlarged nuclei and cytoplasm. Core cells were surrounded by smaller epithelioid-like elongated macrophages (Fig. 3A,C). As expected, IHC-positive staining for *Brucella* confirmed the presence of bacteria in the granuloma core (Fig. 3A,B,E). Higher magnification (1300×; Fig. 3E) indicated the presence of *Brucella* antigen in the cytoplasm of round mononuclear cells and the absence of bacteria in epithelioid macrophages (Fig. 3B vs 3C). *Brucella*-positive signal was located solely inside granulomas, further supporting IHC specificity. Taken together, the livers of infected mice had *Brucella* mainly in the granuloma core and many fewer bacteria in Kupffer residential macrophages. *Brucella* was also detected in the spleen red pulp of acutely infected mice, whereas the white pulp was negative (not shown). Granuloma formation was not observed in bone marrow or spleen.

After 3 weeks of infection, bone marrow of all studied bones – including small bones of the hind and fore paws, tibia, femur, ileum, sacrum, vertebral bodies and processes – were examined for *Brucella* (Fig. 4). Interestingly, *Brucella*-positive cells were consistently located in the marrow of epiphyseal and metaphyseal regions and not detected in diaphyseal regions (Fig. 4B). Similarly, in vertebrae, homologous bone parts were infected that included endplates and subchondral/sub-growth plate areas (Fig. 4A,E,F). Articular cartilage of synovial joints, synovial cavity, nucleus

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**Fig. 1. Distribution of *Brucella melitensis* in BALB/c mice following i.p. infection.** BALB/c mice were inoculated intraperitoneally with 1×10⁷ CFU and imaged at the designated time points. Color scale represents bioluminescent signal intensity. (A) One of four animals is shown, on selected days. Bacteria disperse from the injection site (lower abdomen) to reach peak luminescence levels around day 6 and then are progressively cleared from the animal. A representative of the area used for quantification of photonic intensity – region of interest (ROI) – is depicted in the figure. Arrow indicates residual bacterial FIV, field of view. (B) Closer field of view (FOV; 10 cm) reveals punctuated diffusion of light in the lower limbs and tail of acutely infected mice (day 5; one of three animals is shown). Dispersion pattern is consistent with the segmented skeletal structures of these sites. (C) Spatiotemporal quantification of luminescence dispersal and persistence in body (blue) and tail (red) regions (ROI) of four infected BALB/c mice. Each line represents BLI luminescence quantification from an individual mouse (photons/second/ROI/mouse). Note that the caudal luminescence pattern is more variable than the light dispersion in the animal body. (D) Distribution of luminescence intensity (photons/second) and colony forming units (CFU) in 1.5-cm tail segments of seven BALB/c mice (24 segments total) at day 5 (three animals) and day 13 (four animals) post-infection. The solid line represents the linear regression of log CFU vs log photons/second/segment, and the dotted lines show the 95% confidence interval of the fit.
pulposus and annulus fibrosus of spinal discs were negative for *Brucella* (Fig. 4). Similarly, in bone marrow, ionized calcium binding adaptor molecule 1 [IBA-1; a marker specific for membrane ruffling and phagocytosis in macrophages (Ito et al., 2001)] and *Brucella* IHC staining both exhibited a subchondral location (Fig. 4C), but only a small subset of IBA-1-positive monocytes incorporated *Brucella* (Fig. 4B vs 4C). Bone marrow of both infected and naïve mice contained large numbers of IBA-1-positive cells. Although additional blocking is not required (Invitrogen Life Technologies), a generic blocking buffer (5% normal donkey serum, 2% BSA in 1 × PBS) was used to warrant rigorous specific binding conditions for the primary antibodies.

**Skeletal pathology in infected mice**

Naïve BALB/c mice rarely develop spontaneous inflammation in synovial joints (Adarichev et al., 2008; Farkas et al., 2009). BALB/c females infected with *B. melitensis* for 26 weeks exhibited multiple skeletal involvement with inflammatory and non-inflammatory features. Massive infiltration of inflammatory cells was found in synovial joints of the hind paws (Fig. 5A–C). Developing pannus and bone erosions were prominent features of the brucellosis-induced paw and spine arthritis, whereas *Brucella* itself was not detected in any examined tissues by 26 weeks post-infection. Another site of inflammation was facet joints of the dorsal vertebral processes (Fig. 5D). In both paw and spinal arthritis, leukocyte infiltration and bone erosions were marked. Also, torsion misalignment of the axial skeleton was observed in the caudal region, which was not accompanied with obvious inflammation (data not shown). This complex brucellosis-induced pathology with sterile synovitis resembles reactive arthritis and spondyloarthropathy in human patients.

**DISCUSSION**

In this study, we show rapid systemic distribution of *Brucella melitensis* in wild-type BALB/c mice, with high bacterial loads in multiple infectious sites associated with liver, spleen, bone marrow, spine, bones and joints. Bioluminescent imaging revealed a multitude of infectious foci in the caudal axial skeleton, and infected foci were corroborated using IHC staining specific for...
### Brucella

This extensive bacterial dissemination in the murine host raises novel possibilities for use of this brucellosis experimental model. Osteoarticular complications are particularly common in Brucella-infected humans, and the mouse model of brucellosis is particularly useful for using guided imaging techniques to identify infectious osteoarticular foci.

Osteoarticular involvement in mice is progressive and demonstrates a marked time lag after the peak of infection, thus resembling reactive arthritis in humans. The absence of bacteria is similar to observations of osteoarticular degeneration in some humans previously infected by Brucella (Tuna et al., 2011). In a study of 530 Brucella-infected patients, sacroiliitis was identified in 63 patients and 19 of these were culture negative (Ariza et al., 1993). In humans, Brucella-induced sacroiliitis is usually associated with acute spinal involvement (Ariza et al., 1993), whereas spondylitis, as we observed, is associated with a more chronic form of infection, with a median time of 9 weeks for spondylitis in humans (Dayan et al., 2009).

Additionally, we demonstrate that, in acute infection, numerous granulomas developed in livers by day 7. Morphologically, at least three cell types were readily identified in these granulomas: large core cells positively immunostained for Brucella, epithelioid macrophages, and, in the most outer layer of the granuloma, inflammatory lymphocytes and granulocytes. Over the course of months after Brucella infection, granulomas lost the outer seemingly pro-inflammatory layer of cells. Also, some sporadic remnant granuloma cores possessed very weak positivity for IHC Brucella lipopolysaccharide (LPS) staining. Whether these cells harbored live bacteria or only retained bacterial antigens is uncertain; however, live Brucella has been demonstrated to persist in BALB/c mice for greater than 360 days (Durward et al., 2012).

Recent efforts have initially characterized molecular aspects of Brucella-induced arthritis and inflammatory bone loss using in vitro cell models (Delpino et al., 2012; Scian et al., 2011). Brucella cell infection was found to influence bone metabolism via innate immune sensing, in both human and mouse cells (Delpino et al., 2012), reinforcing the idea that studies of the murine Brucella-infected osteoarticular system will yield valuable insights into the human disease. The intra-articular injection of heat-killed Brucella further suggests that joint infection can induce a pro-inflammatory environment (Scian et al., 2011). However, the nature of the cellular and inflammatory responses in vivo following infection remains to be tested. Our findings suggest that macrophages as well as other cells of the bone marrow and osteoarticular sites are in contact.
with bacteria in vivo and, therefore, it would be important to determine their role in the pathogenesis of osteoarticular brucellosis.

Whereas brucellosis-induced arthritis in wild-type mice is a progressive disease with postponed onset, mice carrying interferon gamma deficiency (IFNγ KO) develop arthritis much earlier, when live Brucella is still present at high concentration in the body (Skyberg et al., 2012). Similar to our results, this type of acute inflammation in IFNγ KO mice was limited to paw joints and tails. Interestingly, inflammation and Brucella foci were independent of infection route, suggesting that the osteoarticular site is a preferred location for bacterial persistence in the host and the most inflammation-susceptible structure (Skyberg et al., 2012). These studies are complementary, and suggest that murine fibroblast-like synoviocytes and monocytes in joints can harbor bacteria and elicit inflammatory mediators following infection. It was shown recently that, at least upon direct infection in vitro with Brucella abortus, human fibroblast-like synoviocytes are able to maintain bacterial replication and increase production of joint-damaging metalloproteases (Scian et al., 2011).

Infection of mice with 10^7 CFU of bioluminescent bacteria increases the numbers of exposed permissible host sites for bacterial localization, increases peripheral sites having detectable levels of bacteria by in vivo imaging, and provides reproducible infection development in peripheral tissues. Infection of mice with lower numbers of bacteria (5×10^5 and 1×10^4 CFU) also causes development of chronic localization of bacteria in tail vertebral joints by day 28 (Rajashekar et al., 2005), supporting chronic infection of this site with lower numbers of bacteria. Similarly, a low infectious dose (2×10^4) of B. abortus has been used in IFNγ−/− mice to induce articular infection at day 42 (Skyberg et al., 2012). However, the frequency of articular infection in these immune-deficient mice was not determined.

The identification of Brucella in the murine osteoarticular tissue substantiates the notion that this site is an important niche for bacterial persistence and pathogenesis. In human osteoarticular involvement, spondylitis is typically observed in the lower lumbar region, whereas arthritis is most prevalent in the knees (Madkour, 1989). Similarly in our studies, the caudal vertebrae were the most frequently affected axial skeletal structures, and knees were also commonly infected. Future studies addressing the immune responses at the osteoarticular tissue foci might elucidate important tissue injury mediators and Brucella survival strategies.

Bioluminescent imaging provides visual localization of osteoarticular brucellosis and permits the further study of infiltrating host cells, cytokines and pathological changes occurring kinetically at these sites. BLI is sensitive to tissue oxygen levels, depth and density, perhaps limiting certain applications. Therefore, it remains possible that other sites harbor Brucella in the host. We could not detect Brucella BLI signal from femur heads, sacrum and cervical, thoracic or lumbar spine, but anticipate these sites as potential infectious foci in vivo. The survival, propagation and dissemination of bacteria to multiple organs results in severe clinical manifestations in brain, heart, liver and osteoarticular tissues. Understanding interactions of Brucella with the host at these diverse infectious foci could elucidate important bacterial tissue-specific pathogenic mechanisms and niches that conceal bacteria and contribute to brucellosis-induced complications. Our consistent observation that Brucella can colonize these foci in this experimental model might help to fill an important knowledge gap by providing the remarkable opportunity to explore the mouse model in novel ways. For instance, focal progression of the infection in peripheral tissues of human patients is not understood. Further exploration of this model should allow us to determine the relevant parallels to human clinical outcomes.

**MATERIALS AND METHODS**

**Bacterial strains, growth conditions and preparation of inocula**

Bioluminescent B. melitensis (strain GR023) was previously constructed by insertion of an EZ::TN transposon containing a promoterless lux operon in gene BMEI0101 of B. melitensis 16M (ATCC23456) (Rajashekar et al., 2005). This strain replicates in vivo with kinetics and virulence similar to the parent wild-type strain (Rajashekar et al., 2005). Brucella was maintained on brucella agar (Becton Dickinson) and grown in brucella broth. Cultures started from isolated colonies were grown for 2 days in brucella broth at 37°C and used for mice infection experiments. Bacterial concentration was estimated indirectly by measuring the optical density at an absorbance of 600 nm and calibrating to known standard curve values. Prior to infection, samples were diluted in phosphate-buffered saline (PBS) for a final volume of 0.1-0.2 ml per mouse.

**Mouse experiments**

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Wisconsin-Madison, who evaluates the ethics of animal experiments (Permit Number: V00554). All in vivo imaging was performed under isofluorane gas anesthesia. All efforts were made to minimize animal suffering, and animals were housed in Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facilities. Animals received a standard rodent diet and water ad libitum.

Groups of BALB/c females (n=36) (Harlan, Indianapolis, IN, and Charles River Laboratories) were infected intraperitoneally with 10^7 bacteria (or indicated dose in the text). This inoculum dose permits detectable levels of bacteria by in vivo imaging, and provides reproducible infection development in peripheral tissues. The day of inoculation was designated day 0 and tissue was harvested at 3, 6, 9, 12, 26 and 27 weeks post-infection. Bioluminescence images were obtained using an IVIS 100 system (Caliper Biosciences) with animals under isofluorane gas anesthetic, administered at ~1-2% in oxygen. Images had an integration time of 1-10 minutes, binning 1-16, field of view 10-20 cm. Higher-sensitivity images, meaning higher ability to capture luminescence, were taken with longer integration time and greater binning, whereas higher-resolution images, meaning more pixels per square inch, were taken with shorter integration time and lesser binning. Higher resolution allowed pinpointing the source of luminescence, but capturing less signal. Data analyses were performed using Living Image software version 3.2. Bioluminescence in a desired anatomical region was determined by outlining the desired anatomical region followed the Living Image software defining the photons per region of interest (ROI). Luminescence intensity is expressed as a calibrated...
measurement of the photon emission from the subject (photons/second/cm²/steradian). For CFU determination, serially diluted tissue lysate prepared from 1.5 cm tail segments homogenized in 0.1% Triton X-100 (in water) with a tissue grinder (VWR) was plated in brucella agar Petri dishes. The CFU counts/tail segment was estimated as the number colonies recovered adjusted for the corresponding dilution factor.

Histopathology and immunochemistry

For histopathological analysis, hind limbs from digits to femur, including knee joint, were collected. Also, axial skeleton from thoracic to caudal regions was harvested. Tissues were fixed in 10% neutral-buffered formalin, decalced using Immunocal reagent (Decal Chemical Corp.) and embedded in paraffin at the Albert Einstein College of Medicine Histopathology and Comparative Pathology Facility, Bronx, NY. Tissues were sectioned at a thickness of 5 μm and stained with hematoxylin and eosin (H&E) or alcian blue and nuclear fast red for histological confirmation of the arthritis. For IHC analysis of bacteria, IgG antibodies to Brucella (#TC-7011, Tetracore Inc.) were labeled using N-hydroxysulosuccinimide ester of biotin (#21425, Thermo Fisher Scientific Inc.) according to the manufacturer’s protocol. After desalting, the degree of biotin incorporated into antibodies was measured using avidin conjugated to 4'-hydroxyazobenzene-2-carboxylic acid (HABA; #21425, Thermo Fisher Scientific Inc.). Paraaffin-embedded sections were heated in a drying oven at 60°C for 55 minutes and deparaffinized with xylene and then rehydrated using 100%-30% gradient ethanol baths. Antigen retrieval for IHC Brucella cell-wall detection was achieved with 0.25% trypsin (Mediatech) incubation for 25 minutes at room temperature. Slides were rinsed with 1×PBS and endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide in 1×PBS for 5 minutes. Nonspecific binding was blocked for 1 hour at room temperature using 2% BSA (Sigma), 2% normal non-immune donkey serum (Vector Labs), and 0.05% Tween-20 in 1×PBS. Sections were rinsed briefly with 1×PBS and additionally blocked using streptavidin-biotin blocking kit (#SP-2002, Vector Labs). Finally, sections were incubated overnight at 4°C with biotinylated antibodies to Brucella (1.0 μg/ml; #TC-7011, Tetracore Inc.). After three washes for 5 minutes with 1×PBS containing 0.05% Tween-20 (PBST), sections were incubated with streptavidin-conjugated to horseradish peroxidase (St--HRP; Pierce, Thermo Fisher Scientific Inc.) for 1 hour at room temperature. After three washes with PBST, slides were finally stained with ImmPact 3,3’-diaminobenzidine (DAB; Vector Labs) and counterstained with hematoxylin (Vector Labs) or alcian blue (Thermo Fisher Scientific Inc.). Sections were dehydrated and mounted with Permount (Thermo Fisher Scientific Inc.).

For IHC detection of the IBA-1 marker, paraaffin-embedded sections were deparaffinized in xylene followed by rehydration in graded alcohols similar to the protocol described for IHC detection of Brucella. Antigen retrieval was performed for 20 minutes in 10 mM sodium citrate buffer at pH 6.0 heated to 96°C. Endogenous peroxidase activity was quenched using 3% hydrogen peroxide in 1×PBS for 10 minutes. Blocking was performed by incubating sections in 5% normal donkey serum with 2% BSA in 1×PBS for 1 hour at room temperature. The primary antibody to IBA-1 (#019-19741, Wako Chemicals USA, Inc.) was used at 1:500 for 1 hour at room temperature. After rinsing with PBST, sections were incubated with SuperPicture HRP rabbit polymer conjugate (#87-9263, Invitrogen Life Technologies) for 15 minutes, rinsed with PBST and developed using DAB as the final chromogen. Immunostained sections were lightly counterstained with hematoxylin mounted with Permount (Thermo Fisher Scientific Inc.).

Statistical analysis

Data graphs were generated by using the statistical software Prism (GraphPad Software, Inc.), and statistical tests are indicated when appropriate. Analysis of Pearson’s correlation was performed using statistical package SPSS v15.0 (SPSS, Inc.). P-values of <0.05 were considered significant.

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COMPETING INTERESTS

The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS


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SUPPLEMENTARY MATERIAL

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