Uncorrected Version. Published on February 4, 2005 as DOI:10.1189/jlb.0604356

Borrelia burgdorferi, an extracellular pathogen, circumvents osteopontin in inducing an inflammatory cytokine response

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Abstract: A classic proinflammatory T helper cell type 1 (T_H1) response directed against intracellular pathogens includes the cytokine osteopontin, which acts predominantly on macrophages, where it induces the secretion of interleukin (IL)-12 and suppresses the secretion of IL-10. As cell-mediated immune responses play an important role in the resistance to Lyme arthritis, a manifestation of infection by the extracellular pathogen *Borrelia burgdorferi*, we tested the hypothesis that osteopontin may be required to induce T_H1 responses and inflammation. The role of osteopontin was tested in vivo and using ex vivo macrophages in B6129F3 mice susceptible to experimental Lyme arthritis. Mice of this genetic background and those fully backcrossed to C57BL/6, which lacked osteopontin expression (*spp1*$^{-/-}$), were as susceptible to *B. burgdorferi*-induced arthritis as littermate controls. Furthermore, equal numbers of spirochetes, as measured by quantitative polymerase chain reaction of the *B. burgdorferi* gene *recA* in *spp1*$^{-/-}$ and B6129F3 wild-type littermates, suggested that susceptibility to infection was not dependent on this cytokine. Neither of the B6129F3 parental mouse strains lacked the ability to secrete osteopontin. *spp1*$^{-/-}$ mice and controls had immunoglobulin G_2 titers, suggestive of a T_H1 response. *B. burgdorferi* was able to directly stimulate the secretion of the proinflammatory cytokines IL-12 and tumor necrosis factor α from wild-type and *spp1*$^{-/-}$ macrophages alike. These results indicate that the usually critical role of osteopontin in the induction of cellular immune responses to intracellular pathogens was circumvented by the ability of the extracellular pathogen *B. burgdorferi* to induce macrophages directly to produce proinflammatory cytokines. *J. Leukoc. Biol.* 77: 000–000; 2005.

Key Words: *mouse · macrophage · IL-12 · TNF-α*

INTRODUCTION

Host immune responses are characterized by predominantly cellular or predominantly humoral components. The former is associated with elevated levels of T helper cell type 1 (T_H1) cytokines, and the latter displays an abundance of T_H2 cytokines. Osteopontin induces macrophages to secrete the type 1 cytokine interleukin-12 (IL-12) and to suppress the generation of the type 2 cytokine IL-10. As such, it often plays an important role in establishing T_H1 responses. Genetic deficiency of the cytokine osteopontin (*spp1*$^{-/-}$) is associated with a type 2 cytokine pattern and increased susceptibility to intracellular pathogens, including *Rickettsia tsutsugamushi, Mycobacterium tuberculosis, Listeria monocytogenes*, and herpes simplex virus-type 1 [1–3]. Osteopontin facilitated macrophage-mediated clearance of *M. tuberculosis*, largely independently of interferon-γ (IFN-γ) or nitric oxide, and expression was associated with survival in human patients [3, 4]. Osteopontin may also contribute prominently to autoimmunity [1, 5, 6]. A role for osteopontin in defense against extracellular pathogens has not yet been defined.

In mice, disease manifestations following infection with the extracellular pathogen *Borrelia burgdorferi* vary widely depending on the host immune response. Experimental Lyme arthritis, which closely recapitulates features of acute Lyme arthritis in humans, is induced in susceptible mouse strains by infection with *B. burgdorferi*. Current evidence suggests that strong proinflammatory responses, coupled with bactericidal humoral responses, control the number of spirochetes in tissues. Susceptible C3H/He mice do not make the acute proinflammatory immune response associated with less severe disease manifestations in other mouse strains [7–9] despite their ability to respond to lipoproteins through Toll-like receptor 2 [10–12]. These mice were previously characterized as bearing a low expression allele of osteopontin [13], and their infection with *B. burgdorferi* consistently induces an early T_H2 cytokine response [8]. Although these results suggested a possible role for osteopontin in host resistance to experimental Lyme arthritis, a previous study concluded that a closely linked gene on mouse chromosome 5, rather than *spp1* itself, might be respon-

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Received June 23, 2004; revised January 10, 2005; accepted January 14, 2005; doi: 10.1189/jlb.0604356

sible for the host defense against *B. burgdorferi* in arthritis-resistant 129S7 mice [14].

A downside of a strong proinflammatory response against *B. burgdorferi* associated with type 1 cytokines is organ involvement, such as arthritis and carditis [15, 16]. Although C3H mice may lack robust, early T_H1 responses, a strong, late T_H1 response in these mice is associated with arthritis [17, 18]. A similar mechanism of delayed T_H1 response might in part explain the course of the group of human patients who develops antibiotic treatment-resistant Lyme arthritis [19]. The inflammation that is seen with *B. burgdorferi* infection may not be dependent on osteopontin, as some interbred mice (B6129F1 and B6129F2) bearing genes from the C57BL/6 and 129S7 parental strains were susceptible to arthritis in the presence and absence of the *spp1* gene [14]. These *spp1*$^{-/-}$ mice produced an immunoglobulin G_2 (IgG_2) humoral response, characteristic of a T_H1 response, suggesting that the immune response had bypassed osteopontin.

To further characterize the possible relationship between osteopontin and inflammation in the immune response to *B. burgdorferi*, we studied cytokine production using ex vivo macrophages and assessed arthritis and bacterial burden in vivo following *B. burgdorferi* infection in arthritis-susceptible mice in the presence and absence of osteopontin.

MATERIALS AND METHODS

Mice

C57BL/6J and C3H/HeJ mice were obtained from Jackson Laboratories (Bar Harbor, ME). Gene-targeted mice bearing a deletion of the osteopontin gene [20] on the 129S7 strain background (129S7^{tm1spp1}) had been backcrossed to C57BL/6J for three generations, and mice bearing the deleted allele were intercrossed to obtain homozygous gene-targeted mice. The mice were weaned between 3 and 4 weeks of age and genotyped by polymerase chain reaction (PCR): B6129S7^{tm1spp1}F3 (*spp1*$^{-/-}$), B6129S7F3 (*spp1*$^{+/-}$), or (*spp1*$^{+/+}$). Littermates of the same sex were housed together in a 14-h light, 10-h dark cycle with chow and water ad libitum.

Spirochetes and antigen preparation

Dr. Stephen Barthold (University of California, Davis) kindly provided the N40 isolate of *B. burgdorferi*. Derivation of clone D10/E9 was described previously [21], and a virulent isolate was obtained by infecting mice with this clone and isolating spirochetes from tissues by outgrowth in Barbour-Stoenner-Kelly (BSK) II medium (Sigma Chemical Co., St. Louis, MO). Soluble antigens were prepared from a 500-ml culture of spirochetes by sonication, as described previously [22]. The supernatant of soluble antigens (lysate) was stored in aliquots at −70°C.

Lysate stimulation of lymph node cells

Single-cell suspensions from pooled lymph nodes of uninfected C57BL/6J or C3H/HeJ mice were washed with RPMI-1640, supplemented with 20 mM HEPES, 1 mM sodium pyruvate, a 1× concentration of nonessential amino acids, and 2 mM l-glutamine (serum-free, complete RPMI), and were plated at 5×10^5 cells/well in 96-well plates in the presence of 10 μg/ml soluble antigen in serum-free, complete RPMI. Supernatant was harvested from cultures after 17–40 h and frozen at −70°C.

Mouse infection

Low passage isolates of *B. burgdorferi* strain N40 were frozen into single experiment aliquots in BSK II containing 30% glycerol. To infect the mice, frozen *B. burgdorferi* were thawed rapidly, transferred into 10 vol BSK II, and cultured overnight at 32°C prior to enumeration of motile spirochetes using dark field microscopy. Mice were inoculated in the right hind footpad with 5×10^4 motile spirochetes in 50 μl BSK II or were mock-infected with BSK II alone. All animal experiments were performed according to National Institutes of Health (NIH) guidelines under an Institutional Animal Care and Use Committee-approved protocol.

Lyophilized, purified recombinant mouse osteopontin was obtained from R&D Systems (Minneapolis, MN), reconstituted with sterile phosphate-buffered saline (PBS), and stored at −70°C. B6129S7^{tm1spp1}F3 and C3H/HeJ mice were injected subcutaneously (s.c.) on days 0, 2, 4, 6, 8, and 10 postinfection with 10 μg osteopontin.

Joint swelling and inflammation

Ankle swelling and histopathologic changes are under control of different genes in mice [23]. Tibiotarsal joint swelling was measured on a weekly basis with a spring-loaded microcaliper (Federal, Providence, RI). After sacrifice at 7 weeks postinfection, the right tibiotarsal joint was fixed in 10% formalin (Fisher Scientific, Pittsburgh, PA). Fixed tissue was decalcified, sectioned, and stained with hematoxylin and eosin by Suzanne White (Beth Israel Medical Center, Boston, MA). K. Craig-Mylius and L. Glickstein independently scored each ankle from zero (no arthritis) to four (severe arthritis) and averaged scores as described previously [22].

Quantitative PCR (Q-PCR)

B. burgdorferi- and mock-infected mice were killed at 5 weeks postinfection, and the joint tissue was stored at −80°C until DNA extraction was performed as described [24]. Q-PCR was performed in a spectrofluorometric thermal cycler (MX4000, Stratagene, La Jolla, CA) The final reaction concentration was 1× Brilliant SYBR Green master mix (Stratagene), 3 mM $MgCl_2$, 0.25 μM each primer, and 200 ng target DNA. The primers used to detect *B. burgdorferi recA* were nTM17F (5′-GTG GAT CTA TTG TAT TAG ATG AGG CTC TCG-3′) and nTM17R (5′-GCC AAA GTT CTG CAA CAT TAA CAC CTA AAG-3′) [24]. The primers used to detect mouse *nidogen* were nido.F (5′-CCA GCC ACA GAA TAC CAT CC-3′) and nido.R (5′-GGA CAT ACT CTG CTG CCA TC-3′) [24]. The thermal profile conditions used were 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 s and 60°C for 1 min. Emitted fluorescence for each reaction was measured three times during the annealing/extension phase, and amplification plots were analyzed using the MX4000 software version 3.0 (Stratagene). A series of standards was prepared using DNA prepared from *B. burgdorferi* using the QIAmp DNA mini kit (Qiagen, Valencia, CA). The DNA concentration of the standard sample was used to calculate *B. burgdorferi* genome equivalents (1 μg/ml DNA=6.1×10^5 genomes/μl), which were then diluted into DNA from an uninfected mouse to make a standard curve. As it has been shown that 200 ng mouse DNA contains ~10^4 *nidogen* copies, the number of *recA* copies amplified was normalized to this as described [24].

Spirochete infection of macrophages

Primary cultures of bone marrow-derived macrophages (BMMϕ) were prepared as described previously [25]. Briefly, the BM from four femurs (two mice) per strain was pooled for each experiment. A single-cell suspension was plated at 4×10^6 cells per 100 mm untreated plastic dish in 10 ml RPMI-1640 medium, supplemented with 10% fetal bovine serum (FBS), glutamine to 2 mM, penicillin G to 100 U/ml, and streptomycin sulfate to 100 ug/ml (components from Gibco/Invitrogen, Gaithersburg, MD), and 30% L cell-conditioned medium (BMMϕ medium) and was incubated at 37°C, 5% CO_2. Plates were fed on day 3 by adding 10 ml fresh BMMϕ medium. On day 5, the medium and nonadherent cells were discarded, and adherent cells were lifted with 2 ml PBS with 5 mM EDTA per plate. The lifted cells were then plated at 5×10^5 cells/well (six-well tissue-culture plates) in RPMI 1640 plus 10% heat-inactivated FBS, glutamine to 2 mM, penicillin G to 100 U/ml, and streptomycin sulfate to 100 ug/ml (L/Mϕ medium). Plates were incubated at 37°C, 5% CO_2, for 2 days before infection on day 7.

B. burgdorferi strain N40 clone D10/E9 was grown to 5×10^7–1×10^8 per ml in modified Kelly-Pettenkofer medium supplemented with human serum in place of rabbit serum at 33°C in tightly capped tubes as described previously [21, 26]. The cultures were then supplemented with sterile glycerol to 20% and frozen at passage 11 at −80°C until use.

Frozen spirochetes were thawed, washed twice at ambient temperature in PBS, supplemented with 0.2% bovine serum albumin (BSA), and resuspended at 1.67×10^7 spirochetes/ml [to achieve a multiplicity of infection (MOI) of 100 bacteria per macrophage] in L/Mϕ medium without antibiotics. Ten-fold serial dilutions of this suspension into the same medium achieved MOIs of 10 and 1. L/Mϕ medium alone (without antibiotics) served as the control and is designated as MOI = 0. Macrophages were infected in serum-free L/Mϕ medium for osteopontin Western blot analysis. Spirochetes were added to prewashed, plated macrophages (3 ml/well each six-well plate), and plates centrifuged (540 *g*) for 15 min at room temperature to maximize bacteria-macrophage contact. Plates were returned to the incubator for 3 h infection (Western blot) or 4 h infection (flow cytometry). Cell-culture supernatants were removed for Western blot, centrifuged (210 *g*) to remove cells and debris, and frozen at −80°C until analysis.

Western blot

Western blotting was performed after separation of 40 μl tissue-culture supernatant on nonreducing, denaturing sodium dodecyl sulfate-polyacrylamide mini-gels and transfer to nitrocellulose membranes. The membranes were blocked with 5% BSA and 0.05% Tween-20 in PBS and probed with biotinylated antiosteopontin antibody (R&D Systems), followed by streptavidin-peroxidase and luminescent detection. Purified recombinant mouse osteopontin (10 ng, R&D Systems) served as a positive control.

Flow cytometry

Monensin (3 μM, Sigma Chemical Co.) was added to six-well plates after 3 h incubation, and plates were returned to incubator for 1 h. Washed, adherent cells were harvested by digestion with 0.5 ml trypsin/EDTA (Gibco/Invitrogen) for 5 min at 37°C, scraping from plates and pooling duplicate wells. Cells (10^5/well) were aliquoted into a 96-well V-bottom plate for staining with the intracytoplasmic cytokine kit from BD PharMingen (San Diego, CA). Briefly, cells were fixed in Cytofix/Cytoperm for 15 min at 4°C, washed once with Perm/Wash, and then incubated with anticytokine antibody [1:100, anti-IL-12 phycoerythrin (PE) or anti-tumor necrosis factor α (anti-TNF-α) PE, from BD Pharmingen) for 15 min at 4°C. Cells were washed twice with Perm/Wash and resuspended in PBS (2% FBS, 0.1% sodium azide, Sigma Chemical Co.) for analysis on a BD Pharmingen FACSCalibur cytometer.

Ig enzyme-linked immunosorbent assay (ELISA)

B. burgdorferi antigen-specific ELISA was performed as described previously [22]. Briefly, diluted sera were incubated in plates previously coated with *B. burgdorferi* lysate and blocked with milk buffer. Isotype-specific secondary antibodies were added after washing, and plates were developed with appropriate reagents. Microplates were read at 405 nm. Titer was calculated by extrapolation from the linear regression of the absorbance from three dilutions of each serum sample. The titer was defined as the point where this line crossed that of the mean plus 3 SD of a pool of negative control sera.

Statistics

Means of groups were compared using Student's *t*-test. We tested the datasets for all groups of mice at all time-points for normal distribution according to the test by David, Pearson, and Stephens [27, 28]. *P* values that were less than or equal to 0.05 were considered significant.

RESULTS

Genetic deficiency of osteopontin does not affect the susceptibility to experimental Lyme arthritis

We obtained mice carrying one targeted allele on the 129S7 background that had been backcrossed to C57BL/6 for three generations, and we derived the three genotypes ($spp1^{+/+}$, $spp1^{+/-}$, $spp1^{-/-}$) by intercrossing these mice. It is important, however, that the 129S7 strain contributes the closely linked genes to the *spp1* locus in mice carrying the targeted allele. We infected $spp1^{-/-}$ mice or their littermates with N40 strain *B. burgdorferi* or injected them with BSK medium as a control. Wild-type mice and heterozygotes infected with *B. burgdorferi* developed moderate swelling of the tibiotarsal joints, one sign of arthritis, which reached statistically significant values in comparison with uninfected control mice 14 days after infection. The mice lacking the osteopontin gene were affected similarly to the $spp1^{+/-}$ and $spp1^{+/+}$ mice (**Fig. 1A**). It had previously been reported that mice with the same targeted mutation at the second backcross to C57BL/6 exhibited a bimodal distribution of susceptibility to *B. burgdorferi* arthritis because of the mixed genetic background [14]. However, in the present experiment, the arthritis data within each group were distributed normally with a probability of error of less than 5% (Fig. 1B). The statistical test used was sensitive enough to detect a non-normal distribution when mock-infected animals of all three genotypes were combined into a single group. In addition, after further backcrossing to C57BL/6 (10th generation, 99.8% congenic with C57BL/6), the susceptibility to arthritis in the $spp1^{-/-}$ mice was still indistinguishable from their littermate controls (data not shown).

We also prepared tissue sections from the ankles of the infected mice and their controls and scored cellular infiltrates as a second marker for arthritis severity. In concordance with the data on ankle swelling, no differences were detected between $spp1^{-/-}$ mice and their controls (Fig. 1C). Histology of inflamed joints was typical and included infiltrates in synovium, muscle, and tendon (Fig. 1D). Thus, the B6129F3 mice were susceptible to experimental Lyme arthritis in the presence and absence of osteopontin.

Exogenous osteopontin does not ameliorate experimental Lyme arthritis

It was still possible that a role for osteopontin was obscured by other susceptibility alleles expressed in these mice. To ascertain whether the cytokine plays a protective role against Lyme arthritis, we infected arthritis-susceptible $spp1^{-/-}$ mice and C3H/He mice with *B. burgdorferi* (strain N40) and injected 10 μg recombinant-purified osteopontin in PBS or PBS alone, s.c. above the right hind limb on days 0, 2, 4, 6, 8, and 10. This dose of osteopontin has previously been shown to reconstitute T_H1 responses in $spp1^{-/-}$ mice [1]. As expected, the C3H/He mice developed more severe arthritis than the $spp1^{-/-}$ mice. In neither case was a protective effect achieved by exogenous osteopontin (**Fig. 2**). In fact, the only significant difference between the groups was a more rapid onset of arthritis (day 14) in the osteopontin-treated C3H/He mice.

Osteopontin deficiency does not increase spirochete burden

Two principal components of Lyme disease are the replication and dissemination of the pathogen, which is limited by cellular as well as humoral immunity, and the tissue damage manifested as arthritis or carditis, which is presumably exacerbated by cellular immunity. These factors can segregate independently [23]. We asked whether the lack of osteopontin led to an increased number of spirochetes after infection. DNA was prepared from excised joint tissue, portions of the *B. burgdor-*

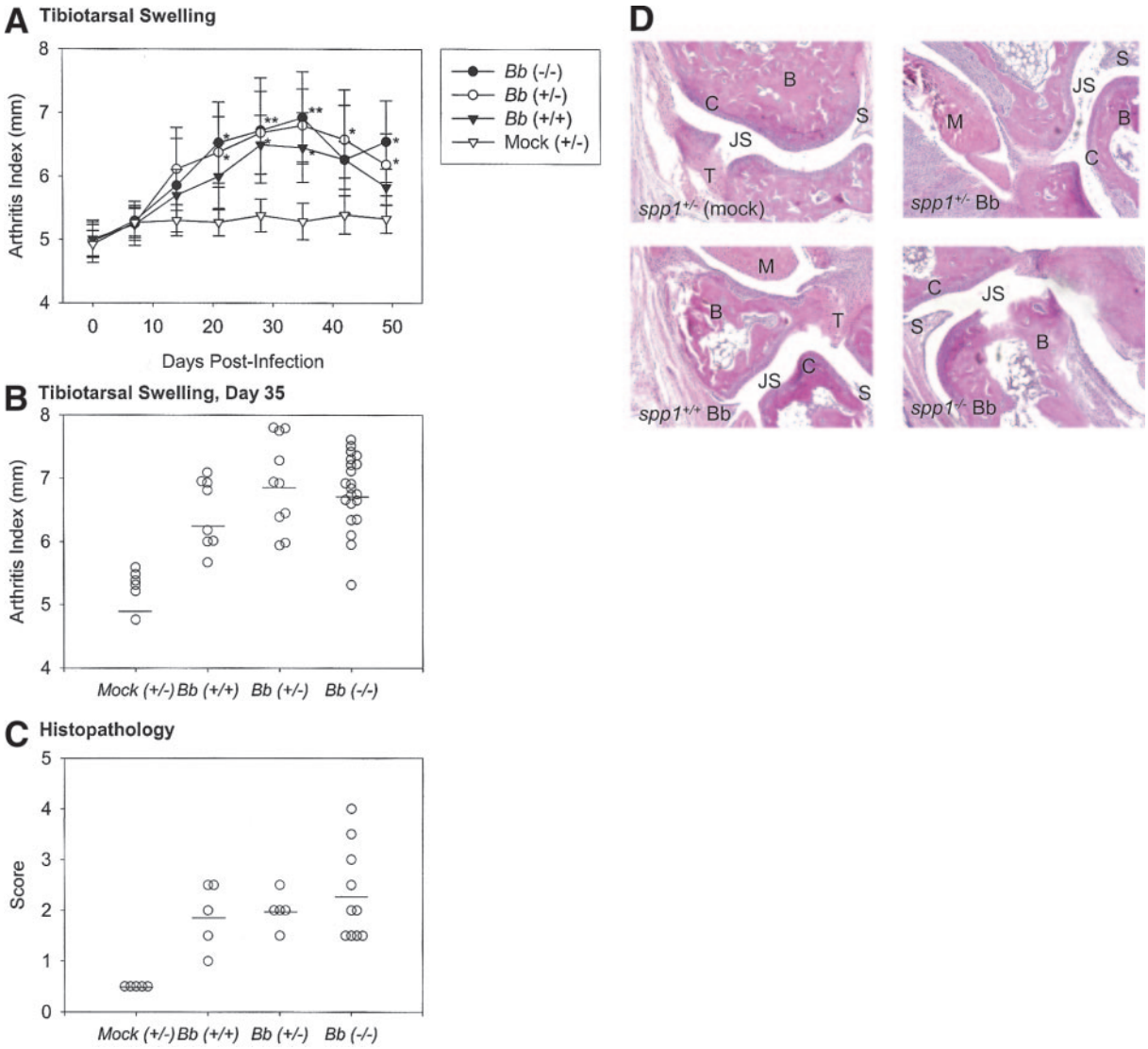

Fig. 1. Genetic deficiency of osteopontin does not affect the susceptibility to *B. burgdorferi*-induced arthritis. (A) Time-course of ankle swelling following infection by *B. burgdorferi*. Arthritis index was calculated as the sum of both tibiotarsal diameters. Mean ± SD of six to 22 mice per group is shown for each time-point. Values that are significantly greater than mock-infected mice are indicated (*). (B) Ankle swelling 35 days after infection, the time of peak arthritis; each point represents an individual animal. A black bar in each column indicates mean value. (C) Histopathology scores of ankle inflammation at sacrifice (49 days) after infection by *B. burgdorferi*. Mean value is indicated. Mock, Injected with medium; Bb, infected with *B. burgdorferi*. (D) Histologic sections of ankles of infected mice and mock-infected controls. *spp1* genotype of mice indicated as homozygous wild-type (+/+), deficient (−/−), or heterozygous (+/−). B, Bone; C, cartilage; JS, joint space; M, muscle; S, synovium; T, tendon.

feri recA and mouse *nidogen* genes were amplified by quantitative PCR, and copies per sample interpolated from a standard curve [24]. According to the ratio of *recA/nidogen* copies, there was no difference between *spp1*$^{-/-}$ mice and their littermate controls in the numbers of *B. burgdorferi* in situ in joints (**Fig. 3**). The numbers ranged from several to several hundred spirochetes per 5000 cell equivalents, as has been observed by others [14].

Osteopontin is secreted by lymphocytes and macrophages in response to *B. burgdorferi* lysate

It was possible that the lack of effect of osteopontin was a result of a failure of mice to secrete this cytokine constitutively or when induced with spirochetal antigens. We infected macrophages from the inbred C57BL/6 and 129S7 parental strains to avoid genetic heterogeneity of the B6129F3 strain and C3H/He mice in vitro with *B. burgdorferi* strain N40 at controlled cell density and known MOI, and we performed Western blot analysis for osteopontin secreted into tissue-culture supernatant. In addition, analysis of secreted osteopontin from lymph node cells of C57BL/6 and C3H/He mice was performed after in vitro stimulation with *B. burgdorferi* lysate.

Consistently, no detectable osteopontin was found in the supernatant from the C3H/He lymph node cells or macrophages following exposure to *B. burgdorferi* or its antigens (data not shown). Arthritis-susceptible C3H/He mice recognize *B. burgdorferi* antigens [17], but they have the osteopontin allele (*spp1*$^{b/b}$), which is associated with low expression levels [13].

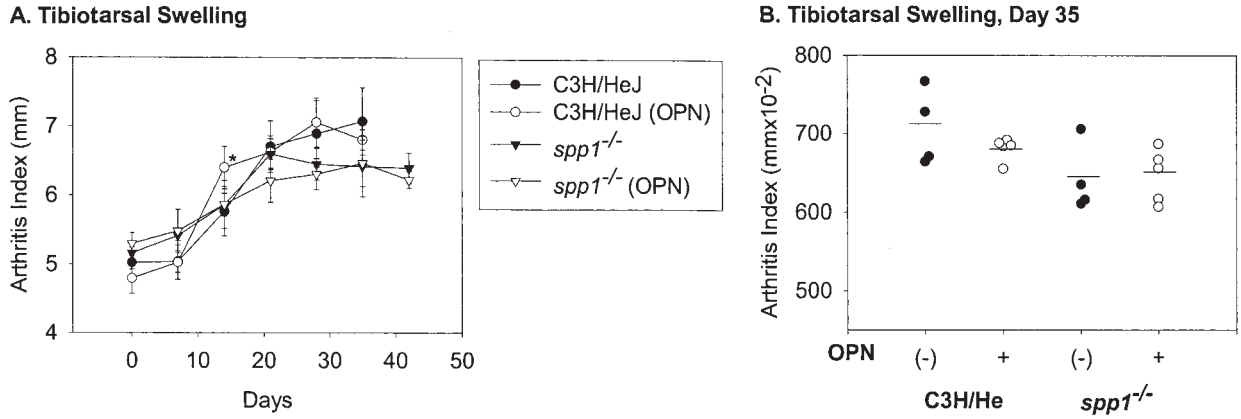

Fig. 2. Exogenous osteopontin does not reduce ankle swelling in C3H/He or *spp1*$^{-/-}$ mice. All mice were infected with *B. burgdorferi* in this experiment. Indicated mice [osteopontin (OPN)] received injections of recombinant osteopontin every other day from days 0 to 10. (A) Time-course of ankle swelling following infection by *B. burgdorferi*; each point represents the mean swelling of four to five animals per group. Values for treated groups that differ significantly from syngeneic infected mice are shown (*). (B) Ankle swelling on day 35 after challenge; each point represents an individual animal. The black bars indicate mean value for each group.

Osteopontin was constitutively secreted in macrophages from the C57BL/6 strain and induced in the 129S7 strain following in vitro infection with live *B. burgdorferi* (**Fig. 4**). Macrophages from the C57BL/6 mice expressed a form of osteopontin that had slower electrophoretic mobility, most likely a result of differential glycosylation [29, 30]. Recombinant osteopontin served as a positive control for antibody specificity.

B. burgdorferi antigens stimulated osteopontin secretion from the lymph node cells of C57BL/6 mice as early as 17 h after activation and accumulating through 40 h of culture (Fig. 4). Supernatant from cells incubated without antigen served as a negative control; no other bands were detectable on the blot (data not shown). This set of experiments demonstrated that spirochetal antigens could induce osteopontin production in cells derived from C57BL/6 and 129S7 mice.

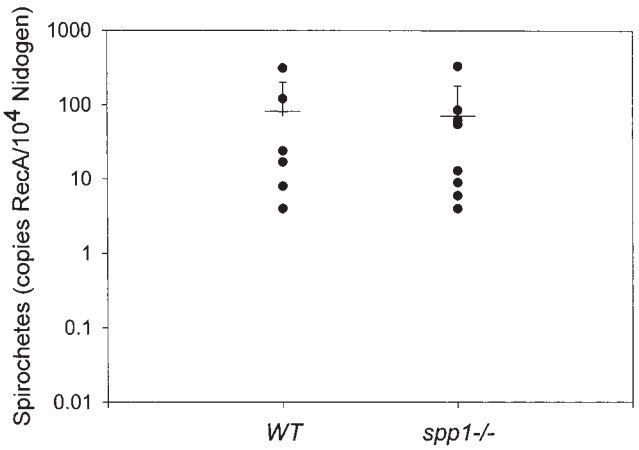

Fig. 3. Osteopontin deficiency does not increase spirochete burden following infection. Spirochete burden was determined by quantification of *B. burgdorferi recA* DNA copies in joints by Q-PCR. DNA was prepared from joint tissue and amplified with *recA* and murine *nidogen*-specific primers. Copy number was determined for each by comparison to a standard curve, and values expressed as copies *recA*/10^4 copies *nidogen*. Results (●) are presented for individual *spp1*$^{-/-}$ mice and wild-type (WT) littermates; black bars indicate mean value and SD.

Immune responses to *B. burgdorferi* do not depend on osteopontin

Class switching in the mouse, in particular, production of IgG_{2a}, is dependent in part on IFN-γ [31]. We examined *B. burgdorferi* antigen-specific Ig isotypes in the sera of infected mice. Although pathogen-specific IgG_{2a} and IgG_{2b} are considered to be indicative of T_H1-driven immunity, high titers of IgG_1 reflect T_H2 responses. We found all isotypes to be induced after challenge with no differences between *spp1*$^{-/-}$ and wild-type mice (**Fig. 5a**).

As the IgG isotype titers were not affected by osteopontin deficiency, we examined cytokine responses further. A previous study observed IL-10, but not IL-12, secretion induced by *B. burgdorferi* OspA lipoprotein from arthritis-susceptible B6129F2 mice [14]. We infected BMMϕ in vitro with live *B. burgdorferi* and measured the production of the T_H1 cytokine IL-12 and the arthritogenic cytokine TNF-α by intracellular staining and flow cytometry. Both cytokines were induced dose-dependently in macrophages from wild-type B6129F3 or *spp1*$^{-/-}$ mice (Fig. 5b). The macrophages from the *spp1*$^{-/-}$ mice produced higher levels of IL-12 and TNF-α in this experiment. The possibility of a negative feedback mechanism involving osteopontin might warrant further study.

DISCUSSION

Osteopontin, a cytokine previously shown to play a dominant role in controlling T_H1 responses in intracellular bacterial as well as herpes viral infections [1], was a candidate cytokine to play such a role in the host response to *B. burgdorferi* infection. In a multifactorial disease, a number of distinct genes contribute to the manifestation and severity of the symptoms, and this is certainly true for experimental Lyme arthritis [23, 32]. Mouse strains display a wide range of susceptibility to infection

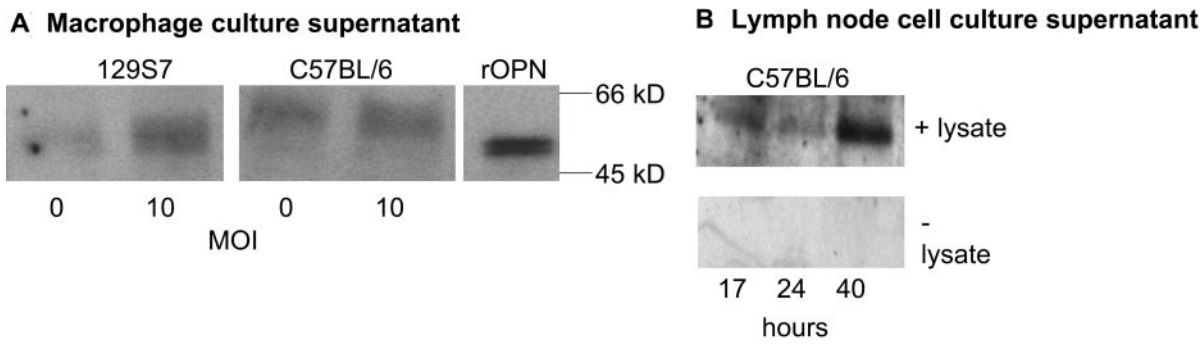

Fig. 4. Osteopontin secretion in response to *B. burgdorferi* in C57BL/6 and 129S7 mice. (A) Equal numbers of macrophages from 129S7 or C57BL/6 mice were infected with 0 or 10 *B. burgdorferi*/cell for 4 h in serum-free media, and the supernatants from the macrophage cultures were collected for Western blot analysis. Purified recombinant mouse osteopontin (rOPN; 10 ng) served as a positive control. (B) Equal numbers of primary lymphocytes from lymph nodes of uninfected C57BL/6 mice were treated with *B. burgdorferi* lysate for 17–40 h in serum-free media, and the supernatants from the culture wells were collected for Western blot analysis.

and arthritis. Osteopontin might have been expected to impact arthritis duration or severity quantitatively or to impact host mechanisms of arthritis resistance or arthritis pathogenesis. As was previously shown, osteopontin deficiency did not affect mechanisms of arthritis resistance in 129S7 mice [14]. In the present study, we demonstrate that osteopontin was also dispensable for arthritis pathogenesis in B6129F3 mice (or in C57BL/6 mice), which were uniformly susceptible, rather than resistant, to this disease manifestation. Furthermore, although C3H mice have the *spp1*$^{b/b}$ allele associated with low expression and develop severe experimental Lyme arthritis, reconstituting osteopontin exogenously in this strain or in the *spp1*$^{-/-}$ mice did not ameliorate this manifestation. These results confirm that osteopontin is not sufficient to prevent experimental Lyme arthritis. The ability of *B. burgdorferi* to cause arthritis in the absence of osteopontin can be explained by our finding that live *B. burgdorferi* induced the production of the proinflammatory cytokines IL-12 and TNF-α by macrophages, independently of osteopontin, a cellular response that was sufficient to generate humoral immunity and arthritis.

Although neither other groups nor ours identified any defect in the response to *B. burgdorferi* infection in vivo by *spp1*$^{-/-}$ mice, exposure to *B. burgdorferi* antigens led to substantial osteopontin secretion from lymphocytes of C57BL/6 and 129S7 mice that bear the *spp1*$^{a/a}$ allele. It is possible that osteopontin does affect symptoms other than arthritis. Our investigations have focused on the multiplicity of organisms in situ and on the development of acute arthritis as readouts for host responses to *B. burgdorferi* infection. We did not examine the murine carditis or cardiac conduction abnormalities associated with *B. burgdorferi* infection [33, 34]. Other signs of infection in humans, such as skin affliction (erythema migrans, acrodermatitis chronica atrophicans), eye, and nervous system involvement (meningoradiculoneuritis, dementia) or treatment-resistant Lyme arthritis, a syndrome of putative infection-induced autoimmunity, do not occur in mice, and thus, the potential effects of osteopontin on these disease manifestations could not be tested using the mouse model. In addition, the relatively low number of *B. burgdorferi* cells per mouse cell during in vivo infection might be below a critical threshold required to induce osteopontin in quantitities sufficient to alter the immune response.

Based on our results, however, we would argue that a role for osteopontin is unlikely, as a classic T_H1 response with cytotoxic T lymphocytes is not the critical arm of the immune response to extracellular pathogens. In the conventional T_H1 priming pathway, activated T cells secrete osteopontin, which induces macrophages to secrete IL-12 and inhibits IL-10 production, both of which drive T_H1 differentiation. For intracellular pathogens, generation of a cytotoxic T cell response is critical, as the immune system faces a challenge in that the pathogens are hidden within host cells. Thus, a mechanism involving T cell recognition and osteopontin-mediated priming of macrophages is needed to induce such a response. In contrast, *B. burgdorferi* expresses a number of lipoproteins that are highly immunostimulatory and proinflammatory [35–37]. In this setting, in which macrophages are activated directly, there is no need for osteopontin, and cytotoxic T cells are not required to clear infection. Rather, antibody responses are critical.

Circumvention of the conventional T_H1 priming pathway by direct induction of IL-12 from macrophages by lipoproteins or other spirochetal components may have implications for the pathology seen in murine and human infection. A lack of osteopontin production, as observed in susceptible C3H/He mice, might be expected to result in overproduction of IL-10. Excess IL-10 has been shown to impede the host antispirochetal immune response [38] and may contribute to chronic infection. Furthermore, if the infection is not cleared by a successful immune response or antibiotic therapy, chronic T cell-independent IL-12 and TNF-α production by macrophages and possibly other cells, induced by *B. burgdorferi* components, would lead to joint pathology and might prime susceptible individuals to develop autoimmunity. It is most important that dependence versus independence from osteopontin may define two variant innate-immune responses to intracellular versus extracellular bacterial infection. This may have important implications for disease manifestations, resolution, and therapeutic strategies.

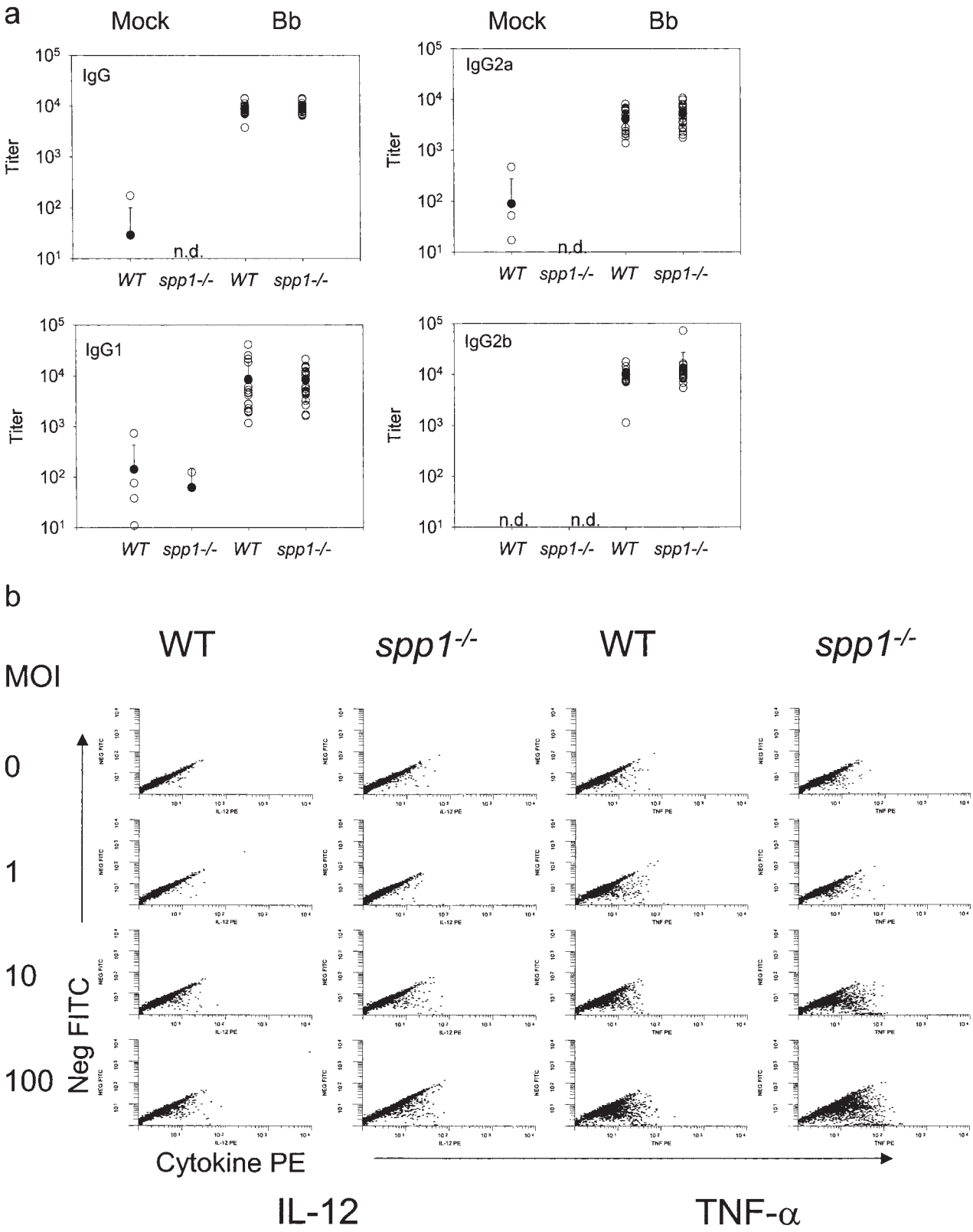

Fig. 5. *B. burgdorferi* induces IgG_2 isotypes and proinflammatory cytokines in macrophages independently of osteopontin. (a) *B. burgdorferi*-specific Ig isotype ELISA. Sera from *B. burgdorferi*-infected (Bb) and mock-infected mice were subjected to Ig isotype-specific ELISA against plate-bound *B. burgdorferi* lysate, and titer was calculated from serially diluted samples. Results for individual animals (○) and mean + SD (●) are presented for *spp1*$^{-/-}$ mice and wild-type (WT) littermates. n.d., None detected. (b) Intracellular cytokine staining. Macrophages from uninfected *spp1*$^{-/-}$ mice and WT littermates were infected ex vivo and subjected after 4 h to flow cytometry analysis for intracellular IL-12 and TNF-α. Histograms are shown for MOI = 0 and MOI = 1, 10, and 100. Neg FITC, Negative fluorescein isothiocyanate.

ACKNOWLEDGMENTS

This study was supported by NIH Research Grants CA76176 to G. F. W. and AI40938 and AI51407 to J. C. In addition, the authors were supported by grants from the Harold G. and Leila Y. Mathers Foundation (L. G.), the Arthritis Foundation (Biomedical Science Grant to J. C.), and by Public Health Service Grant P30DK39428, awarded by the National Institute of Diabetes and Digestive and Kidney Diseases to the Center for Gastroenterology Research on Absorptive and Secretory Processes at Tufts-New England Medical Center. Drs. Susan Rittling and David T. Denhardt generously provided the *spp1*$^{-/-}$ mice. The authors thank Dr. Allen Steere for kindly reviewing the manuscript.

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