Supporting Information

Hawley et al. 10.1073/pnas.1112078109

SI Materials and Methods

Generation of Bone Marrow Macrophages. Bone marrow cells were collected from the femoral shafts and cultured in 20% L929-conditioned RPMI 1640 supplemented with 10% FCS (HyClone Thermo Scientific), 2.4 mM L-glutamine, and 10% penicillin–streptomycin (Invitrogen) in 100 × 15-mm Petri dishes (Fisher Scientific) for 8 d at 37 °C with 5% CO_2. Following incubation, nonadherent cells were eliminated and adherent macrophages scraped, counted, and resuspended in serum-free RPMI medium 2 h before use.

Phagocytosis. CHO cells or macrophages (10^6/mL) were cultured in serum- and antibiotic-free medium with *B. burgdorferi* at different m.o.i. for 4 or 6 h, except where indicated. The cells were then washed extensively, resuspended in PBS supplemented with 1% FCS, and analyzed by flow cytometry using an LSR II flow cytometer (BD Biosciences) or were further prepared for microscopic analysis. The data were analyzed with FlowJo for Mac, version 8.6 (Tree Star). Live cells were gated according to their forward vs. scatter profile.

Inhibitor Treatments. RAW264.7 and CHO-CR3/CD14 cells (10^6/mL) were preincubated for 1 h with 50 μM of a MyD88 inhibitory peptide (InvivoGen), 10 μM LY294002 (Cell Signaling Technology), or 2 μM wortmannin (Millipore) or for 30 min with 5 mM methyl-β-cyclodextran (Sigma Aldrich) at 37 °C in serum- and antibiotic-free medium, followed by incubation with *B. burgdorferi* (m.o.i. = 25) for 4 h.

Microscopy. To confirm phagocytosis, cells were examined by epifluorescence (ApoTome) and confocal microscopy. Following incubation with bacteria, the cells were washed and fixed in 3.7% paraformaldehyde for 7 min. The cells were then permeabilized with 0.1% Triton X-100 for 5 min and washed. Following blocking of nonspecific binding with 5% BSA for 60 min, the cells were stained with rhodamine phalloidin to visualize the actin cytoskeleton for 30 min at 37 °C and DAPI to stain the nuclei (Invitrogen-Molecular Probes) for 5 min at 37 °C. The cells were mounted with Prolong Gold Anti-fade mounting reagent (Invitrogen-Molecular Probes). Photomicrographs were taken using a Zeiss Axiovert 200M inverted microscope equipped with Apotome and a Hamamatsu Orca camera. When noted, the samples were analyzed with a Zeiss LSM 510 Meta Confocal System.

To assess the localization of CD11b to lipid rafts, 5×10^4 RAW264.7 cells were incubated with Bb914 (m.o.i. = 25) for 1 h. The cells were incubated with CTxB the last 20 min of the incubation period, washed, and fixed. The preparations were then incubated with anti-CD11b conjugated to Alexa fluor 647 (M1/70) for 2 h at room temperature in the dark. Upon extensive washing, the slides were mounted and analyzed by confocal microscopy.

Analysis of CR3 Affinity Conformation. A total of 0.5×10^6 RAW264.7, CHO-CR3, and CHO-CR3/CD14 cells were preincubated in media for 10 min at 37 °C, followed by incubation with Bb914 (m.o.i. = 25) or 1 μM $MnCl_2$ for 30 min. The cells were washed and stained with the mAb CBRM1/5 labeled with Alexa Fluor 647 for 45 min on ice. After incubation, the cells were incubated on ice for 10 min with 1% paraformaldehyde, washed, and analyzed by flow cytometry.

Infection with *B. burgdorferi*. Six- to 8-wk-old mice were infected by s.c. injection with 10^5 *B. burgdorferi* strain 297 in the midline of the back. At 2–3 wk of infection, which represents the acute phase of disease, the mice were killed. Arthritis and carditis were evaluated histologically in formalin-fixed sections processed for H&E staining. The joints were also decalcified. The hearts were cut in half through bisections across the atria and ventricles before sectioning. Signs of arthritis were evaluated on the basis of a combined assessment of histological parameters of *B. burgdorferi*-induced inflammation, such as exudation of fibrin and inflammatory cells into the joints and alteration in the thickness of tendons or ligament sheaths, as well as hypertrophy and hyperplasia of the synovium. Signs of carditis were evaluated on the basis of the cardiac inflammatory infiltrate, including the infiltration of connective tissue with macrophages at the base of the heart, including surrounding the aortic valve and the atria. Carditis was scored on a scale of 0 (no inflammation), 1 (mild inflammation with less than two small foci of infiltration), 2 (moderate inflammation with two or more foci of infiltration), or 3 (severe inflammation with focal and diffuse infiltration covering a large area). Both joint and heart tissue sections were blindly examined.

The relative expression of TNF in cardiac tissue was determined from total RNA extracted from the heart, using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The RNA was treated with DNase I (Promega) and reverse transcribed using oligo(dT) primers (Invitrogen) and SuperScript II reverse transcriptase (RT) (Invitrogen). The cDNA was amplified using primers specific for *actin* (5′-GAC GAT GCT CCC CGG GCT GTA TTC-3′ and 5′-TCT CTT GCT CTG GGC CTC GTC ACC-3′) and *tnf* (5′-AGC CCA CGT CGT AGC AAA CCA C-3′ and 5′-ATC GGC TGG CAC CAC TAG TTG GT-3′) in an Mx3005P QPCR System (Stratagene) and SYBR green-containing reaction buffer (Roche). Relative expression of the gene was calculated relative to actin levels using the equation . Bacterial burdens were determined using DNA extracted from the heart by qPCR. The primers used corresponded to *B. burgdorferi recA*: 5′-TCT CGG CAT TGG CGG ATA TCC TAG-3′ and 5′-CCC ACC TTC TTT TTG CAC CTC AGC-3′. The levels of *recA* targets were calculated relative to actin, as before.

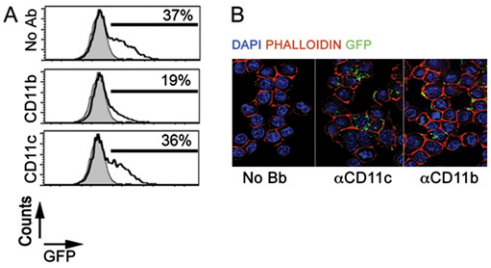

Fig. S1. CR3 is a phagocytic receptor for Bb. (*A*) Flow cytometry of Bb914 phagocytosed by RAW264.7 cells in the presence of 10 μg/mL anti-CD11b or anti-CD11c at an m.o.i. of 50 for 4 h. A 4 °C control was used to determine background binding levels. (*B*) ApoTome microscopy of RAW264 cells incubated with Bb914 (m.o.i. = 10) in the presence of the anti-CD11b mAb, clone M1/70 (*Right*) or an antibody control (*Center*). Rhodamine phalloidin was used to stain actin filaments (red); cell nuclei (blue) were stained with DAPI.

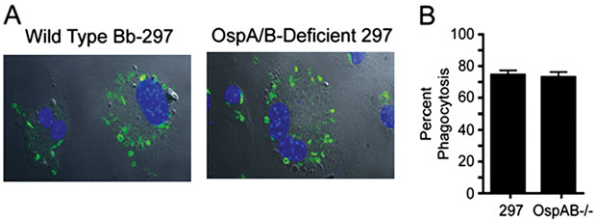

Fig. S2. *B. burgdorferi* phagocytosis is independent of OspA/B expression. (*A*) Representative ApoTome fluorescence micrographs show BMMs with internalized wild-type (*Left*) and OspA/B-deficient *B. burgdorferi* (*Right*). The cells were incubated for 6 h with the spirochetes at an m.o.i. of 50 and fixed. The non-GFP *B. burgdorferi* were stained by incubating with infected mouse serum (1:500) for 1 h at 37 °C, followed by anti-mouse IgG 488 (1:1,000). After extensive washing in PBS, the nuceli were stained with DAPI (1:1,000 dilution). (*B*) Quantification of Alexa Fluor 488$^+$ BMMs. One hundred cells were assessed for the presence of intracellular spirochetes. The data shown represent the average ± SE of three experiments.

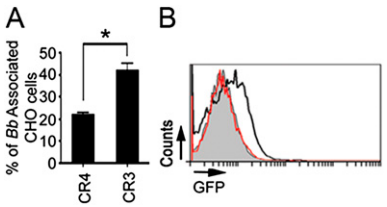

Fig. S3. CR3 mediates the binding of *B. burgdorferi*. (*A*) Average percentage of *B. burgdorferi*-associated CHO-CR4 and CHO-CR3 cells obtained from three separate experiments. *Student's *t* test, $P < 0.05$. (*B*) CHO-CR3 cells were incubated with *B. burgdorferi* at an m.o.i. of 100 for 4 h, followed by gentle (black histogram) or vigorous (red histogram) washing and analysis by flow cytometry.

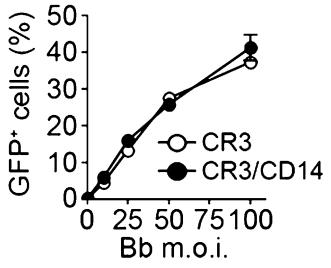

Fig. S4. CD14 does not contribute to *B. burgdorferi* binding. CHO-CR3 (open circles) and CHO-CR3/CD14 (solid circles) were incubated with increasing m.o.i. of Bb914 and then analyzed by flow cytometry. The results represent the mean percentage of GFP$^+$ cells ± SE for three independent experiments.

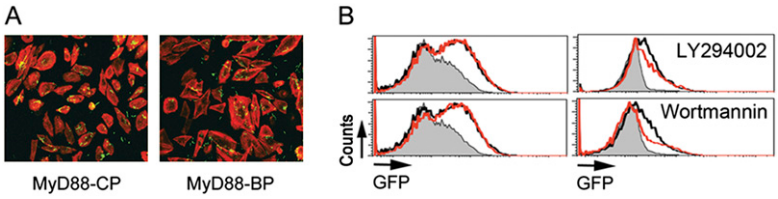

Fig. S5. Phagocytosis of *B. burgdorferi* mediated by CR3/CD14 is independent of MyD88/PI3K. (*A*) Confocal images of *B. burgdorferi* phagocytosis by CHO-CR3/CD14 cells in the presence of a blocking MyD88 peptide. Chamber-grown cells were incubated with 50 μM of the blocking MyD88 peptide (MyD88-BP) or a peptide control (MyD88-CP) for 1 h, followed by incubation with Bb914 for 4 h. The cells were then washed, fixed, and stained with phalloidin–Alexa fluor 594. CHO-CR3 cells grown in eight-well chamber slides were incubated with Bb914 at an m.o.i. of 100 in the presence of 10 μg/mL anti-CD11b blocking antibody or an antibody control. The cells were fixed and stained with phalloidin (red) and DAPI (blue). (*B*) Flow cytometry of phagocytosis by CHO-CR3/CD14 (*Left*) and RAW264.7 cells (*Right*). The cells were preincubated with the PI3K inhibitors, LY294002 (10 μM) and wortmannin (2 μM) (red histograms) or DMSO (control, black histograms). The cells were incubated with the inhibitors for 1 h, followed by Bb914 (m.o.i. = 25). Phagocytosis was allowed to proceed for 4 h. The results are representative of at least three experiments performed.

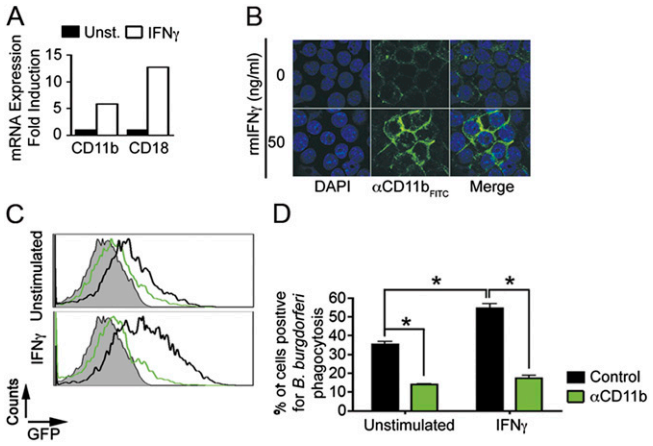

Fig. S6. IFN-γ increases CR3 expression and phagocytosis of *B. burgdorferi* in RAW264.7 cells. (*A*) Quantitative PCR analysis of *cd11b* and *cd18* expression following activation of RAW264.7 cells in the absence (black bars) or presence (white bars) of rmIFN-γ (50 ng/mL) for 16 h. Total RNA was extracted from harvested cells using the TRIzol reagent (Invitrogen), treated with DNase I (Promega), and reverse transcribed using random primers (Invitrogen) and SuperScript II reverse transcriptase (RT) (Invitrogen). The cDNA was amplified using primers specific for glyceraldehyde 3-phosphate dehydrogenase (*gadph*; 5′-CCA TCA CCA TCT TCC AGG AGC GAG-3′ and 5′-CAC AGT CTT CTG GGT GGC AGT GAT -3′), *cd18* (*cd18*; 5′-CGG TCT TCG ACT TGA AGT GAC CTG-3′ and 5′-GGG TCC ATG ATA TCA TCG GC-3′), and *cd11b* (*cd11b*; 5′-GGG TCC AGA AAC CTA ACT ACG TC-3′ and 5′-CCA GTG TAT AAT TGA GGC GC-3′) in a SYBR green-containing reaction buffer (Roche). Relative expression of the gene is referred to nonactivated cells. (*B*) Determination of CD11b expression by RAW264.7 cells by ApoTome fluorescence microscopy following activation with 50 ng/mL of rmIFN-γ for 16 h. Following activation, RAW264.7 cells were washed extensively, fixed, and blocked with 5% BSA in PBS. The cells were then stained with FITC-conjugated rat anti-mouse CD11b (Invitrogen) for 60 min at 37 °C. The nuclei were stained with DAPI and are shown in blue. (*C*) Flow cytometry of RAW264.7 cells stimulated with 50 ng/mL of rmIFN-γ for 16 h (*Lower*) or unstimulated (*Upper*), followed by phagocytosis for 4 h with Bb914 (m.o.i. = 50) in the presence of 10 μg/mL of the anti-CD11b mAb, M1/70 (green histogram) or an mAb control (black histogram). The gray histogram is the 4 °C control. (*D*) Quantification of the percentage of GFP^+ RAW264.7 cells from C. The results represent the average ± SE of three experiments. *Student's *t* test, $P < 0.05$.